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Yeasts in Food and Beverages

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Preface

As a group of microorganisms, yeasts have an enormous impact on food and beverage production. Scientific and technological understanding of their roles in this production began to emerge in the mid-1800s, starting with the pioneering studies of Pasteur in France and Hansen in Denmark on the microbiology of beer and wine fermentations. Since that time, researchers throughout the world have been engaged in a fascinating journey of discovery and development – learning about the great diversity of food and beverage commodities that are produced or impacted by yeast activity, about the diversity of yeast species associated with these activities, and about the diversity of biochemical, physiological and molecular mechanisms that underpin the many roles of yeasts in food and beverage production. Many excellent books have now been published on yeasts in food and beverage production, and it is reasonable to ask the question – why another book?

There are two different approaches to describe and understand the role of yeasts in food and beverage production. One approach is to focus on the commodity and the technology of its processing (e.g. wine fermentation, fermentation of bakery products), and this is the direction that most books on food and beverage yeasts have taken, to date. A second approach is to focus on the yeasts, themselves, and their biology in the context of food and beverage habitats. During the past 25 years, there have been major advances in understanding the basic biology of yeasts, and their cellular and molecular responses to environmental influences. We believe that there is sufficient knowledge and understanding, now, to present a book on food and beverage yeasts, which has a specific focus on the organisms, themselves, and their biology. It will fill a gap in current resources, integrating the technology of yeasts in food and beverage production with the latest understanding of their genomics. This book contains 13 chapters written by an international collection of contributors who are recognized authorities in their field. Chapter topics have been selected to demonstrate the broad significance of yeasts in food and beverage production, the diversity of yeast species involved, and the fundamental ecology, biochemistry, physiology and genomics of their activities.

The impact of yeasts on food and beverage production extends beyond the popular notions of bread, beer and wine fermentations by *Saccharomyces cerevisiae* (Chap. 1). We now know that they contribute to the fermentation of a broad range of other commodities where, in addition to *S. cerevisiae*, many other types of yeasts may work in concert with bacteria and filamentous fungi (Chaps. 2, 4). With increasing consumer demands for more natural foods, there is increasing interest in using microorganisms, including yeasts, as new sources of food ingredients and additives,

such as flavors, colors, antioxidants and vitamins (Chap. 10), and as novel agents for the biocontrol of food spoilage (Chap. 4). Although spoilage of foods and beverages by yeasts is well documented, new spoilage species and new strategies for their control have emerged in recent years (Chap. 11). Food safety and the linkage between diet and health are issues of major concern to modern consumers. Consequently, the public health significance of yeasts in foods and beverages is a topic of emerging interest where, in one context, yeasts could be novel probiotic species but, in other species circumstances, they could lead to infections and other adverse consumer responses (Chap. 12). The impact of yeasts on the quality and safety of foods and beverages is intimately linked to their biological activities. These activities are determined by physical and chemical properties of the ecosystem, and how yeasts respond according to their physiology, biochemistry and genetics. Drawing upon the latest advances in molecular biology and genomics, Chaps. 5, 6 and 8 describe how the different yeast species adapt, evolve, grow and function under the diversity of environmental conditions and stresses presented by food and beverage ecosystems. This fundamental knowledge and understanding, combined with new genomic technologies (Chap. 7), are providing the platform for genetic improvement strategies (Chap. 13), the development of novel bioreactor and biocatalytic technologies (Chap. 9) and new molecular methods for yeast identification and characterization (Chap. 3), that will underpin future innovation in food and beverage yeasts.

Amparo Querol
Graham Fleet
July 2005

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Chapter 1

The Commercial and Community Significance of Yeasts in Food and Beverage Production

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1.1 Introduction

The history of yeast association with human society is synonymous with the evolution of bread, beer and wine as global food and beverage commodities, originating some 5,000 years ago. The microbial science of these products commenced in the mid-1600s with the first observations of yeast cells being reported by Antonie van Leeuwenhoek (The Netherlands). The significance of these findings laid dormant until the classic studies of Pasteur (France) and Hansen (Denmark) during 1850–1900, which heralded the beginnings of the disciplines of microbiology and biochemistry. Subsequent studies by Guilliermond (France) and Kluyver (The Netherlands) in the early 1900s established yeasts as a unique group of microorganisms that had a major role in food and beverage production (Rose and Harrison 1969; Rose 1977). Since the 1950s, several classic texts have specifically highlighted the commercial and social significance of yeasts in foods and beverages (Cook 1958; Rose and Harrison 1970, 1993; Phaff et al. 1978; Skinner et al. 1980; Spencer and Spencer 1990; Reed and Nagodawithana 1991; Deak and Beuchat 1996; Boekhout and Robert 2003).

Today, the impact of yeasts on food and beverage production extends beyond the original and popular notions of bread, beer and wine fermentations by *Saccharomyces cerevisiae* (Table 1.1). In a positive context, they contribute to the fermentation of a broad range of other commodities, where various yeast species may work in concert with bacteria and filamentous fungi. Many valuable food ingredients and processing aids are now derived from yeasts. Some yeasts exhibit strong antifungal activity, enabling them to be exploited as novel agents in the biocontrol of food spoilage. The probiotic activity of some yeasts is another novel property that is attracting increasing interest. Unfortunately, there is also a darker side to yeast activity. Their ability to cause spoilage of many commodities, with major economic loss, is well known in many sectors of the food and beverage industries, while the public health significance of yeasts in foods and beverages is a topic of emerging concern. This chapter defines the scope and diversity of the many beneficial and

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Table 1.1 The commercial and community significance of yeasts in food and beverage production

- Production of fermented foods and beverages.
- Production of ingredients and additives for food processing.
- Spoilage of foods and beverages.
- Biocontrol of spoilage microorganisms.
- Probiotic and biotherapeutic agents.
- Source of food allergens.
- Source of opportunistic, pathogenic yeasts.

detrimental aspects of yeasts in foods and beverages. Subsequent chapters in this book will provide more detailed and specific coverage of these concepts.

1.2 The Informative Process

To effectively exploit and manage the growth and activities of yeasts in foods and beverages, a structured process of knowledge development and understanding is needed, the concepts of which are outlined in Table 1.2 (Fleet 1999). Obtaining this information is a challenging task, and requires the collaborative interaction of microbiologists, chemists, biochemists, molecular biologists, and food scientists. Application of molecular technologies to the detection and identification of yeasts in food and beverage ecosystems (Fernandez-Espinar et al., Chap. 3) and to determining the genetic bases of their biochemical and physiological responses to these habitats (Walker and van Dijck, Chap. 5; Barrio et al., Chap. 6; Bond and Blomberg, Chap. 7; Dickinson and Kruckeberg, Chap. 8) have greatly facilitated acquisition of this fundamental knowledge and understanding. Moreover, there is now a broad recognition and acceptance that many yeasts species other than *Saccharomyces cerevisiae* are intimately and significantly involved in food and beverage production Romano et al. (Chap. 2) and that their individual contributions can be moderated and impacted by interactions with bacteria and filamentous fungi (Viljoen, Chap. 4). For most commodities, the chain of knowledge linking yeast ecology, yeast activity, product chemistry and product quality is very incomplete. Wine could be singled out where most progress has been made in this context.

1.3 Production of Fermented Foods and Beverages

Most individuals, whether they have a scientific or nonscientific background, have a positive image of yeasts because of their well-known association with the production of bread, beer, wine and other alcoholic beverages. Some will know that there are differences between baker's yeast, brewer's yeast, wine yeast and distiller's yeast, and the more learned will know these as either *S. cerevisiae*, *S. bayanus*, or *S. pastorianus*, according to current taxonomic classifications (Vaughan-Martini and Martini 1998; Kurtzman 2003). However, there is increasing awareness that many species other than those of *Saccharomyces* make positive contributions to the fermentations of foods and beverages, and the diversity of these associations are described in Chap. 2.

Table 1.2 Information needed to exploit and manage yeasts in food and beverage production

- Taxonomic identity of species and strains that contaminate and colonize the food throughout the total chain of production and sale.
- Growth profiles of individual species and strains throughout the chain of production and sale.
- Physical location and spatial distribution of species within the product.
- Biochemical, physiological and molecular explanation of how yeasts colonize the product and change its chemical and physical properties.
- Impact of intrinsic, extrinsic and processing factors on yeast growth and metabolic activity in the product.
- Correlation between growth and activity of individual species/strains, and product quality and safety.

In addition to *S. cerevisiae* and *S. bayanus*, it is now well established that various species of *Hanseniaspora*, (*Kloeckera*), *Candida*, *Pichia*, *Metschnikowia*, *Kluyveromyces*, *Schizosaccharomyces* and *Issatchenkia* can make positive contributions to the fermentation of wine from grapes and cider from apples (Fleet 1998, 2003a; Pretorius 2000). *Dekkera* (*Brettanomyces*) species, in addition to *S. pastorianus* and *S. cerevisiae* are significant in the production of some styles of beer (Dufour et al. 2003), while *Schizosaccharomyces pombe* can be important in rum fermentations (Fahrasmane and Ganou-Parfait 1998).

Although the microbiology of dairy products is generally dominated by discussions of lactic acid bacteria, there is now substantial literature describing the important role of yeasts in flavour and texture development during the maturation stage of cheese production, and in the production of fermented milks such as kefir and koumiss (Fleet 1990; Frohlich-Wyder 2003). The most predominant and important species in these associations are *Debaryomyces hansenii*, *Yarrowia lipolytica*, *Kluyveromyces marxianus* and *S. cerevisiae*, but *Galactomyces geotrichum*, *Candida zeylanoides* and various *Pichia* species are also significant. In addition to lactic acid bacteria, micrococci and staphylococci, yeasts also play an important role in the fermentation of meat sausages and the maturation of hams. *D. hansenii*, other *Debaryomyces* species, *Y. lipolytica* and various *Candida* species are involved, and contribute to flavour and colour development in these products (Lucke 1998; Samelis and Sofos 2003).

Yeasts other than *S. cerevisiae* are found in the fermentation of various cereal products, including sourdough breads, where their activities impact on product flavour and rheology. Prominent contributors are *S. exiguus*, *C. humicola*/*C. milleri*, *Torulaspora delbrueckii*, various *Pichia* species and other species of *Candida* (e.g. *C. krusei*/*Issatchenkia orientalis*) (Jenson 1998; Meroth et al. 2003; Hammes et al. 2005). Coffee beans and cocoa beans (chocolate) undergo natural, indigenous fermentations in the primary stages of their processing, where the growth and activities of a diversity of *Hanseniaspora*, *Candida*, *Pichia*, *Issatchenkia*, *Kluyveromyces* and *Saccharomyces* species have been reported. Essentially, these yeasts assist in degradation of bean pulp and contribute to the production of chocolate flavour precursors (Schwan and Wheals 2003, 2004). *Zygosaccharomyces rouxii*, *C. versatilis*, and *C. etchellsii* are important osmotolerant species that play a key role in soy sauce fermentation (Hanya and

Nakadai 2003). Finally, a vast range of traditional, fermented products are produced in Africa, Asia and Latin America, where, along with bacteria, a diversity of yeast species make important contributions (Steinkraus 1996; Nout 2003).

With very few exceptions (e.g. beer), most fermented foods and beverages involve a mixed ecology of yeasts, bacteria, filamentous fungi in some cases, and their viruses. Consequently, complex microbial interactions are likely to be involved (Chap. 4). The ultimate goal is to understand which species are important to product quality and process efficiency, and to develop operational parameters that maximize their positive contributions. Novel bioreactor technologies could be developed to improve process efficiency (Strehaiano et al., Chap. 9) and targets for genetic improvement of strains could be identified (Verstrepen et al., Chap. 13).

1.4 Yeasts as Sources of Ingredients and Additives for Food Processing

Because yeasts have a positive image with consumers, they are considered as a safe source of ingredients and additives for food processing (Demain et al. 1998). Preparations of baker's and brewer's yeasts have been available for many years as dietary, nutrient supplements because of their high contents of B vitamins, proteins, peptides, amino acids and trace minerals. Also, yeasts and are often considered as an alternative source of protein for human consumption (Peppler 1970; Harrison 1993). Many products are now derived from yeasts and, according to Stam et al. (1998), about 15–20% of the global industrial production of yeasts is used for this purpose. Abbas (Chap. 10) describes the production of antioxidants, aromas, flavours, colours and vitamins by yeasts. Other detailed accounts of these topics may be found in Halasz and Laszity (1991) and Reed and Nagodawithana (1991).

Flavour ingredients based on yeast extracts, yeast autolysates and dried yeast preparations represent the most commercially significant products extracted from yeasts, and are used extensively in the food industry as a source of savoury, roasted, nutty, cheesy, meaty and chicken flavours. In addition, some extracts are specifically enriched in their contents of glutamic acid and nucleotides that function as strong flavour enhancers (Dziezak 1987; Nagodawithana 1992; Kollar et al. 1992; Stam et al. 1998). While baker's and brewer's yeasts have been the traditional sources of these products, their diversity and functionality are being expanded by the use of other yeasts such as *C. utilis* (*Pichia jadinii*) and *K. marxianus* (Lukondeh et al. 2003). Yeasts are frequently mentioned as potential sources of high value aroma and flavour substances such as vanillin (*S. cerevisiae*, *Rhodotorula glutinis*), citronellol, linalool and geraniol (*K. marxianus*), and γ- and δ-decalactones (*Sporidiobolus sulmonicolor*, *Y. lipolytica*) (Hagedorn and Kaphammer 1994; Vandamme and Soetaert 2002).

The yeast cell wall, composed principally of β-(1→3) and β-(1→6)-glucans and mannoprotein, represents about 20–30% of the cell dry weight (Fleet 1991; Nguyen et al. 1998). The β-glucans have gelling, thickening and fat-sparing functional properties that offer a range of applications in food processing (Seeley 1977) and, moreover, they have been reported to have anticancer, (Bohn and Be Miller 1995) immunomodulating (Sandula et al. 1999) and cholesterol-lowering activities

(Bell et al. 1999). They also absorb mycotoxins and could offer a method for removing these substances from beverages such as wine (Yiannikouris et al. 2004; Bejaoui et al. 2004).

Food colorants such as astaxanthin and other carotenoid pigments (Lyons et al. 1993; Johnson and Schroeder 1995) and a diversity of vitamins (Reilly 1991; Sauer et al. 2004) can also be derived from yeasts.

1.5 Spoilage of Foods and Beverages by Yeasts

There is an extensive literature on yeasts as food and beverage spoilage agents and, no doubt, this reflects the enormous commercial and economic significance of this problem. Stratford (Chap. 11) gives an updated account of this topic. Earlier, comprehensive discussions include those of Ingram (1958), Walker (1970), Deak (1991), Fleet (1992), Tudor and Board (1993), Thomas (1993), Deak and Beuchat (1996) and Loureiro and Querol (1999). Yeast spoilage is a constant threat and widespread problem in the food and beverage industries that can only be managed by employing educated staff and implementing effective quality assurance programs.

Yeast spoilage is very predictable, principally occurring in those products where bacterial growth is either retarded or prevented by the intrinsic, extrinsic and processing that prevail. Without this competition, yeasts will grow and spoil the product. Typically, high-acid, low-pH foods, products with high sugar (e.g. more than 10% w/v) or high salt (more than 5% NaCl) content, and products preserved with weak organic acids (e.g. sorbic, benzoic, acetic) are prone to yeast spoilage. Fruits, fruit juices, and fruit drinks, fruit pulp, fruit juice concentrates, sugar and flavour syrups, confectionery products, alcoholic beverages, carbonated beverages, vegetable salads with acid dressings, salt- and acid-based sauces, fermented dairy products and fermented or cured (salted) meat products represent prime candidates for yeast spoilage (Walker 1970; Tudor and Board 1993; Deak and Beuchat 1996). Some yeasts (e.g. *Cryptococcus* and *Rhodotorula* spp.) grow better than bacteria at subfreezing temperatures, and will spoil frozen meat, poultry, seafood and other products stored for lengthy periods. Some high-fat, low-water-activity commodities such as margarine and butter can support the surface growth of yeasts (e.g. *Y. lipolytica*). While there is significant diversity in the yeast species associated with food and beverage spoilage, some specific associations are frequent and often predictable. For example, these include *Z. rouxii* in very high sugar products, *D. hansenii* in salted meat products, *Z. bailii* in products preserved with weak organic acids and *Y. lipolytica* in high-fat products. However, an open and enquiring outlook should be maintained, because new spoilage and food processing species may be present and await discovery – for example, *Z. lentus* (Steels et al. 1999) and *Tetrapisispora fleetii* (Kurtzman et al. 2004).

Controlling the growth and activity of spoilage yeasts requires good understanding of their physiology (Chap. 5), biochemistry (Chaps. 8, 9) and genetic responses (Chaps. 6, 7). Unfortunately, there remain large gaps in this knowledge, especially for yeasts other than *S. cerevisiae*. Factors affecting growth and survival, and being able to predict yeast response to these factors, are particularly important at the practical levels of quality control and assurance. For most yeasts, the growth and survival limits, and inactivation kinetics for basic technological parameters such as temperature,

pH, sugar concentration and salt concentration, are not well defined, and require more careful, systematic investigation (Praphailong and Fleet 1997; Betts et al. 2000). Also, new food and beverage processing technologies such as high hydrostatic pressure, exposure to low- and high-intensity electric fields, and treatment with novel antimicrobial plant extracts (Gould 2000) are emerging and new information on the growth, survival and inactivation responses of individual yeast species to these factors is needed. Finally, the ecological origin or source of spoilage yeasts remains a mystery for many species and requires further research. The first line of defense in controlling food spoilage by microorganisms is the prevention of contamination, but the importance of microbial ecology in quality assurance programs is often underestimated.

1.6 Yeasts as Biocontrol Agents

In Chap. 4, Viljoen notes that most food and beverage habitats present complex ecosystems where a diversity of microbial species and interactive responses are likely to occur and impact on product quality. These responses can be beneficial, antagonistic or neutral to individual species within the product. Antagonistic interactions have given rise to the concept of biocontrol, whereby one species could be deliberately exploited to inhibit the growth and survival of another, less desirable species. During the last 20 years, several yeast species that exhibit strong antagonistic activity against filamentous fungi have been discovered. These yeasts have been investigated as potential agents for the biocontrol of fungi that cause pre- and postharvest spoilage of fruits and vegetables (e.g. *Botrytis*, *Penicillium*, *Aspergillus*, *Rhizopus* spp.), thereby enabling a lesser use of chemical fungicides (Fleet 2003b; Punja and Utkhede 2003; Spadaro and Gullino 2004). *C. oleophila* and *Pseudozyma flocculosa* have been commercialized for such use, and other species with biocontrol potential include *Metschnikowia pulcherrima*, *P. guillierlmondii*, *C. sake*, *Sporobolomyces roseus*, *Aureobasidium pullulans* and various *Cryptococcus* species (Fleet 2003b). *P. anomala* has been well studied for its biocontrol of fungi that spoil cereal silages (Druvefors et al. 2002). Various mechanisms have been proposed to explain the antagonistic activity of yeasts towards other fungi, and these include production of killer toxins and other inhibitory proteins and peptides, competition for nutrients and space, production of fungal cell wall lytic glucanases and chitinases, production of toxic metabolites such as ethanol, acetaldehyde, ethyl acetate and fatty acids, and induction of fungal resistance or defense reactions within the plant (Punja and Utkhede 2003).

It should not be forgotten that some yeasts influence the growth and survival of other yeasts by the simple mechanisms of ethanol and killer toxin production (Shimizu 1993). These properties may also impact on bacteria and filamentous fungi (Fleet 1999, 2003a), and highlight the fact that yeasts probably have much greater potential as biocontrol agents than currently recognized. More research is needed on this topic. Ethanol and killer toxin production are significant properties in the selection and commercialization of yeasts for wine production (Degre 1993).

1.7 Public Health Significance of Yeasts in Foods and Beverages

With respect to the field of food safety, yeasts have an impeccably good record and this topic is discussed by Fleet and Roostita in Chap. 12. Unlike bacteria, viruses and some filamentous fungi, yeasts are rarely associated with outbreaks of foodborne gastroenteritis or other foodborne infections or intoxications. As part of normal, daily food consumption, humans are unknowingly and inadvertently ingesting large, viable populations of a diversity of yeast species without adverse impact on their health (e.g. yeasts in many cheeses, fermented and cured meats, fruits and fruit salads, home-brewed beer and wine). Nevertheless, an open mind and vigilance on yeasts and foodborne disease are required – several bacterial species (e.g. *Escherichia coli*) not considered to be serious foodborne pathogens 25 years ago, are now classified in the high risk category.

There is a significant body of "lay" and "alternative" literature that connects yeast presence in foods to the onset of a broad range of allergic and hypersensitive reactions in humans. Migraines, respiratory problems, chronic fatigue syndrome, dysfunctional gut syndrome, irritable bowel syndrome and gut dysbiosis are prominent among these disorders (Crook 1986; Eaton 2004). The linkage between human disorder, food and yeast is largely based on dietary observations – when the suspect food is removed from the diet, the disorder disappears, and returns when the food is reintroduced into the diet. The underlying mechanisms of the human response require systematic, scientific research and could reflect adverse reactions to the yeast cells themselves, or metabolites they have produced (e.g. proteins, biogenic amines, sulphur dioxide).

Unlike many bacteria and viruses, yeasts are not known as aggressive infectious pathogens. However, some yeast species fall into the category of opportunistic pathogens. *C. albicans* and *Cryptococcus neoformans* are prominent in this context, and cause a range of mucocutaneous, cutaneous, respiratory, central nervous, systemic and organ infections in humans (Hazen and Howell 2003). Usually, healthy, immunocompetent individuals are not at risk of such infections. Generally, individuals with weakened health and immune function are at greatest risk, and include cancer and AIDS patients, hospitalized patients and those undergoing treatments with immunosuppressive drugs, broad-spectrum bacterial antibiotics and radio-chemotherapies. The increased frequency of such individuals in the community in recent years has lead to a significant increase in the reporting of yeast infections. Moreover, increasing numbers of yeast species, other than *C. albicans* and *Cryp. neoformans* have been associated with these infections and are now considered in the list of opportunistic pathogens (Hazen 1995; Hobson 2003; Georgiev 2003). These include yeast species that are frequently found in foods such as *C. krusei*/*I. orientalis*, *P. anomala*, *K. marxianus*, *S. cerevisiae* and various *Rhodotorula* species. Murphy and Kavanagh (1999) have drawn specific attention to the pathogenic potential of *S. cerevisiae*.

Epidemiological statistics suggest that many yeast infections in hospitalized patients originate from yeast contamination of catheters (Douglas 2003; Kojic and Daroviche 2004), and there is increasing concern that foods could be a significant

source of yeasts in the general hospital environment. It is also possible that foods could be a source of yeasts that colonize the intestinal tract, from where they translocate to the blood system, resulting in fungaemia and distribution to infect various organs (Cole et al. 1996). Greater understanding of the yeast ecology of the human gastrointestinal tract is needed.

1.8 Probiotic Yeasts

Probiotics are viable microorganisms that are beneficial to the host when consumed in appropriate quantities. Lactic acid bacteria are widely recognized as the main probiotic species but there is increasing interest in adding other organisms to the probiotic list, including yeasts (Klaenhammer 2001). Live *S. cerevisiae* preparations have been used as supplements to animal and poultry feeds for many years, and have been reported to improve the growth and health of these hosts (Lyons et al. 1993). Also, there is an expanding interest in using yeasts as probiotics in the aquaculture industry (Gatesoupe 1995). With respect to humans, *S. cerevisiae* var. *boulardii*, has been successfully used over the last 20 years as an oral, biotherapeutic agent to treat patients with severe cases of diarrhea and other gastrointestinal disorders (McFarland and Bernasconi 1993; Czerucka and Rampal 2002). The yeast colonizes the intestinal tract and, in this context, acts in a probiotic function. Research to establish its credentials as a probiotic agent that can be added to foods is still in progress (van der Aa Kuhle et al. 2005). However, there are significant concerns about its public health safety because of increasing reports of its association with cases of fungaemia (Cassone et al. 2003). Generally, the concept of using yeasts as human probiotics is at an early stage of development and further research is required. Apart from health benefit and safety issues, probiotic yeasts will also require certain technological properties for use in foods, namely, to remain viable in the food, not to grow in and spoil the food, and not to adversely affect sensory acceptability of the food (Heenan et al. 2004).

1.9 Future Prospects

As mentioned already, harnessing and exploiting the activities of yeasts in food and beverage production requires fundamental knowledge of their ecology, physiology, biochemistry and molecular biology. This knowledge provides the base for genetic improvement strategies (Chap. 13) and the discovery of novel bioreactor and biocatalytic technologies (Chap. 9) that are likely to drive the next generations of product and process development.

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Chapter 2

Taxonomic and Ecological Diversity of Food and Beverage Yeasts

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2.1 Introduction

Fermentation has been used for several thousand years as an effective and low-cost resource to preserve the quality and safety of foods. Apart from this primary role, fermentation adds value and enhances nutritional quality and digestibility through biological enrichment, and provides dietary enrichment through aroma and flavour production and modifying textures of food substrates. All these changes are operated by microorganisms, which, naturally present (spontaneous fermentations) or added (inoculated fermentation) in raw materials, break down complex carbohydrates and proteins into more easily digestible elements.

Among the fermentation microorganisms, yeasts are undoubtedly the most important group of microorganisms that are exploited for commercial purposes. Yeasts used in food fermentation processes modify original materials organoleptically, physically and nutritionally and for this they have been used for millennia in bread-making and production of alcoholic beverages.

A diversity of fermented foods, which vary according to geographical area and cultural preference, are produced across the globe and the individual and peculiar characteristics which distinguish and typify each food are determined by the expression of the wide biodiversity of the fermenting microorganisms, such as yeasts. In Western countries yeasts are involved in the production of kefir from milk, beer from barley and hops, and wine from grapes, while in developing countries, where the lack of resources limits the use of techniques such as vitamin enrichment of food and the use of capital-intensive processes for food preservation, food fermentation contributes substantially to food security. In these countries there is a wide diffusion of traditional fermented foods, produced from both edible and inedible raw materials, based on local customs and generally consumed as dietary staples. Here we report the ecology and the role of yeasts involved in fermented food and beverages, focusing on the dominant species and their effect on the product quality, summarised in Table 2.1.

The Yeast Handbook
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Table 2.1 Distribution and principal activities of yeast species in fermented foods and beverages

More frequent yeast species	Origin (Food and beverage)	Major functions
Saccharomyces species	Wine, beer, sourdoughs, cider, sherry, cheese, indigenous fermented foods and beverages	Sugar fermentation Production of secondary metabolites Pectinase and glycosidasic activities Inhibitory effect on the growth of mycotoxin-producing moulds Degradation of some fractions of kasein CO_2 evolution Lipolytic, proteolytic and urease activities
Debaryomyces hansenii	Cheese, salami	Increase of pH Production of growth factors of importance for bacteria
Hanseniaspora (*Kloeckera*) species	Wine, cider, indigenous fermented foods and beverages	Proteolytic, glycosidasic and pectinolytic activities Production of secondary metabolites
Candida-fermenting species	Wine, sourdough, indigenous fermented foods and beverages	Proteolytic, glycosidasic and pectinolytic activities Production of secondary compounds Inhibitory effect on the growth of mycotoxin-producing moulds
Yarrowia lipolytica	Cheese, salami	Lypolytic, proteolytic and urease activities Reduction of fat rancidity

2.2 Yeasts in Dairy Products

For dairy products yeasts are mainly used in cheese production but may also be involved in the production of fermented milk products such as kefir. Yeasts are primarily used as single starter cultures but many products are still produced by back-slopping or spontaneous fermentation. A mixture between the used starter culture and a dominant indigenous flora is also seen in many dairy products. Yeasts are in most cases used as secondary starter cultures in order to enhance the aroma production or to facilitate the growth of other microorganisms. Unfortunately, the functions of yeasts during cheese production and their influence on the cheese quality are in general poorly investigated. For cheese manufacturing *Debaryomyces hansenii*,

Saccharomyces cerevisiae and to some extent *Galactomyces geothricum* and *Yarrowia lipolytica* are the dominant yeast species (Jakobsen et al. 2002). But also other yeasts might be seen, such as *Kluyveromyces lactis*, which has been found to occur in high numbers in soft cheeses such as Camembert (Beresford et al. 2001).

The halophile yeast *D. hansenii* (perfect form of *Candida famata*) is a highly diverse yeast species as shown by phenotypic differences such as the ability to assimilate/ferment different carbon compounds, differences in technological properties such as lipase and protease activity (Sørensen and Jakobsen 1997) and growth under different environmental conditions (Petersen et al. 2002). According to the present taxonomy *D. hansenii* is divided into two varieties, *D. hansenii* var. *fabryi* and *D. hansenii* var. *hansenii*. The two varieties can only be discriminated by different electrophoretic mobilities of their glucose-6-phosphate dehydrogenase and maximum temperatures for growth (Nakase et al. 1998). Apparently the predominant variety seen in cheese is *D. hansenii* var. *hansenii* (Petersen et al. 2001). The type strain (CBS767) of the variety *D. hansenii* var. *hansenii* has previously been reported to be haploid (van der Walt et al. 1977), whereas information on the ploidy of other strains belonging to the species *D. hansenii* is lacking.

D. hansenii is especially of importance during the production of surface-ripened cheeses such as Brick, Limburger, Port Salut, Taleggio, Tilsitter, Trappist, and the Danish Danbo cheese. The surface smear is found to consist of a mixed microbial population comprising both yeasts and bacteria. For these types of cheese the yeasts initiate the ripening by degradation of lactate, thereby increasing the pH on the cheese surface and allowing the growth of a more acid-sensitive bacterial population comprising, amongst others, *Brevibacterium linens* (Leclercq-Perlat et al. 1999; Petersen et al. 2001). Further, *D. hansenii* might produce growth factors of importance for the bacteria as well as aroma components and lipolytic and proteolytic enzymes that contribute to the ripening process (Jakobsen and Narvhus 1996). For the Danish cheese Danbo, the osmotolerant yeast *D. hansenii* has been found almost exclusively (Petersen et al. 2002), whereas in other types of surface-ripened cheeses, including cheeses such as Camembert and blue veined cheese, other yeasts such as *C. zeylanoides*, *Y. lipolytica* and *K. lactis* have been found (Eliskases-Lechner and Ginizinger 1995; Addis et al. 2001; Corsetti et al. 2001a). As mentioned, many of the yeasts occur as a positive part of the indigenous microbial population. However, the benefits of moving from spontaneous fermentations to controlled fermentations are many and therefore there seems to be growing interest in the use of *D. hansenii* as a purified starter culture. Consequently, the use of starter cultures with appropriate technological properties generates the need for fast and simple methods for identification of *D. hansenii* at strain level. Mitochondrial DNA (mtDNA) restriction fragment length polymorphism (RFLP), which was originally developed for differentiation of wine and beer strains of *Saccharomyces* spp., has been used for strain typing of *D. hansenii* (Petersen et al. 2001) and determination of chromosome length polymorphism (CLP) by pulsed-field gel electrophoresis (PFGE) has been used to clarify the genetic diversity of *D. hansenii* and for typing of dairy isolates (Petersen and Jespersen 2004). Figure 2.1 shows the chromosome profiles of 16 isolates of *D. hansenii* from Danish dairies producing surface-ripened cheeses. As seen from the figure, also at the genetic level *D. hansenii* shows an extensive degree of diversity.

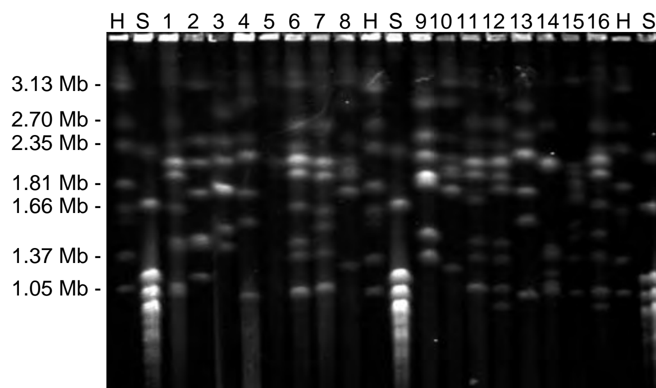

Fig. 2.1. Chromosome profiles of *Debaryomyces hansenii* isolated from Danish dairies producing surface-ripened cheeses. *H* marker "*Hansenula wingei*" (*Pichia canadensis*), *S* marker *Saccharomyces cerevisiae*, lanes 1–16 isolates of *D. hansenii* from brine and surface smear sampled from seven different Danish dairies

S. cerevisiae has been used as a starter culture in the production of, especially, Gorgonzola, but it apparently also occurs spontaneously, together with other yeasts, as an integral part of the microbial population of both blue veined cheeses and some types of soft cheese (Beresford et al. 2001; Hansen and Jakobsen 2001). On cheese substrate some strains of *S. cerevisiae* have been able to degrade α_{si}-kasein and β_{a2}-kasein as determined by capillary electrophoresis (Hansen and Jakobsen 2001). When used as a starter culture in Mycella, a Danish Gorgonzola-type cheese, *S. cerevisiae* was reported to stimulate sporulation and improve the mycelial growth of *Penicillium roqueforti*; however, the stimulation appeared to be strain-dependent (Hansen et al. 2001a). The use of *S. cerevisiae* as a starter culture further resulted in a softer texture and a significantly higher concentration of aroma compounds of the final cheese. The study also indicated the possibility of *S. cerevisiae* to compete with *D. hansenii* in the interior of the cheese.

Even though previously considered as a mould, *G. geotrichum* (perfect form of *Geotrichum candidum*) is now considered as a yeast species (de Hoog et al. 1998). Synonyms include former names like *Oidium lactis* and *Oospora lactis*, indicating the link to milk and dairy products. *G. geotrichum* is known as a starter culture for several types of mould-ripened cheeses, e.g. Camembert, surface-ripened cheeses and cheeses such as the French St. Albray where it is used in combination with other microorganisms such as *Penicillium camemberti* and *B. linens* (Molimard et al. 1995). On the other hand, *G. geotrichum* has also been shown to be a potential spoilage organism especially for blue veined cheeses. It has been shown to have antimicrobial activity and can, because of its rapid growth, effectively compete with other yeasts and moulds in cheeses (Jakobsen et al. 2002).

Y. lipolytica (perfect form of *C. lipolytica*) is often found in soft, blue veined and surface-ripened cheeses even though it is not used as a starter culture. *Y. lipolytica* is characterised by having a quite pronounced lipolytic and proteolytic activity (Guerzoni et al. 2001) that might be difficult to control if it is used as a starter culture

for cheese production. However, it has been reported to have a positive effect on the flavour of Raclette cheese as well as low-fat cheeses (Wyder et al. 1999; Jakobsen et al. 2002). It is rather sensitive to high NaCl concentrations, which might be a limitation for its growth in, for example, blue veined cheeses. Further *Y. lipolytica* is known to produce brownish pigments in cheeses.

For fermented milk, yeasts within the genera *Candida*, *Galactomyces*, *Kluyveromyces*, *Saccharomyces* and *Torulaspora* are generally used (Oberman and Libudzisz 1998). *G. geotrichum* is used as commercial starter culture in the production of *Villi*, a Scandinavian fermented milk product and *S. unisporus* and *K. marxianus* (*C. kefyr*) are used as commercial starter cultures in the production of milky kefir. *S. cerevisiae* has also been reported to be involved in the fermentation of a number of indigenous African fermented milk products known under names such as *amasi*, *nono* and *rob* (Okagbue and Bankole 1992; Gadaga et al. 2001; Abdelgadir et al. 2001).

2.3 Yeasts in Fermented Sausages

Fermented sausages could be classified as intermediate moisture meat products, in which the removal of available humidity is an effective strategy for inhibiting the growth of spoilage microorganisms. They represent foods prepared from raw materials, involving processes of fermentation and chemical curing. Fermentation is a crucial phase of the curing phase of sausages, since at this stage the principal physical, biochemical and microbiological transformations take place (Beriain et al. 1993). These changes are influenced by characteristics of the raw materials and process conditions, which influence the organoleptic properties of the final product, such as flavour, colour and texture, as well as its preservation and safety. The predominant microorganisms involved in the production of fermented meats are lactic acid bacteria, coagulase-negative staphylococci and micrococci, but also moulds and yeasts can be involved, reaching significant numbers and therefore playing a technological role. Yeasts are usually present in low numbers in fresh meat, but counts may increase during low-temperature storage and they may eventually dominate the microflora (Cook 1995).

The surface of dry fermented sausages is covered by a white superficial coat constituted by moulds and yeasts. Yeasts are naturally found on the hides of animals, from where they are easily spread to the fresh meat during slaughtering. The yeast presence is related to natural contaminating yeasts present in the meat (Dillon and Board 1991), in processing equipment and on workers' hands and aprons.

Studies on the yeast flora of fermented sausages are limited, compared with those focused on bacteria and, although yeasts do not form part of the starter cultures usually employed for salami processing, an increase in yeast number during ripening has been observed (Abunyewa et al. 2000). Yeast counts remain low during the fermentation stages when bacterial numbers increase, but their numbers rapidly increase during the maturation stage. The high number of yeasts observed during the later stages of maturation suggests that the yeasts play an important role in the ripening of salami. The progressive growth of lactic acid bacteria during the fermentation stages and yeasts during the ripening stages indicates competition between the different microorganisms for available substrates. However, the interaction between yeasts

and lactic acid bacteria at the later stages appears to be synergistic, since both populations continue to survive at high numbers with neither being inhibited by the other. The high salt concentration and, therefore, the low water activity, combined with the acidic environment, a typical characteristic of fermented sausage, favours the growth of certain yeast species since competing bacteria are repressed.

In the final products yeast cells have been detected in concentration up to 10^5 cfu/g (Encinas et al. 2000; Samelis et al. 1993), numbers that suggest a significant involvement of yeast activity in the salami production. According to Geisen et al. (1992) yeasts' requirements for oxygen restrict them to mainly growing near the surface of fermented sausages. In fact, Coppola et al. (2000) found 10^4 cfu/g yeast cells in samples from the core of Naples-type salami. This value remained almost unchanged until the end of the ripening period. In contrast, samples from the external part of the product reached the maximum level of 10^6 cfu/g after 3 weeks. Probably, higher oxygen availability in the external part of the product could facilitate the growth of these microorganisms.

Fermentative yeast species, however, can thrive under low oxygen conditions since they only require oxygen for production of cell wall constituents, such as sterols and fatty acids. The confirmation of this is also in the results of Gardini et al. (2001), who found increased yeast counts during the first few days of fermentation up to 10^5–10^6 cfu/g, whereas before casing yeast counts ranged from not detectable to about 10^4 cfu/g.

Although the initial flora present in the sausage emulsion and raw meat is extremely variable, strains of *D. hansenii* are frequently isolated during the fermentation and ripening stages. This species appeared to be the most frequent yeast species associated with the processing of salami: Abunyewa et al. (2000) found a frequency of this species of 20.37% of the total number of yeast strains isolated during a pilot scale production of commercial salami. Gardini et al. (2001) found *D. hansenii* and its anamorph *C. famata* with a frequency of 52% of the total isolates from production and ripening of typical salami in southern Italy. Other species frequently isolated are *Rhodotorula mucillaginosa* (15%), a typical air contaminant, described as a frequent food isolate, *Cryptococcus albidus* (10.18%), *Trichosporon beigelii* (*moniliiforme*) (9.26%), *Y. lipolytica* (13.89%), and *C. zeylanoides* and *D. occidentalis* both with about 5% (Abunyewa et al. 2000). Other species isolated, representing less than 5% of the total number of yeasts, are *C. haemulonii*, *C. gropengiesseri*, *D. polymorphus*, *D. vanrijiae*, *G. geotrichum*, *Pichia farinosa*, *P. philogaea*, *Rh. minuta*, *Sporobolomyces roseus*, *Sterigmatomyces halophilus*, and *Torulaspora delbrueckii*.

The incidence of the different yeast species varies depending on the salami processing. The species *C. gropengiesseri*, *P. farinosa*, *P. philogaea*, *Rh. minuta*, *Sp. roseus* and *St. halophilus* are isolated only during the fermentation stage, suggesting an inhibition of these yeast species due to the reducing water activity. Other species, such as *C. haemulonii*, *D. polymorphus*, *D. occidentalis* and *T. delbrueckii*, are not frequently isolated during processing, so they do not constitute the typical microflora of salami processing.

C. zeylanoides, *Cr. albidus*, *D. hansenii*, *Rh. mucillaginosa* and *Y. lipolytica* represent the species found most frequently. Parameters such as their tolerance to low temperatures, high salt concentrations and low pH levels could offer a fundamental ecological advantage for the growth of these species in fermented sausage. Different treatments during salami processing, such as smoking, variety of spices added and

method of ripening, could influence the yeast count. Encinas et al. (2000) found differences statistically significant in yeast counts between smoked and nonsmoked sausages. In particular, after the second stage of the manufacture, the mean counts in smoked sausages were lower than in nonsmoked ones. On the other hand, yeasts have been described as being affected by smoking and also by factors such as time and temperature (Leistner 1995). Other reports underline the influence of spices on yeasts, in particular a significant inhibitory effect of garlic on their growth (Ghamnnoum 1990). The addition of garlic powder strongly affects both *D. hansenii* and *C. utilis* (Olesen and Stahnke 2000), but *D. hansenii* is completely inhibited, whereas *C. utilis* seems to be more resistant. Therefore, dried garlic powder exerts at least a fungistatic potential, and perhaps also a fungicide potential.

Other compounds, such as sorbates, allowed by numerous legislations and frequently present in industrial sausages, seem to decrease the yeast counts, as a consequence of their known effect on the control of yeasts in food and drinks (Fleet 1990).

Also ripening conditions affect yeast growth and, for example, when an accelerated process is used, yeast counts decreased by 1 log unit/g (Encinas et al. 2000).

The role of yeasts in the manufacture of fermented sausage is mainly related to the development of colour (by removing the oxygen) and flavour, as a consequence of their ability to degrade peroxides, lipolytic activity and, to a lesser extent, proteolytic activity (Lücke 1985). Furthermore, yeasts protect sausages from the adverse effect of light. In fact, it is believed that yeasts delay rancidity and protect the red nitrosomyoglobin from breakdown by degrading peroxides and consuming oxygen, thus stabilising the appealing red colour of fermented sausages (Lücke and Hechelmann 1987), which is an indication that the product is fully cured.

Yeasts play an important role in sausage fermentation as well as in maturation of hams (Cook 1995) and bring about characteristic flavours and surface appearance. The contribution of these organisms to the typical aroma of the products is based on their primary and secondary metabolites and lipases and proteinases are key activities. Among the flavour products identified in dry sausages, the oxidation products of lipids account for about 60% of the total compounds which influence the flavour (Berdagué et al. 1993). The distinctive flavours of these products were found to be related to hydrolytic and oxidative changes occurring in the lipid fraction during ripening. The species *Y. lipolytica* has been found to be characterised by a higher lipolytic potential (Gardini et al. 2001), with isolates of this species exhibiting the capability to reduce the content of total free fatty acids after 6 days of incubation in a medium containing pork fat (5%).

The decrease of total free fatty acids content could be related to the activity of *Y. lipolytica*, which can further metabolise or oxidise unsaturated free fatty acids to flavour compounds (Ordoñez et al. 1999). Furthermore, lipases from isolates of *Y. lipolytica* favour at pH 5.5 the liberation of saturated free fatty acids rather than unsaturated ones. This tendency could have a positive effect by reducing the possibility of rancidity, which principally involves polyunsaturated free fatty acids.

D. hansenii and *C. zeylanoides* are able to reduce the fat rancidity of salami by hydrolysing lipids through lipolytic activity (Metiva et al. 1986), even if some authors (Sørensen 1997) report that the lipolytic activity is inhibited by low pH and low temperature. The anamorphic yeast *Cr. albidus* exhibits proteolytic activity

(Huerta et al. 1988) and may cause spoilage by hydrolisation of salami proteins. Most of these species have the ability to utilise organic acids produced by lactic acid bacteria (Besancon et al. 1992; Roostita and Fleet 1996), resulting in an increase of pH by the production of amines and ammonia, and thereby favouring the growth of spoilage bacteria. In contrast, data reported by other authors (Abunyewa et al. 2000) did not support the role of yeast to provide a means of spoilage by assimilating organic acid, such as lactic acid. It may be that the loss of water results in concentrating the acids and thus counterbalances their assimilation.

The introduction of starter cultures has become essential during industrial production of fermented sausages in order to shorten the ripening period, ensure colour development, enhance the flavour and improve product safety (Gardini et al. 2001). The inclusion of bacteria in starter cultures has frequently been investigated, while little attention has been focused on the role of yeasts in the fermentation of sausages. The use of commercially available starters, mainly constituted of lactic acid bacteria and micrococci, may also produce an impoverishment of flavour and aroma and a loss of peculiar organoleptic characteristics found in naturally fermented sausages.

Starting from the earliest studies on salami (Capriotti 1954), *D. hansenii* is the most commonly isolated yeast among the yeast population of fermented sausages. On the basis of this, *D. hansenii* has been used as a starter with positive effects on the development of a characteristic yeast flavour and stabilisation of the reddening reaction. *D. hansenii* and its imperfect form *C. famata* are now used in starter preparations and should be added to the sausage mixture at a concentration of 10^6 cfu/g (Hammes and Knauf 1994). The yeast *Y. lipolytica*, the perfect form of *C. lipolytica*, is also frequently isolated from fresh beef (Fung and Liang 1990; Dalton et al. 1984) and sausages (Viljoen et al. 1993). Owing to its lipolytic activity, *Y. lipolytica* could have significant technological interest (Sinigaglia et al. 1994) as a starter for the production of traditional dry fermented sausages by shortening the ripening time in relation to the evolution of the lipid fraction and also to protein breakdown, related to its elevated proteolytic activity.

2.4 Yeasts in Sourdough Breads

Bread-making, together with the making of wine, beer and sake, and the production of yogurt and cheese, is among the oldest biotechnology processes. Spontaneous souring with natural microflora and the use of special starter cultures are both used in sourdough bread baking. Sourdough is an important part of cereal fermentation and its preparation is based on traditional customs of each country: sourdough breads vary from a large number of different rye and flat breads to San Francisco sourdough bread made from wheat flour. In Italy, in which sourdough is used in more than 30% of bakery products, there are more than 200 different types of sourdough breads (INSOR 1995) (Corsetti et al. 2001b). Most of these breads are made by following old traditions and differ in the type of flour, other ingredients, type of sourdough, technology and shelf life. In all cases, the routine is to save a portion of the raw dough (or "leaven") from each batch, and this is then used to inoculate the next lot of dough. The leaven contains both yeasts and lactobacilli: in fact, sourdough could be considered a complex biological system which includes a mixed

microbial population mainly represented by yeasts and lactic acid bacteria. Since the mid-nineteenth century, yeast cultures have been available commercially and the use of inoculated fermentation has turned into common practice.

In doughs that are spontaneously fermented, the yeast population can range from 7×10^7 to 6.1×10^9 cfu/g of sourdoughs (Paramithiotis et al. 2000), while the ratio between yeast and lactic acid bacteria varies from 1:8 to 1:73. Gobbetti et al. (1994b) analysed the microflora composition of wheat sourdoughs from semi-industrial bakeries in an Italian region and reported an average ratio between lactic acid bacteria and yeasts of about 100:1, with the yeast count ranging from 3×10^4 to 5×10^7 cfu/g. These values are similar to results of other authors (Roecken and Voysey 1995) and seem to indicate that these products are really naturally fermented, without addition of commercial yeasts. In products from bakeries, where commercial yeasts are added to accelerate the leavening process, yeast populations are much higher than those of lactic acid bacteria.

The most frequent yeast species detected in sourdoughs are *S. cerevisiae*, *C. krusei*, *C. milleri*, *P. anomala*, *P. subpellicosa*, *S. exiguus*, *T. holmii* and *C. humilis* (Rossi 1996; Gullo et al. 2002).

In a study conducted on samples of homemade sourdoughs from small bakeries in Sicily (Italy) (Pulvirenti et al. 2004), among the wild yeast strains, identified by molecular techniques such as PCR/RFLP analysis of internal transcribed spacer regions, the dominant species was *S. cerevisiae*, followed by *C. milleri*, *C. humilis*, *S. exiguus* and *Issatchenkia orientalis*. Also Paramithiotis et al. (2000) analysed wild yeast strains isolated from traditional Greek wheat sourdoughs, both household and semi-industrial types, and reported *S. cerevisiae* as the predominant species in all the samples examined. Depending on the origin of the sample, *P. membranifaciens* or *Y. lipolytica* was found. When *S. cerevisiae* is associated with *P. membranifaciens* the ratio generally ranges from 1:0.5 to 1:0.31 with *S. cerevisiae* as predominant species. This is correlated to the capability of *S. cerevisiae* isolates to ferment all the sugars present in the dough, i.e. glucose, fructose, sucrose and maltose, whereas *P. membranifaciens* can only ferment glucose, but about 8 times slower than *S. cerevisiae* isolates.

The ratio between *S. cerevisiae* and *Y. lipoytica* is 1:0.14, with *S. cerevisiae* dominant. This is related to the absence of fermentative metabolism in *Y. lipolytica* isolates, while they only oxidise glucose. The occurrence of *S. cerevisiae* strains in every sourdough tested leads to the conclusion that this species represents the indigenous yeast microflora of traditional Greek sourdoughs, while *P. membranifaciens* and *Y. lipolytica* might represent an occasional microflora of these products. The frequent presence of *S. cerevisiae* in sourdoughs is reported by different authors (Ottogalli et al. 1996; Rossi 1996; Gobbetti et al. 1994a) and in many cases this species seems to constitute part of indigenous microflora, whereas in others its presence is related to the extensive use of this yeast in bakeries. The presence of *P. membranifaciens* strains in sourdough microflora is also mentioned by Rossi (1996) and it has been found in traditional Portuguese bread doughs (Almeida and Pais 1996). The data reported by Paramithiotis et al. (2000) represent the first evidence of the presence of *Y. lipolytica* in sourdoughs, a species mostly associated with meat and dairy products.

The numbers and the yeast species present in sourdough are influenced by the degree of yeast tolerance to the organic acid produced by lactic bacteria, particularly

lactic and acetic acids derived from homo- and heterofermentative bacteria. The yeasts *S. cerevisiae* and *S. exiguus*, very frequent species in sourdough, exhibit different behaviour for the resistance to these two acids: *S. cerevisiae* is very sensitive, while *S. exiguus* is very resistant. The sensitivity to these organic acids is related to the presence in sourdough of the undissociated form of acetic acid at low pH values (3.5–3.9). For this reason in some products, such as Italian Panettone obtained by a natural fermentation system, *S. exiguus* may be exclusively present during certain production phases, yielding satisfactory results (Rossi 1996).

However, technical parameters such as temperature, flour composition, degree of dough hydration, sodium chloride content and time between rebuildings are significant variables for the selection of microorganisms (Gobbetti et al. 1994a). Another critical point for the microbial development in sourdough is represented by the available sources of carbon (Lues et al. 1993; Gobbetti et al. 1994b). The available carbohydrates in wheat flour are (in decreasing order) maltose, sucrose, glucose and fructose, with some trisaccharides (maltotriose, raffinose). They may either increase during fermentation, as in the case of glucose, or decrease, as in the case of sucrose in the presence of yeasts possessing high invertase activity. This enzyme catalyses the hydrolysis of sucrose into glucose and fructose, thus increasing osmotic pressure. Therefore, baker's yeast strains with low invertase activity have been selected for sweet dough applications because they are suitable to elaborate dough containing low concentrations of sucrose (less than 8–10%). However, these strains do not show intrinsic osmotolerance and at the higher sugar contents the osmotic pressure inhibits their activity (Myers et al. 1997). The presence of *S. exiguus* in the dough is associated with a rapid consumption of soluble carbohydrates with the exception of maltose. In any case *S. cerevisiae* and *S. exiguus* are not in nutritional competition for maltose because *S. exiguus* does not metabolise maltose.

In bread-making many different functional properties have been defined for yeasts. The most important function of baker's yeasts is leavening (Paramithiotis et al. 2000), by producing CO_2 via the alcoholic fermentation of the sugars. In the initial stages of bread-making, the CO_2 produced stays in solution until the water phase becomes saturated. At this point, all further CO_2 diffuses into the existing gas cells produced during mixing. Increased production of CO_2 increases the dough volume, giving bread with characteristic a light, spongy texture. Baker's yeast also influences the development of the dough gluten structure, brought about by expansion of the dough owing to CO_2 production.

Furthermore, yeasts produce primary and secondary metabolites, such as alcohols, esters and carbonyl compounds which contribute to the development of the characteristic bread flavour (Damiani et al. 1996; Hansen and Hansen 1994; Martinez-Anaya 1996). Some of these compounds are volatile and are baked out of the bread. In addition, through their enzymatic activities, such as proteases, lecithinase, lipases, α-glucosidase, β-fructosidase and invertase, yeasts can affect not only the organoleptic characteristics, but also the overall appearance of the final product. In fact, these enzymatic activities have an influence on the dough stickiness and rheology, as well as on the crust colour, crumb texture and firmness of the bread (Antuna and Martinez-Anaya 1993; Collar et al. 1998).

The great potentiality of sourdough bread is related to the interaction between lactic acid bacteria and yeasts. Like other fermented foods produced by mixed microflora, the organoleptic, health and nutritional properties of this product depend on the cooperative activity of lactic acid bacteria and yeasts. In fact, each sourdough can be considered a microhabitat in which yeasts and lactic acid bacteria exist together in a dynamic equilibrium. The yeasts are responsible for the leavening process, while the bacteria determine the souring of the dough. The combined metabolic activity of these microorganisms leads to final products with particular sensorial properties and a prolonged shelf life (Gänzle et al. 1998).

Numerous pieces of research have reported the effect of the interaction between sourdough lactic acid bacteria and yeasts on the metabolism of carbohydrates, the production of CO_2 and other volatile compounds. The utilisation of soluble carbohydrates by lactic acid bacteria and, consequently, their energy yield, lactic and acetic acid production are greatly influenced by the associated yeasts and vary according to the type of sugars. In a continuous sourdough fermentation the association between *Lb. sanfranciscensis* and *S. cerevisiae* is optimal for producing acetic acid, while yeast extract does not produce the same effect (Vollmar and Meuser 1992). *Torulopsis holmii* has been found to improve dough acidification by *Lb. sanfranciscensis*, while *S. cerevisiae* enhances acid production by *Lb. sanfranciscensis* and *Lb. plantarum* (Spicher et al. 1982). The lack of competition between *Lb. sanfranciscensis* and *S. exiguus* for maltose is fundamental for the stability of this association in San Francisco bread (Gobbetti 1998). On the other hand, the lack of competition for the main carbon source seems to be one of the prerequisites for the stability of lactic acid bacteria/yeast association in food fermentation. When *Lb. plantarum* is associated with *S. cerevisiae* and *S. exiguus* in the presence of sucrose as a carbon source, cell yield and lactic acid production increase (Gobbetti et al. 1994b). The hydrolysis of sucrose by yeasts liberates glucose and fructose, which are then more rapidly depleted than the sucrose by lactic acid bacteria (Gobbetti 1998). Furthermore, yeasts hydrolyse sucrose about 200 times faster than the released hexoses which are fermented (Martinez-Anaya 1996), causing the rapid disappearance of sucrose during sourdough fermentations (Gobbetti 1998).

The relationship between yeasts and lactic acid bacteria seems to provide more favourable conditions for CO_2 production. Although yeast cell concentrations and the type of yeast are the major parameters determining gas production rates (Akdogan and Ozilgen 1992), the growth of lactic acid bacteria can influence the yeast leavening and CO_2 production (Gobbetti et al. 1995). The production of CO_2 by other yeasts, such as *S. exiguus*, is not comparable to the high gassing power of *S. cerevisiae*. Besides, compared with *S. cerevisiae* alone, the associative growth of *S. cerevisiae* and *Lb. sanfranciscensis* decreased to one third of the time necessary to reach the maximum production of CO_2 by the yeast. The same increase has also been observed with the associated growth of *S. exiguus* and *Lb. sanfranciscensis*. The associated growth of *Lb. plantarum* with *S. cerevisiae* caused an increase in CO_2 produced and improved the capacity of the dough to retain the gas.

The flavour of leavened baked goods is influenced by the raw materials (Hansen and Hansen 1994), sourdough fermentation, proofing, baking and by the starters. Even if the greatest number of compounds influencing aroma is formed during

baking, sourdough fermentation is essential for achieving an acceptable flavour. Each type of metabolism (heterolactic, homolactic and alcoholic) which characterises the sourdough fermentation is defined by typical volatile compounds (Damiani et al. 1996).

The differentiation is mainly related to 2-methyl-1-propanol and 2,3-methyl-1-butanol, the principal products of yeast fermentation, diacetyl, mainly produced with other carbonyls by homofermentative lactic acid bacteria, and ethyl acetate, mainly produced with some alcohols and carbonyls by heterofermentative lactic acid bacteria. The sourdough process starts with the association of *Lb. sanfranciscensis* and other homo- or heterofermentative lactic acid bacteria and/or *S. exiguus* characterised by a balanced profile. The sourdough produced with mixed starter composed by *Lb. sanfranciscensis*/*S. cerevisiae* has higher concentrations of yeast fermentation products (1-propanol, 2-methyl-1-propanol and 3 methyl-1-butanol) and fewer bacterial compounds (Damiani et al. 1996; Gobbetti et al. 1995). Annan et al. (2003) obtained similar results in a study aimed at comparing volatile compounds associated with Ghanaian maize dough samples prepared by spontaneous fermentation and by the use of added starter cultures. The starter cultures added were *Lb. fermentum*, *S. cerevisiae* and *C. krusei*. The amount of ethanol, which is the alcohol produced in the highest amounts, is higher in dough fermented spontaneously and with *S. cerevisiae* than with *C. krusei* or *Lb. fermentum*. The fusel alcohols 1-propanol, 2-methyl-1-propanol and 3-methyl-butanol are found in the highest amounts in fermentation with *S. cerevisiae*, while phenylethyl alcohol is found in the highest amounts in fermentations with *C. krusei*. Among esters, ethyl acetate, the most abundant ester formed, is generally found in higher amounts in fermentations with *S. cerevisiae*. Higher levels of acetic acid are formed in fermentations with *C. krusei* than with *S. cerevisiae*, which produces amounts not significantly different from those found in spontaneously fermented maize dough.

2.5 Yeasts in Grape Wines

Wine is a natural product resulting from several biochemical reactions, which start during ripening of the grapes and continue during harvesting, throughout the alcoholic fermentation, clarification and after bottling. Even if the grape must represents a complete growth medium, only a limited number of microbial species are able to grow as a consequence of its low pH values and high sugar content. Thus, of all the microorganisms present on the grapes, the yeasts are the principal agents of the biochemical transformation of grape must.

The variety and proportion of different yeasts in the must can depend on numerous factors, such as geographic location, climatic conditions, grape variety, physical damage caused by moulds, insects and birds and viticultural practices (Pretorius et al. 1999).

In traditional winemaking, spontaneous fermentation of grape juice is performed by a sequential development of different yeast species that originate from the grapes and the winery equipment. The initial population of yeasts in freshly extracted grape juice is 10^3–10^6 cfu/ml. *Hanseniaspora* (*Kloeckera*) spp. are often the predominant species on the surface of grape berries, accounting for roughly 50–75% of the total yeast population. Numerically less prevalent there are species

of *Candida* (e.g. *C. stellata* and *C. pulcherrima*), *Brettanomyces* (*B. anomalus* and *B. bruxellensis*), *Cryptococcus*, *Kluyveromyces*, *Metschnikowia* (*M. pulcherrima*, the perfect form of *C. pulcherrima*), *Pichia* (*P. membranifaciens*), the so-called film yeast, as well as species previously assigned to the *Hansenula* genus, e.g. *H. anomala* and the pink yeast *Rhodotorula* (*Rh. minuta*).

On immature grape berries a very low yeast population has been detected ($10–10^3$ cfu/ml) with the predominance of *Rhodotorula*, *Cryptococcus* and *Candida* species, along with the yeast-like fungus *Aureobasidium pullulans*. These species are also found on surface of ripe grapes, but at this stage *Hanseniaspora* and *Metschnikowia* species are mostly predominant. Damage to grapes increases the yeast population (above 10^6 cfu/ml), especially of the *Hanseniaspora/Kloeckera*, *Metschnikowia* and *Candida* species, as well as species of *Saccharomyces* and *Zygosaccharomyces* (Fleet et al. 2002).

The population of the main wine yeast *S. cerevisiae* in grape juice is initially very low: generally it reaches populations less than 10–100 cfu/g. The limited number of *S. cerevisiae* on grapes (Martini 1993) and the evidence that this yeast does not appear to be specifically associated with the vineyard or other natural substrates induced Martini et al. (1996) to conclude that grapes are not the primary source of *S. cerevisiae*, reported as strictly associated with the winery and fermentation plants; the few, if any, cells of *S. cerevisiae* resident on grapes should provide an extremely limited contribution to spontaneous must fermentation (Ciani et al. 2004).

Different conclusions have been reached by other authors who, even though recognising the exiguity of *S. cerevisiae* cells residing on grapes, sustain that vineyard-resident cells do play an important role in natural fermentation and the presence or absence of *S. cerevisiae* differs with each plant and grape cluster. Török et al. (1996) by using more elaborate isolation methods, classical genetic analysis and electrophoretic karyotyping of monosporic clones, demonstrated that the vineyard is the primary source for the wine yeasts and that the strains found on the grapes can be followed through the fermentation process.

While the fermentation process is occurring, the establishment of anaerobic conditions, the antimicrobial activity of sulphur dioxide added, the depletion of nutrients and the increasing levels of ethanol enlarge the selectivity of the medium. The non-*Saccharomyces* yeasts present in grape juice, such as *Hanseniaspora* (*Kloeckera*), *Candida*, *Pichia*, *Kluyveromyces* and *Metschnikowia* could proliferate to final populations of about $10^6–10^7$ cfu/ml, and started to decline by mid-fermentation, when the ethanol production by *S. cerevisiae* exceeds 5–7% (Heard and Fleet 1988; Gao and Fleet 1988). Ethanol production by *S. cerevisiae* is the major factor affecting the growth of non-*Saccharomyces* yeasts. However, *Hanseniaspora* and *Candida* species, when the fermentation is performed at temperatures lower than 15–20°C, decrease their sensitivity to ethanol, giving a significant contribution to wine flavour (Erten 2002).

During the latter stages of natural wine fermentation the strongly fermentative strains of the *Saccharomyces sensu stricto* group of wine yeasts, which are more ethanol-tolerant and more competitive for growth in media with high sugar content, become predominant ($10^7–10^8$ cfu/ml) and complete the fermentation. The *Saccharomyces sensu stricto* group is an evolutionary lineage, which is distinct from the species belonging to the *Saccharomyces sensu lato* group (Kurtzman and

Robnett 2003). Actually, the *sensu stricto* group comprises *S. cerevisiae*, *S. paradoxus*, *S. bayanus* and *S. pastorianus*, which are associated with wine fermentation, and three newly defined species, *S. cariocanus*, *S. mikatae* and *S. kudriavzevii* (Naumov et al. 2000). Apart from the well-known and recognised prevalence in winemaking of the species *S. cerevisiae*, *S. bayanus* plays a particular role in primary winemaking, especially when must fermentation takes place at low temperatures, and *S. pastorianus* is thought to originate from the hybrid cross between *S. cerevisiae* and *S. bayanus* (Tamai et al. 1998). In a recent work (Redžepović et al. 2002), in the indigenous population of *Saccharomyces sensu stricto* strains in Croatian vineyards, *S. paradoxus*, possessing potentially important oenological characteristics, occurs in much higher numbers than *S. cerevisiae*.

Other yeasts, such as species of *Brettanomyces*, *Kluyveromyces*, *Schizosaccharomyces*, *Torulaspora* and *Zygosaccharomyces*, may also be present during the fermentation and ageing of the wine, and some of these yeasts influence adversely sensory quality. *Schizosacharomyces pombe*, *Zygosaccharomyces bailii* and *Zygosaccharomyces fermentati* are characterised by a high level of ethanol tolerance (more than 10%) and, although frequent in a winery environment, their contribution to grape must fermentation is rarely reported. The reason is probably related to their slower growth in comparison with that of other wine yeasts or to the production of inhibitory factors by other yeasts. These species could be very interesting in winemaking in consequence of their ability to utilise malic acid.

Numerous studies on grape juice fermentation reported that non-*Saccharomyces* species (especially *K. apiculata* and *C. stellata*) survive during fermentation at a significant level (Heard and Fleet 1988; Ciani 1997), but owing to their low ethanol tolerance these yeasts are generally undetectable at the end of fermentation.

The persistence of non-*Saccharomyces* yeasts during fermentation depends upon many factors, such as fermentation temperature, nutrient availability, inoculum strength of *Saccharomyces*, use and levels of sulphur dioxide and the quantity and identity of microorganisms initially present on the grapes (Fleet 2003).

The growth of non-*Saccharomyces* species affects both the kinetics of growth and the metabolism of *Saccharomyces* (Lema et al. 1996). These yeasts are capable of anaerobic as well as aerobic growth and may persist during the fermentation, competing with *Saccharomyces* for nutrients. In addition, non-*Saccharomyces* yeasts seem to be less tolerant to very low oxygen availability than *S. cerevisiae*. Therefore, removal of residual oxygen from fermenting grape juice by the vigorous growth of *S. cerevisiae* could contribute to the early death of non-*Saccharomyces* species (Hansen et al. 2001b). Other studies report that *K. apiculata* (*H. uvarum*) could strip the grape juice of thiamine and other micronutrients, leading to deficient growth of *S. cerevisiae* (Mortimer 2000). However, some non-*Saccharomyces* species, such as *K. apiculata* (*H. uvarum*) and *M. pulcherrima* possess a significant proteolytic activity (Charoenchai et al. 1997; Dizzy and Bisson 2000) and can generate amino acids useful for *S. cerevisiae*. The autolysis of these non-*Saccharomyces* yeasts (Hernawan and Fleet 1995) represents a possible source of nutrients for *S. cerevisiae*.

The presence of killer interactions represents another factor affecting yeast species and strain evolution during wine fermentation. The killer phenomenon is based on the secretion of polypeptide toxins which kill sensitive cells of their own

species and frequently those of other yeast species and genera. However, killer strains are immune to the toxin they produce.

A wide literature deals with the killer character in *S. cerevisiae* isolates from fermenting grape juice (Musmanno et al. 1999; Guriérrez et al. 2001). Killer strains of *S. cerevisiae* sometimes predominate at the end of the fermentation, suggesting that they take over the fermentation by asserting their killer activity. Killer strains have been found among wine isolates of *Candida*, *Pichia* and *Hanseniaspora* and some of these can exert their killer action against wine strains of *S. cerevisiae* (Fleet and Heard 1993).

It is expected that the inoculated cultures of *S. cerevisiae* will suppress indigenous microflora, both non-*Saccharomyces* and *S. cerevisiae* strains, thus dominating the fermentation.

The transformation of grape juice in wine is a biochemical process, in which the enzymes play a fundamental role, being the major force catalysing the numerous biotransformation reactions which characterise the complex event of winemaking. These enzymes originate from the grapes, from the indigenous microflora present on the grapes and from the microorganisms present during winemaking. Pectinase, proteases and glycosidases are some of the enzymes secreted by yeasts that are of interest in winemaking for their technological effects and their contribution to aroma formation. Pectinolytic enzymes cleave long pectin chains into shorter, more soluble chain segments that facilitate pressing of the grapes, contribute to the clarification of the musts, may increase extraction of the substances that contribute to colour and aroma and may enhance filtration of the wines. Enzymatic hydrolysis of the proteins into smaller, more soluble nitrogen-containing molecules (peptides and amino acids) facilitates the clarification and stabilisation of the musts and wines, also preventing incomplete fermentations due to a deficiency of assimilable nitrogen in the must. These enzymes also play a major role during the autolysis process in wines kept on yeast lees during ageing.

The aroma and flavour properties of the wine can be enhanced by glycosidases, which hydrolyse nonvolatile glycosidic precursors of the grapes. It must be underlined that yeasts involved in winemaking can be important producers of numerous enzymes (Esteve-Zarzoso et al. 1998). *S. cerevisiae*, the principal wine yeast, is not recognised as a significant producer of extracellular proteases, lipases or proteolytic enzymes, although a few strains have been reported to degrade polygalacturonate (McKay 1990; Gainvors et al. 1994). Various authors have reported glycosidase production by this species (Delcroix et al. 1994). Conversely, non-*Saccharomyces* yeasts, such as species of *Candida*, *Debaryomyces*, *Hanseniaspora* (*Kloeckera*), *Hansenula*, *Metschnikowia*, *Pichia*, *Schizosaccharomyces*, *Torulaspora* and *Zygosaccharomyces*, have been described as potential sources for the commercial production of enzymes, such as proteases, esterases, pectinases, lipases and glycosidases (Rosi et al. 1994; Saha and Bothast 1996; Charoenchai et al. 1997). Regarding wine production, it is now recognised that non-*Saccharomyces* wine species, occurring in the must during the early stages of vinification, contribute to the enzymatic reactions (Heard and Fleet 1986).

Proteolytic activity has been observed in strains of *C. pulcherrima*, *K. apiculata*, *P. anomala* and *P. membranifaciens* (Fernández et al. 2000).

The pectin esterase and polygalacturonase activities increase during grape ripening and are produced by non-*Saccharomyces* yeasts present in must. Fernández et al. (2000) analysed 182 non-*Saccharomyces* yeasts isolated from grape must and found that polygalacturonase was the enzyme most commonly found and was secreted by 45% of the yeasts analysed (*M. pulcherrima*, *B. clausenii*, *P. membranifaciens*, *K. thermotolerans*, *P. anomala*, *C. stellata*). Blanco et al. (1994) reported that at least 75% of oenological strains tested in their study possessed limited proteolytic activity. These results suggest that wine yeasts exert little influence on pectin composition of must/wine.

Difficulties in wine clarification and filtration can also arise from the presence of high molecular weight β-glucans produced by *Botrytis cinerea* in infected grapes. The action of glucanase enzymes can solve these problems. The presence of β-(1,3)-D-glucanases has been reported in many yeast species (Fleet 1991). These enzymes exert endo- and exo-activities and are constitutive glycoproteins. *S. cerevisiae* excretes several β-(1,3)-glucanases and endo-β-(1,3)-glucanases activity has been determined in dried yeasts used in winemaking (Canal-Llaubéres 1988).

Among glycosidase enzymes involved in flavour-releasing processes, β-D-glucosidase has been widely studied because of its widespread occurrence in plants and also in yeasts (Esteve-Zarzoso et al. 1998), such as in *H. vineae* (Vasserot et al. 1989) and in *Candida* species (Günata et al. 1990). A study conduced on 317 strains belonging to 20 wine yeast species indicated that yeast species of *Candida*, *Debaryomyces*, *Hanseniaspora* (*Kloeckera*), *Kluyveromyces*, *Metschnikowia*, *Pichia*, *Saccharomycodes*, *Schizosaccharomyces* and *Zygosaccharomyces* genera possess β-glucosidase activities (Rosi et al. 1994). Saha and Bothast (1996) in a screening of 48 yeast strains of the genera *Candida*, *Kluyveromyces*, *Debaryomyces* and *Pichia* for the production of extracellular glucose-tolerant β-glucosidase activity found that all yeast strains tested produced extracellular β-glucosidase activity, but enzymes from only 15 yeasts showed very high glucose tolerance.

More recently β-D-xylosidase has become of interest in oenology because it is a component of the enzyme complex that degrades the xylan, the major hemicellulolytic component of plant cell walls, but there is a lack of information on β-D-xylosidase activity in wine yeasts. Manzanares et al. (1999) evaluated the β-xylosidase activity in 54 yeast strains belonging to the genera *Candida*, *Dekkera*, *Hanseniaspora*, *Metschnikowia*, *Pichia*, *Rhodotorula*, *Schizosaccharomyces* and *Zygosaccharomyces*, mainly isolated from grapes and wines. The β-xylosidase activity was only detected in eight yeast strains belonging to *H. osmophila*, *H. uvarum* and *P. anomala* species. With respect to the location of the enzyme activity, *P. anomala* strains exhibited extracellular, cell-wall-bound and intracellular β-xylosidase activities. *H. uvarum* strains showed the lowest level of β-xylosidase production, the majority of which was cell-wall-bound. High intracellular β-xylosidase activity was found in *H. osmophila* strains and no extracellular activity could be detected. Furthermore, β-xylosidase preparations from *P. anomala* and *H. uvarum* strains maintained their activities at pH and temperature values and at concentrations of glucose and ethanol typically found during winemaking processes, thus potentially allowing their application for the improvement of the aroma and flavour properties of wine. Recently, Capece et al. (2005)

by analysing numerous *H. uvarum* wine strains found several strains exhibiting β-glucosidase and β-xylosidase activities at a significant level.

Another fundamental parameter which differentiates the wines is the final bouquet, characteristic of each vine cultivar and produced by fermentation and transformation of the aroma during the ageing. The wine aroma appears mainly during yeast fermentation and among the more than 1,000 volatile compounds identified, more than 400 are produced by yeast.

Thus, the various yeast species/strains that develop during the fermentative process metabolise grape juice components to a wide range of volatile and non-volatile end-products that contribute to the aroma and flavour characteristics of the wine. The differences in the composition of the wines resulting from the different yeasts appear to be largely quantitative rather than qualitative: the fermentative products are usually identical, but the relative amounts are different. Therefore, conversion of grape sugars to alcohol and other end-products by a specific yeast population may yield wines with distinct organoleptic quality and the yeast species represents a prominent factor in determining the content of some by-products in wine. Strong polymorphism has been observed within the different wine species, with highly variable by-product formation, resulting as a species-specific pattern. Then, within each species, the level on the by-products is often an individual strain characteristic.

The growth of non-*Saccharomyces* species could be considered quantitatively significant and may produce secondary compounds affecting the analytical composition and quality of the wine. Thus, their potential to contribute to the fermentation of inoculated wines should not be underestimated. Zohre and Erten (2002) analysed the behaviour of *K. apiculata*, *C. pulcherrima* and *S. cerevisiae* with pure and mixed cultures in grape juice. The non-*Saccharomyces* yeasts in pure cultures exhibited very slow rates of utilisation of total sugars without completeness of fermentation in comparison with the performance obtained in mixed fermentation with *S. cerevisiae*. On the other hand, stuck or sluggish fermentations are reported in the literature in cases where *K. apiculata* and other non-*Saccharomyces* yeasts dominated in grape juice. The role of non-*Saccharomyces* yeasts in determining the organoleptic properties of the final product is strictly dependent on the strain and on the extent of its growth. Strains belonging to the species *K. apiculata* and *H. guilliermondii* have been extensively studied in relation to the formation of some fermentation products of oenological interest (such as glycerol, acetic acid, higher alcohols, acetaldehyde, acetoin and ethyl acetate), demonstrating that the amounts of these secondary compounds are strain-dependent (Ciani and Maccarelli 1998; Romano 2002).

Ethanol is the main volatile product of yeast metabolism, followed by diols, higher alcohols and esters. Ethanol determines the viscosity of the wine and acts as a fixer of aroma. Among non-*Saccharomyces* species, good fermentation power is exhibited by *Schizosaccharomyces pombe* and *Saccharomycodes ludwigii* that could achieve 14–15% v/v of ethanol. *T. delbrueckii* shows a large variability of ethanol production. However, some strains are capable of producing considerable amounts of ethanol (13% v/v). A low fermentation power is characteristic of *C. stellata* and of apiculate wine yeasts (*Hanseniaspora*/*Kloeckera*).

Despite the low quantity of organic acids in the wine, they are sufficiently volatile to contribute to its aroma. The most important is acetic acid, whereas propanoic, butanoic and lactic acids are usually below the perception threshold. Acetic acid represents more than 90% of the volatile acid of wine (Henschke and Jiranek 1993; Radler 1993) and it is one of the most important by-products that negatively affect the analytical profile of the wine. In fact, by law its concentration limit in wine may not be higher than 1.0–1.5 g/l, depending on the country. Acetic acid becomes objectionable near its flavour threshold of 0.7–1.1 g/l and values between 0.2 and 0.7 g/l are considered optimal (Dubois 1994). Apiculate strains are well known to produce high concentrations of acetic acid and for this they have been considered for a long time as spoilage yeasts. The wine species of *Hanseniaspora/Kloeckera* usually produce acetic acid at elevated levels, in the range 0.7–3 g/l and above. In synthetic medium, strains of *K. apiculata* and *H. guilliermondii* exhibit great variability in acetic acid production, with strains producing amounts of this compound of less than 1 g/l (Romano et al. 1992). Strains of *K. apiculata* forming low amounts of acetic acid in fermentation of different grape musts have been described (Comi et al. 2001; Capece et al. 2005). As regards *S. cerevisiae* strains, acetic acid formation is affected by sugar concentration, pH, nitrogen and the high/low production varies depending on the strain involved in the fermentative process. Romano et al. (2003a) found that the production of acetic acid was the main variable for the differentiation of 115 wild *S. cerevisiae* strains isolated from Aglianico-Vulture wine (Southern Italy), with values ranging from 130 to 1,610 mg/l. Similar results were obtained by Paraggio and Fiore (2004), who analysed *S. cerevisiae* wild strains of different vine cultivar origin, and found a certain correlation between acetic acid production and strain origin. In particular, in both pieces of research *S. cerevisiae* strains isolated from Aglianico-Vulture were characterised by a high level of acetic acid production.

Two of the most important fermentation by-products affecting the "body" of the wine are succinic acid and glycerol. Succinic acid is the main acid produced by yeasts and its formation is strain-dependent. The non-*Saccharomyces* yeasts are usually higher producers of this acid than *S. cerevisiae*. High amounts of succinic acid are generally produced by *C. stellata* (Ciani 1997). Glycerol is quantitatively a very important wine constituent and contributes significantly to the sweetness and the body and fullness of the wine.

Another important, but not always desirable, secondary compound of wine fermentation is acetaldehyde, accounting for 90% of total aldehydes. In consequence of their low sensory threshold values, aldehydes are important to the aroma of the wine.

Acetaldehyde is the product of the decarboxylation of pyruvate during alcoholic fermentation. This compound plays an important role in the free/combined sulphur dioxide balance and its amount in wine is variable, ranging from 10 to 300 mg/l, depending on grape variety and yeasts involved in fermentation. Wines containing amounts of 500 mg/l are considered unmarketable. In fact, the high concentration of acetaldehyde may be sufficient to cause an undesirable oxidised taste in wines, especially in white wine, whereas in red wines it should be present in amounts up to 100 mg/l. The total aldehyde content varies with the type of yeast species/strain involved in the fermentative process. Some authors (Fleet and Heard 1993) reported that *S. cerevisiae* strains produce relatively high levels. The general behaviour recognised as

commoner in *H. uvarum*/*K. apiculata* strains is the production of acetaldehyde in amounts comparable to those in the case of *S. cerevisiae* (Ciani and Maccarelli 1998), with strains forming about 90 mg/l and strains producing about 200 mg/l. Romano et al. (1994) divided 86 *S. cerevisiae* strains into groups producing low, medium and high amounts of this compound. The low and high phenotypes also differed considerably in the production of other secondary compounds, such as acetic acid, acetoin and higher alcohols. The wide variability recorded among *H. uvarum*/*K. apiculata* strains (Romano 2002) leads to consider this by-product as having individual strain characteristics, as reported for *S. cerevisiae* strains (Romano et al. 2003a). Other data (Fleet and Heard 1993) indicate that non-*Saccharomyces* species, such as *K. apiculata*, *C. krusei*, *C. stellata*, *H. anomala* and *M. pulcherrima*, produce low levels (from undetectable amounts to 40 mg/l) of acetaldehyde. Among non-*Saccharomyces* species evaluated by Ciani (1997), only *T. delbrueckii* is a lower producer of acetaldehyde than *S. cerevisiae*, whereas other species (*C. stellata*, *S'codes ludwigii*, *H. uvarum*, *K. apiculata*) produce consistent amounts of this compound. Granchi et al. (2002) found that *H. osmophila* and *K. corticis* isolates produce this compound at significant concentrations, the anamorph form showing a greater variability among the isolates (from 40 to 100 mg/l).

Higher alcohols represent another important group of secondary products influencing the analytical profile of the wine. They are produced by the Ehrlich pathway in the presence of amino acids and from sugars via biosynthesis by yeasts during alcoholic fermentations. Isoamyl alcohol, active amyl alcohol, isobutyl alcohol and *n*-propanol are the principal higher alcohols. Small or enhanced amounts of these compounds contribute positively to wine quality, giving a certain complexity to wine bouquet, while high levels (above 500 mg/l) influence negatively wine aroma. Their overall presence in wine covers a wide range: from a concentration slightly lower than 100 mg/l to a concentration higher than 500 mg/l (Lambrechts and Pretorius 2000). These compounds are also important precursors for ester formation, and the esters of higher alcohols are associated with pleasant aroma. Higher alcohols are usually present at levels below their detection thresholds, but the presence of numerous wild yeasts may increase the concentration of these compounds until undesirable levels. Higher alcohol production appears to be a general characteristic of yeasts, although the amounts produced depend on cultural conditions, and also on yeast genus, species and strain. Amyl alcohols and isobutyl alcohol have been considered the most significant; the predominant one in commercial fermentations is isoamyl alcohol with a range of 50–300 mg/l. The production of higher alcohols by non-*Saccharomyces* yeasts is generally lower than for *S. cerevisiae*. Apiculate strains show a general pattern of low higher alcohol producers (Herraiz et al. 1990; Gil et al. 1996), even when a considerable variability was recorded among strains tested in different grape must (Comi et al. 2001). In particular, a great variability was found for active amyl alcohol (ranging from 6 to 110 mg/l) and isoamyl alcohol (from 13 to 132 mg/l). Tested in different grape musts, apiculate strains exhibit uniform behaviour for higher alcohol production, resulting generally in low producers. Despite the strain variability, the total amount of higher alcohols produced by apiculate yeasts is always at an acceptable level, thus representing a positive trait of this species, but not a selective strain parameter. Non-*Saccharomyces* yeasts of the early

fermentation phase produce low levels of active amyl alcohol, isobutyl alcohol and *n*-propanol, which are much below that of their flavour thresholds reported in the literature (Zohre and Erten 2002). Only isoamyl alcohol is produced at concentrations which could contribute to the sensory properties of wines.

Other compounds contributing to wine aroma are acetate esters, which impart a mostly pleasant smell. In fact, the fresh, fruit aroma of young wines derives in large part from the presence of the mixture of esters produced during fermentation. They are mainly produced by yeasts during alcoholic fermentation in a reaction between alcohols and acetylcoenzyme A (acetyl-CoA). Ethanol is the main alcohol in wine; therefore, ethyl acetate produced from ethanol and acetyl-CoA is the major ester formed by yeasts. This ester is always present in wines with concentrations below its high threshold taste level of 150 mg/l. At low level (less than 50 mg/l) it may be pleasant and adds to general fragrance complexity (called "fruit esters"), while at levels above 150 mg/l it yields a sour-vinegar off-flavour and can flaw the fragrance of wine. All apiculate yeasts form high amounts of ethyl acetate: Ciani and Picciotti (1995) reported that a strain of *H. uvarum* was able to produce about 380 mg/l, while Zohre and Erten (2002) found *K. apiculata* strains producing 580 mg/l of ethyl acetate. Also *C. pulcherrima* (anamorph of *M. pulcherrima*) strains produce large amounts of this ester (676 mg/l), whereas a lower amount (about 30 mg/l) was produced by the main wine yeast *S. cerevisiae* (Zohre and Erten 2002). In *K. corticis* and *H. osmophila* (Granchi et al. 2002) the average values found were always below the threshold taste level and were lower than the concentrations found with *K. apiculata*/*H. uvarum* strains.

Another secondary compound involved in the bouquet of wine is acetoin, which is present in amounts ranging from 2 to 32 mg/l. In contrast to *S. cerevisiae* wine strains, non-*Saccharomyces* species are high producers of acetoin (Romano et al. 1993, 2003b; Romano and Suzzi 1996), and only *T. delbrueckii* exhibits a low production of acetoin (Ciani 1997). A biometric study on acetoin production in *S. cerevisiae* (Romano et al. 1993) showed that low acetoin production is the dominant pattern of this species. *Hanseniaspora*/*Koeckera* species produce acetoin at concentrations higher than its threshold value (150 mg/l) (Ciani and Maccarelli 1998; Romano et al. 1998; Comi et al. 2001; Granchi et al. 2002). It is ascertained that apiculate yeasts produce high amounts of acetoin that, by means of acetoin reductase, is reduced by *Saccharomyces* wine yeasts to 2,3-butanediol, the second most abundant constituent of wine. High and low production levels of acetoin and 2,3-butenediol are exhibited constantly by each wine species with an inverse pattern (Romano et al. 2003c). Thus, strains of *H. uvarum*/*K. apiculata* always produce low amounts of 2,3-butanediol (from about 50 to 220 mg/l) (Romano et al. 1998) and high levels of acetoin, from about 50 to 250 mg/l. In particular, isolates of *K. corticis* were found as producers of traces of 2,3-butanediol (maximum amounts of about 0.1 g/l) (Granchi et al. 2002).

Phenolic substances can be very important to the taste, colour and odour of wines. Vinylphenols (4-vinylguaiacol, 4-vinylphenol) in white wines and ethylphenols (4-ethylguaiacol, 4-ethylphenol) in red wines are quantitatively the most significant volatile phenols, identified as classic components of wine aroma (Chatonnet et al. 1997). They are present at concentrations from 0 to 6,047 µg/l in wines and can

yield phenolic off-odours, often described as animal, stable, horse sweat, medical, and "elastoplast" when present above their threshold values. The phenolic off-flavours of red wines most often develop during ageing and especially in wines stored in old barrels. *Brettanomyces/Dekkera* species are reported to be involved in wine spoilage by production of these off-flavours (Egli and Henick-Kling 2001). In wine, these yeast species grow typically in low cell numbers, whereas after completion of the alcoholic and malolactic fermentation during the aging of wine in barrels, tanks and bottles, they can grow easily on traces of residual sugars. Only careful hygiene and proper sulphuring of wines and containers can prevent the development of these undesirable yeasts.

Other compounds related to yeast activity are sulphur compounds, which can make a significant contribution to wine flavour in consequence of their reactivity and extremely low threshold values (far below 0.002 ppb). The production of hydrogen sulphide by yeasts has been studied in detail, since its aroma is frequently detected during fermentation. This substance has an unpleasant aroma with a low sensory threshold (10–100 µg/l); amounts above these values cause an off-flavour similar to that of rotten eggs and high amounts of hydrogen sulphide can also lead to the formation of other undesirable volatile sulphur compounds. The production of this compound varies with the strain of *S. cerevisiae*, with some strains producing amounts exceeding 1 mg/l. Rauhut et al. (1996) tested the ability of several commercial wine yeasts to produce volatile S compounds, demonstrating that *S. cerevisiae* strains differ in their capacity to synthesise S compounds. In particular, yeasts are able to form sulphite during wine fermentation so it is very unlikely that SO_2-free wine could be produced. The formation of SO_2 by *S. cerevisiae* is a strain characteristic; in fact this species produces sulphite in the range 10–30 mg/l (Romano and Suzzi 1993). Some strains, called "SO_2-forming yeasts", produce sulphite in amounts exceeding 100 mg/l. Such sulphite, defined "biological sulphite", is of enological interest because it binds to acetaldehyde and other compounds, contributing to unacceptably high levels of SO_2 in wines. In fact, the selection programmes of wine strains consider this aspect and starter cultures are normally tested for their capability to produce SO_2. However, some of the high sulphite producing strains possess a rarely encountered character, defined "stabilising power", which affects the chemical and biochemical stability of the wine in a similar way to the addition of SO_2 to the must, but the precise mechanism has not yet been completely explained.

As a consequence of the wide and extensive reports, wine quality appears to be the direct consequence of the typical yeast microflora which develops during the fermentation. The synergistic interaction among different yeast strains and their effect on wine sensory properties remain to be fully investigated: yeast combinations of different *S. cerevisiae* strains, and possibly *S. cerevisiae* with selected non-*Saccharomyces* strains, might be used to enhance the profile to produce unique-flavour wines. With the important contribution of non-*Saccharomyces* yeasts now fully realised, interest should also be focused on this group, which contains a wide variety of yeasts that have been shown to produce a diverse array of extracellular enzymes compared with *S. cerevisiae*. Actually, there is growing demand to differentiate, among the fermentative yeast flora, autochthonous strains with typical oenological traits which could be considered representative of a particular oenological region. These strains are

better adapted to the different wine-producing regions of the world with their respective grape varietals, viticultural practices and winemaking techniques. As the importance of *S. cerevisiae* in winemaking is long established, the use of commercial strains of yeast cultures in fermentation is becoming one of the commonest practices in order to ensure a reproducible product and to reduce the risk of wine spoilage. However, this practice can determine a progressive substitution of local microflora and a consequent reduction or lack of some desirable and typical organoleptic characteristics of natural or spontaneous alcoholic fermentation.

2.6 Yeasts in Brewing

Two types of *Saccharomyces* yeasts are involved in beer fermentation: ale yeasts (also known as top-fermenting yeasts) and lager yeasts (also known as bottom-fermenting yeasts) (Hammond 1993). Traditionally ale and lager yeasts are differentiated by their ability to ferment melibiose as lager yeasts, in contrast to ale yeasts, produce the extracellular enzyme melibiase (α-galactosidase) and therefore are able to ferment melibiose (Stewart et al. 1984). Further, ale yeasts are able to grow at 37°C, while this is not the case for lager yeasts, which in contrast grow better at lower temperature than ale yeasts. Ale yeasts have, since the last century, been classified as *S. cerevisiae*, whereas lager yeasts have been known under a variety of names such as *S. carlsbergensis*, *S. uvarum* and *S. cerevisiae*. Both yeast species belong to the closely related *Saccharomyces sensu stricto* species all having relatively uniform karyotypes consisting of 16 chromosomes (Hansen and Piskur 2003). Even though closely related, the development of molecular typing techniques has revealed several genetic differences between ale and lager brewing yeasts (Jespersen et al. 2000; Tornai-Lehoczki and Dlauchy 2000) and according to recent classifications, lager yeasts are now considered to belong to *S. pastorianus* (Vaughan-Martini and Martini 1998) even though they are often still referred to as *S. carlsbergensis* (Børsting et al. 1997). It appears to be generally accepted that lager yeasts are allopolyploid and contain parts of two divergent genomes (Kielland-Brandt et al. 1995; Casaregola et al. 2001), one from *S. cerevisiae* and one from another *Saccharomyces* species, most likely *S. bayanus* (Tamai et al. 1998, 2000; Kodama et al. 2001) or a specific strain of *S. monacensis* (Børsting et al. 1997; Joubert et al. 2000), which according to recent taxonomic keys now also belongs to *S. pastorianus* (Vaughan-Martini and Martini 1998). The hybridisation theory is supported by the fact that analysis of individual genes in many lager yeast strains reveals at least two copies of each gene, one closely related to the equivalent gene in *S. cerevisiae* and one that shows a higher degree of divergence. Also a large segment of *S. cerevisiae* DNA on chromosome XVI failed to hybridise to genomic DNA from different lager yeast strains, suggesting that this region may have diverged significantly or that it is absent in the lager yeast strains.

The brewing industry has a long tradition of the use of single starter cultures of brewing yeast based on single cell cultures. Worldwide, up to 1,000 different brewing yeast cultures have been described. The brewing yeast strains vary in their technological properties, including aroma production, rate and degree of attenuation, flocculation, oxygen requirement and reproduction (Hammond 1993; Dufour et al. 2003). As fermentation of carbohydrates in the wort leading to the formation of aroma

components and ethanol is the key event of brewing, the rate and extent of attenuation and the formation of aroma components are in focus when selecting the right strain of brewing yeast. The spectrum of carbohydrates in wort normally comprises sucrose, glucose, fructose, maltose and maltotriose together with some dextrins (D'Amore et al. 1989). However, the use of adjuncts is widespread and will significantly influence the carbohydrate composition of the wort. During brewing fermentation maltose is the most dominant carbon source. Brewing yeast strains have been shown to vary in their ability to utilise maltose and genotypic variations in their number of maltose transporter genes have been reported (Jespersen et al. 1999). The primary yeast metabolites are ethanol and CO_2, which both have an inhibitory effect on the yeast. Secondary metabolites serve mainly as flavour components. Among the most important groups of secondary metabolites are esters, fusel alcohols, aldehydes (acetaldehyde), organic acids, fatty acids as well as vicinal diketones (diacetyl and 2,3-pentanedione) and some sulphur components (hydrogen sulphide and sulphur dioxide). Different strains of brewing yeasts vary significantly in their formation of aroma compounds especially in the formation of esters (Verstrepen et al. 2003).

Flocculation is a cell-wall-mediated phenomenon characteristic of late exponential or stationary phase cells. Interactions between protein, phosphomannan and calcium seem to be involved in the cross-link of the yeast cells (Speers et al. 1992a, b; Hammond 1993). Besides being linked to the genotype of the yeast strain and the surface properties of the yeast cells, flocculation is influenced by fermentation conditions such as temperature and carbon availability. Flocculation is of technical importance for the brewing process as it permits the separation of the yeast from the beer and allows the reuse of the cropped yeast. The yeast is cropped at certain intervals at the later stages of the fermentation and will often be in a poor physiological condition owing to exposure to high levels of ethanol and various other toxic metabolites as well as physical stress. Owing to the anaerobic conditions the yeast will suffer from depletion of sterols and unsaturated fatty acids. Replacement of the yeast culture after a number of generations, often eight to ten, is considered as good practice in order to avoid contaminations and too high a proportion of dead or damaged cells.

Even though *Saccharomyces* yeasts have been shown to be able to utilise sterols and unsaturated fatty acids from the surrounding media (Ness et al. 1998), the brewing wort does not contain sufficient amounts of especially sterols and therefore the presence of free oxygen is required in order to ensure satisfactory yeast proliferation. The amount of oxygen required is dependent on several factors such as wort composition, wort gravity, pitching rate, yeast handling, the physiological condition of the pitching yeast culture including the intracellular pool of sterol esters and finally the brewing strain used. Different strains of lager brewing yeasts have, on the basis of determination of the rate of attenuation at different wort oxygenation levels, been shown to vary in their oxygen requirement (Jakobsen and Thorne 1980). The reason for the variation in oxygen requirement between different strains of brewing lager yeast appears not to be known despite the fact that the oxygen requirement is of great importance for the brewing industry. Also the ability of the yeast strain to cope with hypoxic stress conditions appears to be important during industrial fermentations (Higgins et al. 2003).

Yeasts other than *Saccharomyces* spp. may be involved in the brewing of some special brands of beer such as the Belgian Lambic and Gueuze beers. These brands of beer are mostly fermented spontaneously and include both enterobacteria, *Pediococcus* spp. and yeasts (Dufour et al. 2003; Hansen and Piskur 2003). At the later stages of fermentation yeasts such as *Dekkera* spp. (perfect form of *Brettanomyces* spp.) are involved.

Yeasts occurring as contaminants are in the context of brewing defined as "wild yeasts" and in practice they are distinguished from cultures of brewing yeasts by their ability to grow on a number of well-defined selective substrates (Jespersen and Jakobsen 1996; van der Aa Kühle and Jespersen 1998). Brewing contaminants are traditionally divided into *Saccharomyces* and non-*Saccharomyces* yeasts (Jespersen and Jakobsen 1996), of which *Saccharomyces* spp. in general are considered to be the most hazardous (van der Aa Kühle and Jespersen 1998). The majority of the *Saccharomyces* brewing contaminants detected belong to *S. cerevisiae* but other *Saccharomyces* spp. have also been reported (Jespersen et al. 2000). Infections with these yeasts typically cause phenolic off-flavours and superattenuation of the final beer. The production of phenolic off-flavours is due to the ability of these wild yeasts to decarboxylate different phenolic acids such as ferulic acids and *trans*-cinnamic acids, resulting in the formation of 4-vinylguaiacol (Coghe et al. 2004). Superattenuation is due to the production and secretion of glycoamylases with starch-debranching activity which enable the wild yeasts to use dextrins normally not fermented by the culture yeast (Röcken and Schulte 1986). Infections by *Saccharomyces* wild yeasts can be very difficult to detect owing to their physiological and biochemical similarities with the culture yeast. However different differential techniques have been developed (Jespersen et al. 1993; van der Aa Kühle and Jespersen 1998). Once isolated, the *Saccharomyces* wild yeasts can often be distinguished from lager yeasts by cell morphology and spore formation as *S. cerevisiae* normally rather easily forms spores on sporulation media, which is generally not the case for lager yeasts (Ingledew and Casey 1982). The most important non-*Saccharomyces* wild yeasts are *P. membranifaciens* and *P. anomala* (perfect name of *C. pelliculosa* and formerly known as *H. anomala*) as well as a number of species belonging to such different genera as *Brettanomyces*, *Candida*, *Debaryomyces*, *Filobasidium*, *Hanseniaspora*, *Kluyveromyces*, *Torulaspora* and *Zygosaccharomyces* (Campbell and Msongo 1991; Campbell 1996; van der Aa Kühle and Jespersen, 1998). The non-*Saccharomyces* wild yeasts cause various types of spoilage, e.g. *P. membranifaniciens* is known to produce film, haze and off-flavours such as phenolic, estery and acidic notes. According to Campbell and Msongo (1991) spoilage caused by wild yeasts belonging to the genera *Pichia*, *Hansenula* and *Debaryomyces* is commonly associated with aerobic conditions even though the yeast species to some extent are capable of anaerobic growth.

2.7 Yeasts in Other Alcoholic Beverages (Cider, Sherry Wine, Tequila)

Cider is an alcoholic beverage commonly consumed in numerous European countries, such as France, Spain, Ireland and Slovenia, where it is still produced by a natural

fermentation process involving the sequential development of indigenous yeast species. Wild microflora performing the alcoholic fermentation of apple must into cider typically originate from the fruits or from the surfaces of the process equipment. A great similarity was demonstrated between yeast populations involved in both wine and cider fermentation processes. Recently Morrissey et al. (2004) by using molecular techniques and differential media isolated, identified and tracked the yeast species involved in the overall process of a traditional Irish cider fermentation, finding as predominant yeast species *M. pulcherrima*, *P. anomala*, *B. anomalus*, *B. bruxellensis*, *D. polymorphus*, *H. uvarum*, *P. fermentas*, *P. guilliermondii*, *S'codes ludwigii* and *S. cerevisiae*. The Irish cider fermentation could be subdivided into three principal phases depending on the prevalent yeast species. *K. apiculata*/*H. uvarum* yeasts are the predominant species in the first phase, representing over 90% of the initial yeast count of 6.0×10^6 cfu/ml. As the alcohol level rises to above 4%, the numbers of *Hanseniaspora*/*Kloeckera* yeasts decrease and *Saccharomyces* yeasts begin to dominate, reaching at the fermentation peak (on day 5) 8.3×10^8 cfu/ml. Then a marked decrease occurs in the population, which drops to 5.0×10^6 cfu/ml by day 18.

Naumov et al. (2001) analysed genetic and molecular data of 21 strains, isolated from cider juice produced in France, and found that 18 out of the 21 cider strains belong to the variety *uvarum* of the species *S. bayanus*. The specific ecologic niche of *S. bayanus* var. *uvarum* in winemaking is at low temperatures and also cider production is controlled at low temperatures. It is relevant from a technological point of view that the production of pectinolytic enzymes has been reported as a specific character of *S. bayanus* var. *uvarum*.

The last phase of Irish cider fermentation, the so-called maturation phase, is dominated by *Dekkera*/*Brettanomyces* species, which begin to be detected after day 12, with their overall numbers increasing from 11% of the total yeast population to over 90% of the population by day 22. At the 25th day the fermented cider is racked and submitted to the maturation phase for up to 18 months. During this phase *Brettanomyces*/*Dekkera* are the only yeast species detected. The presence of these species has also been reported in French cider (Le Quere and Drilleau 1996). Owing to their prevalence, these species likely represent the principal contributors to the overall organoleptic properties of this alcoholic beverage. Regarding the sources of these species in these traditional cider-making process, for *Saccharomyces* species the main sources are the apples themselves, with high numbers (2×10^4–5×10^6 cfu/ml), and the process utensils, which have substantial yeast populations even some months after the last pressing. *Hanseniaspora*/*Kloeckera* species are the prevalent yeasts of the fresh must, so it is possible to conclude that the apple might be the principal source for this species, while *Brettanomyces* yeasts could be traced back to the press house and also to the fruit.

The production of sherry wine starts with an alcoholic fermentation of must by yeasts to produce white wine, followed by long ageing (5–12 years) in oak casks. Sherries comprise three different types of white wines, *fino*, *amontillado* and *oloroso*, depending on the different ageing procedures, giving wines with aroma compounds. *Fino* wines result from biological ageing, using the "solera system", under a velum produced by the so-called flor yeasts growing on the wine surface when the ethanol

content is lower than 15% v/v. The aerobic metabolism developed by these yeasts causes changes in the aroma fraction that endows the wine with its typical flavour. In addition, these yeasts protect against browning, allowing the wine to retain its pale colour for years. *Oloroso* wines are obtained by oxidative ageing, after the addition of ethanol up to a content of about 18% v/v, which prevents the growth of flor yeasts. Under these conditions, *oloroso* wine develops a dark colour as a result of the oxidation of phenolic compounds. *Amontillado* wines are obtained by ageing in a two-step process involving biological ageing under similar conditions to those of *fino* wines, followed by an increase in the ethanol content; after that they are subject to oxidative ageing, as for *oloroso* wine. *Amontillado* wines are thus the oldest and the most valued of the three types, in consequence of the development of a more complex flavour than the other two. Yeasts involved in velum formation during ageing have been included into four races formerly described as *S. beticus*, *S. cheresiensis*, *S. montuliensis* and *S. rouxii*. In a recent revision of the *Saccharomyces* genus (Esteve-Zarzoso et al. 2004) the first three yeasts were included in the species *S. cerevisiae*, whereas *S. rouxii* is now included in *Z. rouxii* species. The yeast ecology in sherry wine is affected by overproduction of acetaldehyde and the rate of film formation. A correlation between high acetaldehyde production and specific *S. cerevisiae* strains was found, such as a relationship between the number of days required to form a yeast film on the wine surface and the ability to dominate the flor yeast population. Although molecular techniques has been applied to study film-forming yeasts and for race characterisation (Martinez et al. 1995), the differentiation of four races of flor yeasts is still based on their ability to ferment different sugars. Analysis of the mtDNA restriction pattern (Martinez et al. 1995, Ibeas and Jimenez 1997) of flor yeasts yielded a high genetic variability, whereas the analysis of chromosomal profiles showed less polymorphism (Esteve-Zarzoso et al. 2004). Since ethanol is a powerful inducer of respiration-deficient mutant, the polymorphism found in mtDNA has been attributed to the mutagenic effects of ethanol upon the mitochondrial genome, followed by the selection of those mtDNA sequences which make the mitochondria metabolically active under these conditions.

Yeasts also play an important role in the production of alcoholic beverages typical of Mexico (tequila, mezcal, sotol, bacanora and raicilla), which are obtained from different agave juices. The first part of this process is the transformation by yeast fermentation of agave must in an alcoholic aromatic product, which then is distilled yielding each typical agave beverage. As regards tequila fermentation, a wide variety of yeasts are present at the beginning of the fermentation (Lachance 1995), such as in wine production. Among the fermenting yeasts, the most frequent isolates belong to the species *S. cerevisiae*, *K. africana*, *C. magnolia* and *C. krusei*.

A recent study (Fiore et al. 2005), conducted on *Saccharomyces* and non-*Saccharomyces* yeasts isolated from grape and agave musts, has revealed a correlation between strain technological aptitude and origin, explained as a specific adaptation to fermentation conditions, which probably determine different physiological and enological properties. Thus, the significant differences in β-glucosidase and β-xylosidase activities between *S. cerevisiae* agave and grape strains could indicate a certain specialisation to metabolise different cellulosic materials from grape juice and agave plant.

2.8 Yeasts in Indigenous Foods, Beverages and Cash Crops

Indigenous fermented foods and beverages play a major role in the diet of many people, especially in Africa, Asia and South America. The fermentations are predominantly conducted spontaneously without the use of starter cultures. For some products back-slopping may be used. The food processing normally takes place at household level or on a small industrial scale and the products are consequently often of varying quality and stability. Cereals, legumes and tuber roots are the major raw materials used for indigenous fermentation but milk is also fermented, e.g. in East Africa. Important cash crops such as cocoa and coffee are also produced by spontaneous fermentation. Especially in developing countries where the lack of appropriate storage facilities is a major problem, fermentation is a very effective way of food processing. Also the socioeconomic and cultural effects of the production of traditionally fermented foods and beverages from local crops should not be neglected. An overview of indigenous fermented foods from all over the world has been published previously (Steinkraus 1996).

A wide range of yeast species are involved in the fermentation of indigenous foods and beverages and as these products to a great extent are made by spontaneous fermentation consequently several different yeast species will be present especially at the initial phases of the fermentation. Also at strain level a pronounced biodiversity is found in these products. The yeast population found will primarily depend on the raw materials and processing conditions and also the occurrence of other microorganisms may influence the composition of the yeast population. *S. cerevisiae* is apparently the commonest yeast in indigenous fermented foods and beverages, where it has been shown to be very important especially in the fermentation of cereals and alcoholic beverages (Jespersen 2003). Even though it has not been described in detail, it may further play a role during fermentation of cocoa (Jespersen et al. 2005).

Examples on alcoholic beverages where *S. cerevisiae* plays a dominant role are *pito*, *dolo*, *burukutu* and *otika*, all different names for indigenous fermented beers made from guinea corn (*Sorghum vulgare*). The beers have a fairly thick consistency owing to a large amount of solids (5–7%) and the alcohol content is rather low (1–3% v/v). The beers are consumed in an actively fermenting state and therefore have quite a short shelf life. Owing to their low alcohol content and the large quantity of suspended solids, many consumers consider these indigenous fermented sorghum beers as much as a food as a beverage. For samples of dried yeasts harvested from previous brews and reused as inocula in the next beer fermentation, 99% of the isolates could be identified as *S. cerevisiae* (van der Aa Kühle et al. 2001). The dominance of *S. cerevisiae* in the fermentation of *pito* and other indigenous fermented sorghum beers seems to be a general observation even though the composition of the yeast population responsible for the fermentation may vary depending on regional area and local deviations in the production.

S. cerevisiae also plays a leading role in the fermentation of maize dough that forms the basis for a variety of different foods in Africa and South America where they in certain areas contribute to a large proportion of the daily food intake. From investigations on fermented maize dough used for production of *kenkey* in Ghana, West Africa, it is known that the yeast population on the raw maize, during steeping

and early phases of fermentation, consists of a mixed flora comprising *Candida* spp., *Saccharomyces* spp., *Trichosporon* spp., *Kluyveromyces* spp. and *Debaryomyces* spp. However, after 24–48 h of fermentation *S. cerevisiae* dominates with counts exceeding 10^6 cfu/g and after 72 h of fermentation *C. krusei* is the dominant yeast species (Jespersen et al. 1994). The microbial succession leading to the dominance of *C. krusei* at the advanced stage of fermentation is likely to be due to an increased tolerance of this yeast species against high levels of organic acids present at the later stages of fermentation (Halm et al. 2004).

A tremendous biodiversity at both intraspecies and interspecies level is observed during fermentation of cocoa and coffee. For cocoa production the seeds and pulp from the fruit pods of the tree *Theobroma cacao* Linné are fermented. The first step in cocoa fermentation is the spontaneous fermentation of the cocoa beans including the surrounding pulp. The cocoa beans are either fermented in heaps, boxes, baskets or trays. The methods of fermentation vary considerably from country to country and even adjacent farms may differ in their processing practices, which might influence the composition of the yeast population (Jespersen et al. 2005). During fermentation, microbial activity leads to the formation of a range of metabolic end-products such as alcohols, acetic acid and other organic acids, which diffuse into the beans and cause their death. This induces biochemical transformations within the beans that lead to formation of precursors of the characteristic aroma, flavour and colour, which are further developed during drying and are finally obtained during roasting and further processing (Schwan and Wheals 2004). A recent study (Jespersen et al. 2005) has shown that *C. krusei* is the dominant species during heap fermentation followed by *P. membranifaciens*, *P. kluyveri*, *H. guilliermondii* and *Trichosporon asahii*, whereas *S. cerevisiae* and *P. membranifaciens* are the dominant species during tray fermentation, followed by low numbers of *C. krusei*, *P. kluyveri*, *H. guilliermondii* and some yeast species of minor importance. Isolates of *C. krusei*, *P. membranifaciens*, *H. guilliermondii*, *T. asahii* and *Rh. glutinis* could be found on the surface of the cocoa pods and in some cases on the production equipment, whereas the origin of *S. cerevisiae* isolates was not indicated by the results obtained. For the predominant yeast species determination of chromosome length polymorphism (CLP) by PFGE showed a pronounced biodiversity involving several different strains within each species. During fermentation of coffee *P. kluyveri*, *P. anomala* and *H. uvarum* have been shown to be the dominant yeast species during “wet” processing, which is the method used for removal of the pulp, mucilage, parchment and silver skin covering *arabica* coffee beans (Masoud et al. 2004).

As mentioned previously, biodiversity at the strain level is often observed in spontaneously fermented products. Especially the biodiversity of *S. cerevisiae* strains has been investigated intensively. For strains isolated from fermented maize dough several different chromosome profiles can be observed as well as differences in their assimilation profiles (Hayford and Jespersen 1999; Naumova et al. 2003). The diversity has further been confirmed by PCR amplification using primers against the 5′ termini of the delta elements flanking the Ty1 retrotransposon. Strains that cannot be separated by the PFGE methods can sometime be separated by the PCR method and vice versa (Hayford and Jespersen 1999). For strains from fermented sorghum beer produced in the northern part of Ghana and Burkina Faso the majority of the isolated

S. cerevisiae strains (52.5%) were only able to assimilate glucose and maltose (van der Aa Kühle et al. 2001) and this is thereby not consistent with the accepted description of the species *S. cerevisiae* by Vaughan-Martini and Martini (1998) but is in accordance with the description given by Barnett et al. (2000). By sequence analysis of the D1/D2 domain of the large subunit (26S) ribosomal DNA a deviation from the type strain of *S. cerevisiae* (CBS 1171) of three nucleotides equivalent to 0.5% of the DNA was found, which according to Kurtzman and Robnett (1998) is typical of conspecific strains. Also several physiological variants of *S. cerevisiae* have been found to be involved in the fermentation of palm wine (Owuama and Saunders 1990), during fermentation of *cachaca*, an alcoholic beverage produced from sugar-cane juice in Brazil (Guerra et al. 2001) and during fermentation of *aguardente*, another alcoholic beverage produced from sugar-cane juice in Brazil (Pataro et al. 2000). Also differences in multiple locus genes such as the *MAL* genes have been found between strains of *S. cerevisiae* isolated from cereal-based indigenous fermented foods and strains of *S. cerevisiae* used for industrial applications (Hayford and Jespersen 1999; van der Aa Kühle et al. 2001). In general, a low number of *MAL* genes are seen for the strains isolated from the indigenous products compared with the industrial strains, which can imply that the selection pressure for maltose utilisation has not been as pronounced for these strains as for industrial strains of *S. cerevisiae*. Also other differences are seen, including the fact that *MAL41* has never been observed for *S. cerevisiae* strains isolated from indigenous fermented foods and beverages, whereas *MAL41* is nearly always present in industrial strains of *S. cerevisiae* and *S. pastorianus* (Jespersen et al. 1999). Also for a significant number of isolates from indigenous fermented foods and beverages a yet undescribed *MAL* locus can be observed (Hayford and Jespersen 1999; van der Aa Kühle et al. 2001).

The functions yeasts might have in indigenous fermented foods, beverages and cash crops are several even though the topic has not been investigated in detail for many of these products. In general, yeasts are involved in the fermentation of carbohydrates and in the production of aroma compounds. However, depending on the raw materials and processing, yeasts might further be involved in stimulation of lactic acid bacteria, inhibition of mycotoxin-producing moulds, improvement of the nutritional value, degradation of cyanogenic glucosides, production of tissue-degrading enzymes and some strains might additionally have probiotic properties (Jespersen 2003).

The most intensively studied function of yeasts in the fermentation of foods and beverages is the conversion of carbohydrates into alcohols and other aroma components such as esters, organic acids and carbonyls. For spontaneously fermented maize dough a total of 76 aroma compounds have been identified by gas chromatography–mass spectrometry. The compounds included 21 carbonyls, 19 alcohols, 17 esters, 12 acids, a furan, five phenolic compounds, an alkene and one unidentified compound. In general, alcohols and esters are produced in much higher amounts than other aroma compounds when yeasts are present in high concentrations (Annan et al. 2003).

In indigenous fermentations yeasts often coexist with other microorganisms. Depending on the type of product a microbial succession involving both yeasts and other microorganisms will normally take place. Yeasts have been reported to stimulate the growth of other microorganisms, including lactic acid bacteria by providing essential metabolites such as pyruvate, amino acids and vitamins, and *S. cerevisiae*

has further been reported to utilise bacterial metabolites as carbon sources (Gadaga et al. 2001; Leroi and Pidoux 1993). However, the topic appears to be poorly investigated and the mechanisms appear not to have been described in detail. *S. cerevisiae* together with *C. krusei* have been reported to have an inhibitory effect on the growth of mycotoxin-producing moulds such as *Penicillium citrinum*, *Aspergillus flavus* and *Aspergillus parasiticus*. The inhibitory effects of the yeasts were mainly shown to be due to substrate competition but also inhibition of spore germination might occur owing to the production of high concentrations of organic acids (Halm and Olsen 1996). *S. cerevisiae* as well as several other yeast species have been reported to have pectinase activity that could be of importance for the substrate availability of other microorganisms and subsequent microbial degradation of complex molecules. The presence of pectinase activity in yeasts is especially of importance in the fermentation of coffee where yeasts such as *P. kluyveri* and *P. anomala* have been shown to be able to degrade the pectin in the mucilage layer surrounding the coffee bean (Masoud et al. 2004).

Especially for indigenous fermented products produced in developing areas, bioavailability of vitamins and other nutrients is very important. Even though it has been poorly investigated, yeasts might influence the nutritional value of the fermented products. For products fermented with *S. cerevisiae* and *Lb. plantarum* the crude protein content and the contents of riboflavin, thiamine, niacin and ascorbic acids were found to increase. The contents of some amino acids were improved, while those of others were reduced. The total contents of polyphenols, tannins and phytate were reduced by the fermentation. Also, increased physiochemical properties such as improved starch stability and improved gelatinisation were obtained by the fermentation (Onilude et al. 1999). The last mentioned properties are important in the production of adult foods but might be a drawback in the production of weaning foods. In clinical trials *Saccharomyces* yeasts have been reported to be effective in the treatment of acute infantile gastroenteritis and diarrhoea following treatment with antibiotics, and have been shown to inhibit infections with *C. albicans*, *Salmonella typhimurium* and *Shigella flexneri* as well as *Clostridium difficile* (Berg et al. 1993; McFarland et al. 1994; Ouwehand and Salminen 1998). Also, *Saccharomyces* yeasts have been shown to protect against cholera toxin probably by adhesion of the toxin to receptors on the yeast surface (Brandâo et al. 1998). Further, *Saccharomyces* yeasts have been shown to modulate the host immune response by stimulating sIgA production and the phagocytic system in mice and mammalian cells (Rodrigues et al. 2000) and strains of *S. cerevisiae* isolated from West African fermented maize dough have been shown to lower the expression of proinflammatory cytokines upon exposure to pathogenic bacteria such as toxin-producing *Escherichia coli* (van der Aa Kühle et al. 2005).

2.9 Collections of Food Yeast Cultures

The results of numerous research groups around the world and in the last few years have yielded the isolation, identification and characterisation of yeasts involved in food processing. These strains are often deposited in culture collections of universities or research centres and these collections exist on a variety of scales and with a variety of purposes, but are especially addressed to provide materials and services to scientists

Table 2.2 Principal food yeast collections of the world

Country	Yeast collections Code	Website
Armenia	RCDM	http://wdcm.nig.ac.jp/catalogue/rcdm/rcdm.html
Belgium	MUCL	http://www.belspo.be/bccm
Brazil	CCT	http://www.cct.org.br/
Canada	LCC	
China	CGMCC	http://www1.im.ac.cn/typecc/junzhong/en.html
	YM	http://www.ynst.net.cn/kyjg/kydw/dw15/index.html
Hungary	NCAIM	http://ncaim.kee.hu
Italy	DBVPG	http://www.agr.unipg.it/dbvpg/
Japan	IFO	http://www.ifo.or.jp/index_e.html
	JCM	http://www.jcm.riken.jp/JCM/catalogue.html
Netherlands	CBS	http://www.cbs.knaw.nl/index.htm
Portugal	PYCC	http://www.igc.gulbenkian.pt/
Russian Federation	VKM	http://www.vkm.ru/
Slovak Republic	CCY	http://www.chem.sk/activities/yeast/ccy/
Slovenia	ZIM	http://www.bf.uni-lj.si/zt/bioteh/chair/CIM.htm
Spain	CECT	http://www.uv.es/cect/
Taiwan	BCRC	http://www.bcrc.firdi.org.tw/bcrc/indexe.htm
UK	NCYC	http://www.ifr.bbsrc.ac.uk/ncyc/
USA	ATCC	http://www.atcc.org/
	NRRL	http://wdcm.nig.ac.jp/wdcm1999/a_kurtzman.html
	Herman J. Phaff	http://www.phaffcollection.org/home.asp

Data from WFCC-MIRCEN World Data Centre for Micro-Organisms and from other sources.

and to promote research around the world. Among the numerous yeast collections, we have selected some in different countries, comprising more than 1,000 yeast strains and characterised by the electronic database online (Table 2.2).

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Chapter 3

Molecular Methods to Identify and Characterize Yeasts in Foods and Beverages

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3.1 Introduction

Studies aimed at identifying different yeast species, as well as the strains that belong to one species, have been based on morphological and physiological approaches (Kreger-van Rij 1984; Barnett et al. 1990). As an example, Table 3.1 shows the morphological and physiological characteristics of some of the main yeast species associated with foods and beverages as well as the morphological appearance of the cells of some of them (Fig. 3.1). These characteristics can vary according to growing conditions (Scheda and Yarrow 1966, 1968; Yamamoto et al. 1991) and sometimes the species are defined by a unique physiological characteristic that is controlled by a single gene. Therefore, depending on the physiological state of the yeast, as happens with the fermentation of galactose, which has traditionally enabled oenologists to differentiate the species *S. cerevisiae* and *S. bayanus* (Price et al. 1978; Kurtzman and Phaff 1987). More recently, methods have been developed to differentiate yeasts based on the analysis of total proteins in the cell (Van Vuuren and Van der Meer 1987; Vacanneyt et al. 1991), isoenzymic patterns (Duarte et al. 1999) and fatty acid analysis using gas chromatography (Cottrell et al. 1986; Tredoux et al. 1987; Moreira da Silva et al. 1994). However, the reproducibility of these techniques is somewhat questionable, as in many cases they depend of the physiological state of the yeasts (Golden et al. 1994). By contrast, techniques using molecular biology are seen as an alternative to traditional methods since they analyse the genome independently of the physiological state of the cell. Many techniques have been developed using the tools offered by molecular biology and many of them are useful to identify and characterize yeasts at a molecular level. We will go on to talk about those techniques that have had preferential use in the field of the food yeasts.

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Table 3.1 Morphological and physiological characteristics of some of the main yeast species associated with foods and beverages (according to Kurtzman and Fell 1998)

Morphology	Assimilation	Gal	Glc	Lac	Mal	Raph	Suc	Thre	Fermentation						
									Gal	Glc	Lac	Mal	Raph	Suc	Thre
Candida stellata	Globose to ovoidal	–	+	–	–	+	+	–	–	+	–	–	+/l	+	–
Debaryomyces hansenii	Spheroidal to short ovoidal	+	+	v	+	+	+	+	w/– –	w/– –	–	w/– –	w/–	w/–	w/–
Dekkera bruxellensis	Ellipsoidal, oval and elongated	v	+	–	v	v	v	v	v	+	–	v	–	v	v
Hanseniaspora uvarum	Apiculate, lemon	–	+	–	–	–	–	–	–	+	–	–	–	–	–
Metschnikowia pulcherrima	Globose to ellipsoidal	+	+	–	+	–	+	+	w/– –	+	–	–	–	–	–
Saccharomyces cerevisiae	Globose, ovoidal	v	+	–	+	+	+	+	v	+	–	v	+	+	–
Saccharomycodes ludwigii	Lemon, sausage	–	+	–	–	+	+	–	–	+	–	–	+	+	–
Yarrowia lypolitica	Spheroidal, ellipsoidal to elongated	v	+	–	–	–	–	–	–	–	–	–	–	–	–
Zygosaccharomyces bailii	Spheroidal to ellipsoid	v	+	–	–	–	v	+/w	–	+	–	–	–	v	w/–

+ positive, – negative, v variable, w/– weak or negative, +/w positive or weak, +/l positive or latent.

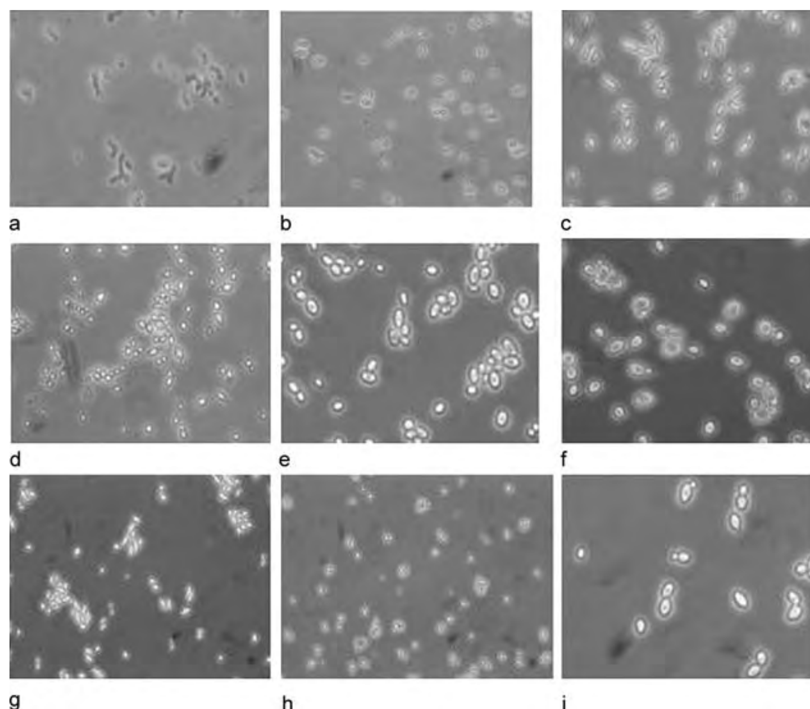

Fig. 3.1. Morphology of the main yeast species in foods and beverages: (**a**) *Candida stellata*, (**b**) *Debaryomyces hansenii*, (**c**) *Dekkera bruxellensis*, (**d**) *Hanseniaspora uvarum*, (**e**) *Metschnikowia pulcherrima*, (**f**) *Saccharomyces cerevisiae*, (**g**) *Saccharomycodes ludwigii*, (**h**) *Yarrowia lypolitica* and (**i**) *Zygosaccharomyces bailii*

3.2 Methods for Species Identification

3.2.1 Methods Based on the Analysis of Ribosomal Regions

The ribosomal genes (5.8S, 18S and 26S) are grouped in tandem forming transcription units that are repeated in the genome between 100 and 200 times (Fig. 3.2). In each transcription unit two other regions exist, the internal transcribed spacers (ITS) and the external ones (ETS), regions that are transcribed but are not processed. In turn, the codifying units are separated by the intergenic spacers, also called NTS. The gene 5S is not included in the previously described transcription unit but is found adjacent in the same repetition unit in tandem in the case of yeasts. The ribosomal genes 5.8S, 18S and 26S, as well as the ITS and NTS, represent powerful tools to establish the phylogenetic relationships and to identify species (Kurtzman and Robnett 1998), owing to the conserved sequences to be found there, as well as their concerted evolution, i.e. the similarity between repeated transcription units is greater within species than between units belonging to different species, owing to mechanisms like the unequal crossing over or genetic conversion (Li 1997).

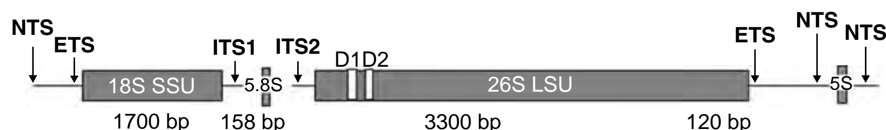

Fig. 3.2. Structure of nuclear ribosomal DNA

Different methods have been developed to identify yeast species using the information contained in these regions, as we will describe in the following sections.

3.2.1.1 Sequencing Ribosomal Regions

One of these methods is based on the determination and comparison of the nucleotide sequences in these regions. The two most commonly used regions are those corresponding to the domains D1 and D2 located at the 5′ end of gene 26S (Kurtzman and Robnett 1998) and the gene 18S (James et al. 1997). The availability of these sequences in databases, especially in the case of the D1/D2 region of gene 26S, makes this technique very useful to assign an unknown yeast to a specific species when the percentage of homology of its sequences is over or similar to 99% (Kurtzman and Robnett 1998). The database comparison is carried out using the program WU-BLAST2 at the internet address http://www.ebi.ac.uk/Blas2/index.html.

Moreover, the development of the DNA PCR, which enables direct sequencing of the regions of interest, together with modern technologies of automatic sequencing make this technology relatively quick to use. In this process, which is outlined in Fig. 3.3, the domain under consideration is amplified by PCR starting off with total DNA. The PCR products are purified using commercial kits to eliminate the primers and the excess of deoxynucleotides that would interfere in the sequencing reaction. In the automatic sequence systems, four fluorescent dyes are used to identify each of the bases (A, G, C and T). The dyes are incorporated by means of PCR amplification using the same primers. The DNA fragments marked in this way are separated in fine capillaries in terms of their size and are simultaneously excited by a laser, producing an emission that is different for each of the dyes. The signals generated are later transformed by software into peaks of colour, each of which corresponds to a nucleotide. The separation is quick and allows approximately 600 nucleotides to be read in 2 or 3 h, according to the sequencer model. Recent applications of the technique are shown in Table 3.2.

3.2.1.2 Restriction Analysis of Ribosomal Regions

With an industrial application in mind, other simpler identification methods were developed in parallel, based on PCR amplification of these regions of the ribosomal DNA and later restriction of the amplified fragment. The basis of the PCR technique is detailed in Sect. 3.3.3. Although it is usual to use DNA as a template in the amplification reaction, in a number of studies a small quantity of an isolated colony

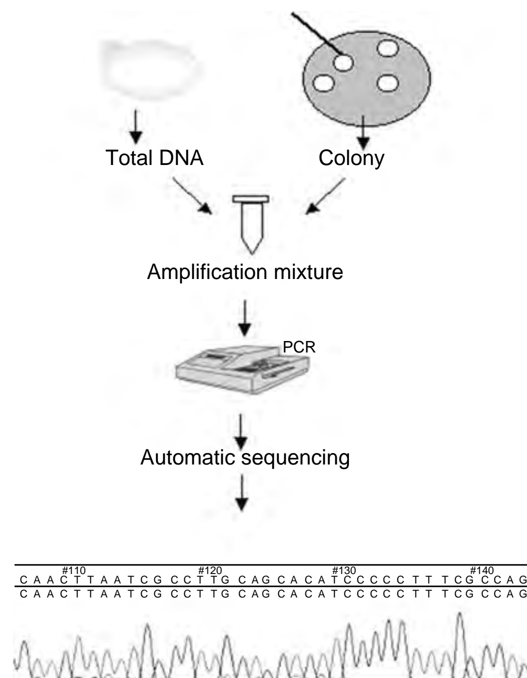

Fig. 3.3. Method for species identification based on PCR amplification and subsequent sequencing of ribosomal regions

has been used as the template. This approach represents a great saving in time and only needs a previous 15-min step at 95°C in the amplification protocol in order to liberate the DNA into the reaction mixture. The amplification products are visualized in agarose gels at 1.4%. The differently sized amplification products correspond to different species; however, when the amplified fragments are the same size they do not always correspond to the same species and it is necessary to resort to the digestion of these fragments to be able to identify them definitively. The digestion of the PCR products is carried out directly without needing a previous purification step and the generated fragments are separated by electrophoresis in agarose gels at 3% and their size is determined by comparison with appropriate markers. This technique, which is schematized in Fig. 3.4, is characterized by its easy execution and its reproducibility. Dlauchy et al. (1999) used this methodology to amplify the ribosomal gene 18S and the intergenic region ITS1 of 128 species mainly associated with foods, wine, beer and soft drinks using the primers ns1 (5′-GTA GTC ATA TGC TTG TCT C-3′) and its2 (5′-GCT GCG TTC TTC ATC GAT GC-3′) and digesting enzymes *Alu*I, *Hae*III, *Msp*I and *Rsa*I. Later, this methodology was used by Redzepovic et al. (2002). Another ribosomal region that is very useful to differentiate at species level is the one that includes the gene 5.8S and the adjacent intergenic regions ITS1 and ITS2, amplified using the primers its1 (5′-TCC GTA GGT GAA

Table 3.2 Molecular techniques most frequently used for identification of yeast species in foods and beverages and their application in the last 5 years

Technique	Target species	Matrix (food or beverage)	References (from 2000 to 2005)
Sequencing	*S. cerevisiae*	Sorghum beer	van der Aa Kuhle et al. (2001)
	S. cerevisiae/*S. uvarum*/non-*Saccharomyces*[a]	Orange juice	Arias et al. (2002)
	S. cerevisiae/*C. humilis*	Sourdoughs	Foschino et al. (2004)
	Non-*Saccharomyces*[b]	Fermentation of coffea arabica	Masoud et al. (2004)
	S. cerevisiae/non-*Saccharomyces*[c]	Fermentation of cocoa beans	Jespersen et al. (2005)
PCR-RFLP of 5.8S ITS	*S. cerevisiae*/non-*Saccharomyces*[d]	Wine	Esteve-Zarzoso et al. (2001), Torija et al. (2001), Beltrán et al. (2002), Rodríguez et al. (2004)
		Poultry	Deak et al. (2000)
	S. cerevisiae/*S. kluyveri*/non-*Saccharomyces*[e]	Wine	Pramateftaki et al. (2000)
	Pichia anomala	Yoghurt	Caggia et al. (2001)
	S. cerevisiae	Sorghum beer	van der Aa Kuhle et al. (2001)
	S. cerevisiae/*S. uvarum*/non-*Saccharomyces*[a]	Orange juice	Arias et al. (2002)
	S. cerevisiae/*S. unisporus*/non-*Saccharomyces*[f]	Orange fruit and juice	Las Heras-Vazquez et al. (2003)
	S. cerevisiae/non-*Saccharomyces*[g]	Wine	Ganga and Martínez (2004)
	S. cerevisiae/non-*Saccharomyces*[h]	Cider	Morrissey et al. (2004)
	S. cerevisiae/*S. exiguous*/non-*Saccharomyces*[i]	Sourdough	Pulvirenti et al. (2004)
	Zygosaccharomyces spp.	Candied fruits and marzipan	Martorell et al. (2005)
PCR-DGGE	*S. cerevisiae*/non-*Saccharomyces*[j]	Cocoa fermentation	Nielsen et al. (2005)
	S. cerevisiae/non-*Saccharomyces*[k]	Coffea fermentation	Masoud et al. (2004)
	Non-*Saccharomyces*[l]	Grapes	Prakitchaiwattana et al. (2004)
	S. cerevisiae/non-*Saccharomyces*[m]	Sourdough	Meroth et al. (2003)
	Saccharomyces/non-*Saccharomyces*[n]	Wine	Mills et al. (2002)
	S. cerevisiae/non-*Saccharomyces*[o]	Wine	Cocolin et al. (2000)

Real-time PCR	*D. bruxellensis*	Wine	Phister and Mills (2003)
	Food spoilage yeasts[p]	Yoghurts, milk, cheese, fruit juice	Bleve et al. (2003)
	Food spoilage yeasts[q]	Fruit juice	Casey and Dobson (2004)
	D. bruxellensis	Wine	Delaherche et al. (2004)

[a]*Candida*, *Clavispora*, *Geotrichum*, *Hanseniaspora*, *Issatchenkia*, *Metschnikowia*, *Pichia*, *Rhodotorula*, *Saccharomycopsis* and *Torulaspora* spp.
[b]*Candida*, *Hanseniaspora*, *Issatchenkia*, *Kluyveromyces*, *Pichia* and *Torulaspora* spp.
[c]*Candida*, *Hanseniaspora*, *Pichia*, and *Trichosporon* spp.
[d]*Dekkera anomala*, *Issatchenkia terricola*, *Kloechera apiculata*, *Kluyveromyces thermotolerance* and *M. pulcherrima*; *Candida*, *Hanseniaspora*, *Schizosaccharomyces* and *Zygosaccharomyces* spp.
[e]*C. stellata*, *H. uvarum*, *M. pulcherrima* and *Torulaspora delbruckii*.
[f]*Candida*, *Clavispora*, *Hanseniaspora*, *Pichia*, *Rhodotorula* and *Trichosporon* spp.
[g]*C. krusei* and *Rhodotorula glutinis*; *Zygosaccharomyces* spp.
[h]*Brettanomyces*, *Debaryomyces*, *Hanseniaspora*, *Metschnikowia*, *Pichia* and *Saccharomycodes* spp.
[i]*I. orientalis* and *Candida* spp.
[j]*H. guilliermondi and P. membranifaciens*; *Candida* spp.
[k]*H. uvarum*, *I. orientalis*, *K. marxianus* and *T. delbrueckii*; *Candida* and *Pichia* spp.
[l]*Aureobasidium pullulans*, *Hanseniaspora* and *Metschnikowia* spp.
[m]*C. humilis*, *D. hansenii* and *S. uvarum*.
[n]*Candida*, *Hanseniaspora*, *Kluyveromyces*, *Pichia* and *Metschnikowia* spp.
[o]*C. ethanolica*, *K. apiculata* and *M. pulcherrima*.
[p]*Candida*, *Debaryomyces*, *Kluyveromyces*, *Pichia*, *Rhodotorula*, *Saccharomyces* and *Zygosaccharomyces* spp.
[q]*Z. bailii*, *Z. rouxii*, *C. krusei*, *R. glutinis* and *S. cerevisiae*.

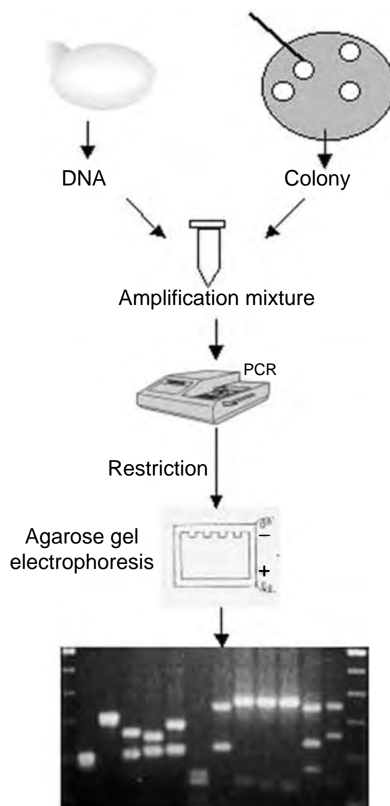

Fig. 3.4. Method for species identification based on PCR amplification and subsequent restriction analysis of ribosomal regions

CCT GCG G-3′) and its4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) as described by White et al. (1990). Guillamón et al. (1998) used this technique to identify wine yeasts quickly and later its use was extended to a total of 191 yeasts (Esteve-Zarzoso et al. 1999; Fernández-Espinar et al. 2000; de Llanos et al. 2004) related to food and drinks. The amplified fragments and restriction profiles of these species with the enzymes *Hae*III, *Hin*fI, *Cfo*I and *Dde*I are currently available online at the address http://yeast-id.com. The utility of the technique has been proved by studying reference strains (Ramos et al. 1998; Fernández-Espinar et al. 2000; Cadez et al. 2002; Esteve-Zarzoso et al. 2003; Naumova et al. 2003) and has been applied by numerous authors for species identification in different foods and beverages. Recent applications of the technique are shown in Table 3.2.

Restriction analysis of other ribosomal regions has also been used to identify yeast species, especially those belonging to the complex *Saccharomyces* "*sensu stricto*." This is the case for the ribosomal regions denominated NTS (Baleiras Couto et al. 1996; Nguyen and Gaillardin 1997; Pulvirenti et al. 2000; Nguyen et al. 2000a, b; Caruso et al. 2002; Romero et al. 2005), the gene 18S with the neighbouring region NTS

(Capece et al. 2003) or ITS (Vasdinyei and Deak 2003), the gene 18S (Tornai-Lehoczki and Dlauchy 2000) and different domains of the gene 26S (Smole-Mozina et al. 1997; Van Keulen et al. 2003). However, the fact that a database is not available means that these techniques using these regions cannot be generalized to the identification of yeasts.

3.2.2 PCR–Denaturing Gradient Gel Electrophoresis

Recently, a genetic fingerprinting technique based in PCR amplification, denaturing-gradient gel electrophoresis (DGGE), was introduced into microbial ecology (Muyzer et al. 1993).

In PCR-DGGE, which is schematized in Fig. 3.5, DNA fragments of the same length but with different sequences can be separated. Separation of DNA amplicons is based on the decreased electrophoretic mobility of a partially melted double-stranded DNA molecule in polyacrylamide gels containing a linear gradient of

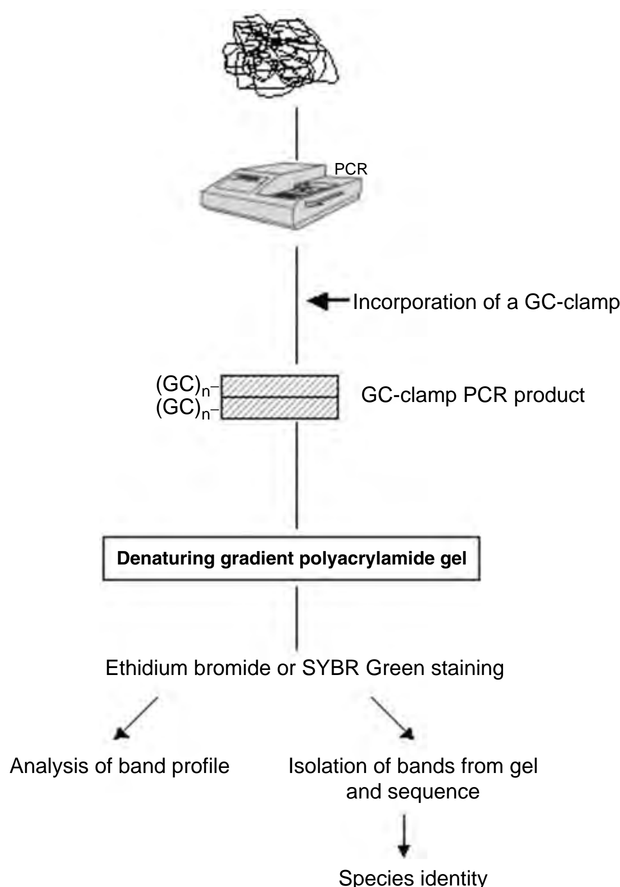

Fig. 3.5. Denaturing gradient gel electrophoresis

DNA denaturants (a mixture of urea and formamide). The mobility of the molecule is retarded at the concentration at which the DNA strands dissociate. Complete strand separation is prevented by the presence of a high melting domain which is artificially created as follows. DNA is specifically amplified by PCR using particular groups of universal primers. A sequence of guanines (G) and cytosines (C) is added to the 5′ end of one of the PCR primers, coamplified and thus introduced into the amplified DNA fragments.

A related technique is temperature-gradient gel electrophoresis (TGGE), which is based on a linear temperature gradient for the separation of DNA molecules. DNA bands in DGGE and TGGE profiles can be visualized using ethidium bromide. Recently, SYBR Green I was introduced as an alternative to ethidium bromide. PCR fragments may be isolated from the gel and used in sequencing reactions for species identification as described in Sect. 3.2.1.

The use of DGGE and TGGE in microbial ecology is still in its infancy, but their future perspectives are promising (Muyzer and Smalla 1998). Their application to yeast identification in food and beverage settings is very recent as is shown in Table 3.2.

3.2.3 Real-time PCR

The real-time PCR technique was developed in 1996 and from then on its use for different applications has increased almost exponentially (Wilhelm and Pingoud 2003). In this technique, the amplification products are observed as the PCR cycles take place. The technique is based on the detection and quantification of a fluorescent donor whose signal increases in direct proportion to the quantity of PCR product in the reaction. The process, which is schematized in Fig. 3.6, is carried out in a thermocycler that has a detection system able to capture and quantify the signal emitted by the donor at the end of each cycle for each sample. The information obtained is represented as an amplification curve that provides the cycle number for which the intensity of donor emission increases compared with the background noise. This cycle number is called the cycle threshold (Ct) and is inversely proportional to the number of copies of the sample; thus, it can be used to evaluate the initial quantity of sample numerically (DNA or cells) with great precision, within a wide range of concentrations.

The fluorescence can be obtained through binding agents or probes. As a binding agent SYBR Green is used, which binds to the double-chained DNA, increasing the

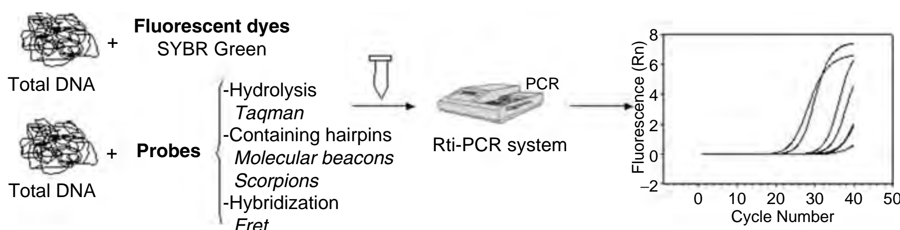

Fig. 3.6. Real-time PCR technique

fluorescence as the quantity of PCR product increases. Regarding the probes, three types can be distinguished: hydrolysis probes, loop-shaped probes and hybridization probes. The most commonly used hydrolysis probe is the so-called Taqman probe and it is characterized by having a donor photochrome binding to an acceptor photochrome. When both photochromes are bound to the probe, the donor does not emit a signal. But, when the probe binds to the sequence of interest during the PCR reaction, the exonuclease activity of the Taq polymerase activates the donor photochrome of the rest of the probe, leading to the emission of a fluorescence signal. The fluorescence signal of the donor is monitored, and it increases in the successive PCR cycles. The loop-shaped probes (Molecular Beacons, Scorpions) have inverted repeated sequences (ITR) at their 5′ and 3′ ends. This design allows a loop shape to be formed owing to the complementarity of the two ITR regions, in the absence of the target sequence. When the probe binds to the target DNA, the separation of the fluorochromes leads to efficient fluorescence. Lastly, the hybridization probes consist of two probes, donor and acceptor, binding to the region to be amplified, each one marked with a fluorophore. Resonance energy transfer only occurs when both probes bind to the target DNA, and they are very close together. All these fluorescence systems have advantages and drawbacks that determine when they are chosen. For example, if one wants a simple, economic and easy-to-use system, SYBR Green is chosen. However, during the PCR reaction this can bind to primer dimers and other nonspecific products, leading to an overestimation of the target DNA concentration. If greater specificity is required, one must resort to the system with probes.

Real-time PCR has numerous advantages compared with other identification techniques. It is necessary to stress its high specificity and sensitivity, its ability to quantify and the fact that analysis after PCR is not necessary (electrophoresis). The latter, together with the use of reduced reaction times and cycles, makes it very fast, which is very useful in the event of routine analysis and especially in applications that require correction measures. Given all the advantages of a system of this type, one must bear in mind that the design of the primers and probes is very demanding because the specificity and sensitivity of the method will depend on them. There is software that helps to design primers and probes that are suitable for the conditions of real-time PCR. The design approach usually starts off with data concerning the sequence of genes or regions whose usefulness in establishing the phylogenetic relationships among yeast species has been demonstrated and that also has the advantage of being easily found through the internet. These sequences are those corresponding to the ribosomal region D1/D2 (Kurtzman and Robnett 1998), to the mitochondrial gene COX2 (Belloch et al. 2000; Kurtzman and Robnett 2003) and to the nuclear gene of actin (Daniel and Meyer 2003). Recent applications of real-time PCR for the identification of yeasts in foods and beverages are shown in Table 3.2.

3.2.4 New Technologies

DNA microchips began to be operative between 1993 and 1995 and were consolidated around 2000–2001 as a research technique, thus representing one of the most recent tools that researchers can count on to face the demands of modern winemaking techniques. With this technique, through the hybridization of nucleic acids,

one can find out which yeast species are present. Until now, the microchip technique has not been used to detect yeasts; however, given its high specificity and sensitivity, as well as the quantity of information it provides, this technique is seen as a good alternative in the near future and therefore it is interesting to know its basis.

Microchips, manufactured by specialized companies, are small devices that contain thousands of fragments of biological material (DNA, RNA, proteins) arranged in an orderly and well-known way on a solid support (slides, glass, plastic). The sample of DNA that will come into contact with the microchip must be marked to allow its detection. The most commonly used markers are fluorescent ones, but radioactive markers or chemio-luminescence can also be used. When they come into contact with a sample, only those chains that are complementary to those on the chip bind and form a characteristic pattern of light, which can be read with a scanner and interpreted with a computer.

Nucleic acid sequence based amplification (NASBA) is a promising diagnostic tool for the analysis of viable microorganisms, since it is based on amplification of RNA rather than DNA. NASBA was first described by Compton (1991). Amplification involves the coordinated activities of three enzymes, AMV reverse transcriptase, RNase H and T7 RNA polymerase. Oligonucleotide primers, complementary to sequences in the target RNA, deoxyribonucleotide triphosphate and ribonucleotide triphosphate are incorporated in the reaction. The first primer allows the reverse transcriptase to form a complementary DNA (cDNA) strand. Then, the RNAse digests away the RNA and the second primer binds to the cDNA, allowing the reverse transcriptase to form a double-stranded cDNA copy. The cDNA is used as a template and as result RNA will be produced exponentially. The reaction is performed at a single temperature, normally 41°C. At this temperature, the genomic DNA from the target microorganism remains double-stranded and does not become a substrate for amplification. The NASBA reaction requires fewer "cycles" than conventional PCR to produce a desirable amplification, only four to five cycles are required. There are several approaches for the detection of products. The simplest one is the use of standard agarose gel electrophoresis and ethidium bromide staining. The use of molecular beacons has recently been developed to allow real-time detection of NASBA products. This procedure facilitates the establishment of strategies for quantification (e.g. similar to that used in Rti-PCR assays).

NASBA for detection of microorganisms is at around the same stage as PCR was a decade or so ago, with a few methods being published sporadically in the scientific press (Cook 2003). Hence, considerable further development is required before NASBA can be used for routine use. However, since the technique can equal the rapidity and accuracy of PCR and has additional potential advantages, NASBA is a very promising tool for detection of viable food yeasts.

3.3 Methods to Differentiate at Strain Level

3.3.1 Pulsed Field Electrophoresis of Chromosomes

In this technique, the alternating application of two transverse electrical fields means the chromosomes are forced to change their migration direction continually,

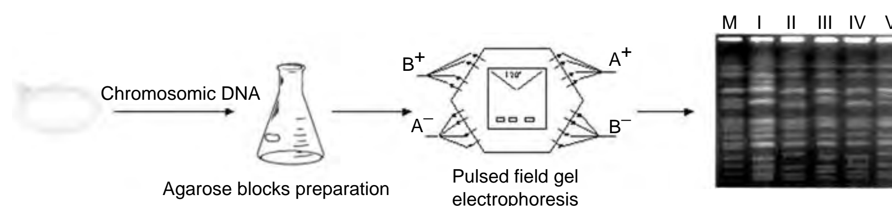

Fig. 3.7. Pulsed field electrophoresis of chromosomes technique

thus avoiding their being retained in the lattice of the agarose gel and enabling large fragments of DNA to be separated (Lai et al. 1989).

The yeasts are grown in liquid medium and then they are combined with melted agarose and placed in small moulds. The absorbed yeast cells undergo lyses in situ and then the free DNA is immobilized in the agarose matrix. The blocks are inserted in agarose gels which are subjected to electrical fields (Fig. 3.7). The parameters that condition the resolution of the bands are the variation intervals in the force of the electrical field, the agarose concentration, the temperature and the angle between the electrical fields.

Karyotype analysis is demonstrated to be a highly efficient technique to differentiate strains of *S. cerevisiae*. The polymorphism revealed by this technique is the result of the addition or elimination of long fragments of DNA in homologous chromosomes during the evolution of the yeast genome (Wolfe and Shields 1997; Casaregola et al. 1998; Keogh et al. 1998).

Numerous authors have applied karyotype analysis to the characterization of reference and commercial yeasts belonging to different species (Blondin and Vezinhet 1988; Degré et al. 1989; Vezinhet et al. 1990; Yamamoto et al. 1991; Querol et al. 1992; Fernández-Espinar et al. 2001; Petersen and Jespersen 2004; Schuller et al. 2004). These works demonstrate that karyotype analysis is an efficient technique to differentiate yeasts at strain level. Recent applications of the technique to *S. cerevisiae* and other yeast species associated with different foods and beverages are shown in Table 3.3.

3.3.2 Restriction Analysis of the mitochondrial DNA

The mitochondrial DNA (mtDNA) of *S. cerevisiae* is a small molecule of between 65 and 80 kb, whose degree of variability can be shown by restriction. The high degree of polymorphism revealed by this technique among strains of *S. cerevisiae* makes it one of the most commonly applied techniques in the characterization of this type of isolate (Table 3.3).

Several methods have been developed to isolate yeast mtDNA (Aigle et al. 1984; Gargouri 1989; Querol and Barrio 1990). However, Querol et al. (1992) have developed a method of mtDNA analysis (Fig. 3.8) that avoids using gradients in ceasium chloride and an ultracentrifuge, factors which restrict use in industry. The simplification of the technique is based on the fact that the mtDNA of yeasts is a molecule

Table 3.3 Molecular techniques most frequently used for yeast strain characterization in foods and beverages and their application in the last 5 years

Technique	Target species	Matrix (food or beverage)	References (from 2000 to 2005)
Karyotyping	*Y. lipolytica/C. zeylanoides*	Poultry	Deak et al. (2000)
	S. bayanus	Wine	Naumov et al. (2000)
	S. cerevisiae/S. cerevisiae "flor"	Wine	Esteve-Zarzoso et al. (2001)
	S. cerevisiae	Cachaca	Guerra et al. (2001)
	S. cerevisiae/S. bayanus	Cider	Naumov et al. (2001)
	S. bayanus	Wine	Naumov et al. (2002)
	S. uvarum	Wine	Demuyter et al. (2004)
	S. cerevisiae	Wine	Martínez et al. (2004)
	S. cerevisiae/non-*Saccharomyces*[a]	Grape and wine	Rodríguez et al. (2004)
	S. cerevisiae	Must	Antunovics et al. (2005)
	S. cerevisiae/non-*Saccharomyces*[b]	Fermentation of cocoa beans	Jespersen et al. (2005)
RFLP mtDNA	*S. cerevisiae*	Grape	Comi et al. (2000)
	S. cerevisiae/non-*Saccharomyces*[c]	Wine	Pramateftaki et al. (2000)
	S. cerevisiae/K. marxianus/K. lactis	Cheese	Suzzi et al. (2000)
	S. cerevisiae/S. cerevisiae "flor"	Wine	Esteve-Zarzoso et al. (2001)
	S. cerevisiae	Must and wine	Fernández-González et al. (2001)
	S. cerevisiae	Wine	Torija et al. (2001)
	S. cerevisiae	Wine	Beltrán et al. (2002)
	S. cerevisiae	Wine	Lopes et al. (2002)
	D. hansenii	Cheese	Petersen et al. (2002)
	S. cerevisiae	Wine	Granchi et al. (2003)
	S. cerevisiae	Wine	Torija et al. (2003)
	S. cerevisiae	Must	Cappello et al. (2004)
	S. cerevisiae "flor"	Wine	Esteve-Zarzoso et al. (2004)
	S. cerevisiae/C. humilis	Sourdoughs	Foschino et al. (2004)
	S. cerevisiae	Wine	Martínez et al. (2004)
	S. cerevisiae/non-*Saccharomyces*[a]	Grape and wine	Rodríguez et al. (2004)
	Z. bailii/Z. rouxii	Candied fruits and marzipan	Martorell et al. (2005)

RAPDs	*Y. lipolytica/C. zeylanoides*	Poultry	Deak et al. (2000)
	Y. lipolytica	Sausages	Gardini et al. (2001)
	S. cerevisiae	Cachaca	Guerra et al. (2001)
	Schizosaccharomyces pombe	Cachaca	Gomes et al. (2002)
	S. cerevisiae	Sourdoughs	Succi et al. (2003)
	Geotrichum candidum/D. hansenii	Dairy products	Vasdinyei and Deak (2003)
	Non-*Saccharomyces*[d]	Cheese	Fadda et al. (2004)
	S. cerevisiae/C. humilis	Sourdoughs	Foschino et al. (2004)
	Z. bailii/Z. rouxii	Candied fruits and marzipan	Martorell et al. (2005)
Micro- and minisatellites	*S. cerevisiae/Kl. apiculata*	Wine	Caruso et al. (2002)
	S. cerevisiae	Wine	Howell et al. (2004)
	Z. bailii/Z. rouxii	Candied fruits and marzipan	Martorell et al. (2005)
δ PCR	*S. cerevisiae*	Wine	Pramateftaki et al. (2000)
			Lopes et al. (2002)
			Ciani et al. (2004)
			Demuyter et al. (2004)
		Must	Cappello et al. (2004)
		Sourdough	Pulvirenti et al. (2004)

[a]*C. guillermondi*, *C. pulcherrima* and *Kl. apiculata*.
[b]*C. krusei*, *H. guilliermondii*, *P. membranefaciens* and *S. kluyveri*.
[c]*C. stellata*, *H. uvarum*, *M. pulcherrima* and *T. delbruckii*.
[d]*C. lambica*, *C. zeylanoides*, *Debaryomyces hansenii*, *G. candidum* and *K. lactis*.

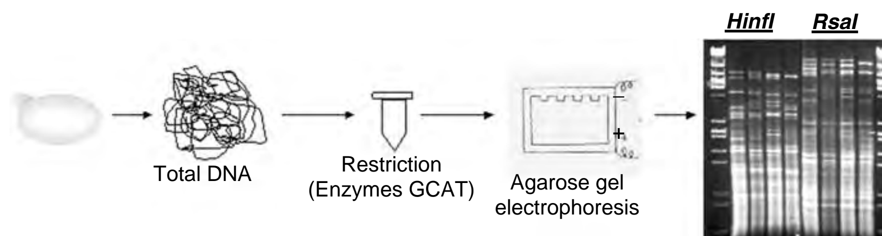

Fig. 3.8. Method based on the restriction analysis of the mitochondrial DNA

with 75% of A and T (Gray 1989) and although it is rich in AT, some 200 regions rich in GC have also been found. Therefore, digestions of total DNA with GCAT-type enzymes do not recognize the sequences rich in either GC or AT. Given the small number of restriction sites in the mtDNA and the high number of cutting sites in the nuclear DNA, the latter breaks into small fragments, which enables one to visualize the bands corresponding to the mtDNA as clearly defined bands, superimposed on the shadow of the nuclear DNA digested. Not all the enzymes reveal the same degree of polymorphism and it depends greatly on the species. In the specific case of *S. cerevisiae* the enzymes that are most suitable to differentiate at strain level are *Hin*fI and *Hae*III (Guillamón et al. 1994).

This rapid technique enables a greater number of strains to be analysed in less time, and is ideal for industry given its speed, safety and economy and because it does not require sophisticated material or very specialized employees. López et al. (2001) simplified the method: the 77 h that was needed to complete the protocol following the original method has been reduced to 25 h with the adapted method.

Numerous authors have applied restriction fragment length polymorphism (RFLP) mtDNA to the characterization of reference and commercial wine yeast strains (Vezinhet et al. 1990; Querol et al. 1992; Guillamón et al. 1996; Mesa et al. 1999; Fernández-Espinar et al. 2001; Esteve-Zarzoso et al. 2004; Schuller et al. 2004) and strains belonging to other species (Romano et al. 1996; Guillamón et al. 1997; Petersen et al. 2001) These works demonstrate that RFLP mtDNA analysis is an efficient technique to differentiate at strain level. Recent applications of the technique to strains from yeast species associated with different foods and beverages are shown in Table 3.3.

3.3.3 Methods Based on the PCR Technique

The quickest molecular techniques are those based on the PCR technique (Saiki et al. 1985, 1988) and they have been used to discriminate between strains of wine yeasts. Some variants of the basic PCR technique have been developed that can be used to detect polymorphisms of DNA fragments without the need to use restriction enzymes. The techniques most frequently used to differentiate yeasts at strain level are randomly amplified polymorphic DNA (RAPD) and microsatellites. Other techniques, such as δ-sequence amplification and amplification of “intron splice sites”, have been developed specifically to differentiate strains of the species *S. cerevisiae*.

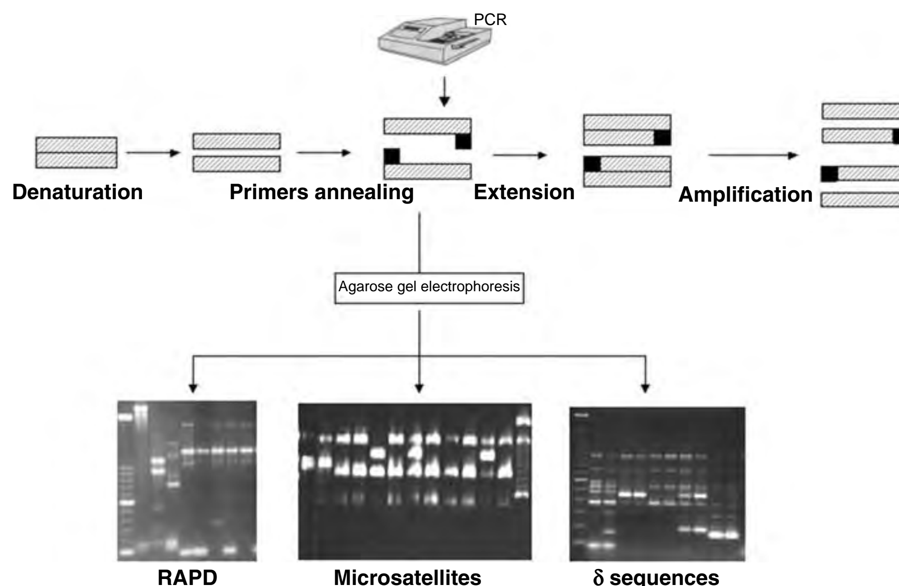

Fig. 3.9. Methods to differentiate at strain level based on PCR amplification

All these techniques use oligonucleotides as primers, which bind to target sequences in each DNA strand of the yeast. The sequence of the primers varies according to the technique, as we will see later. The amplification is carried out with a thermostable polymerase DNA and the amplification protocol always includes a variable number of cycles (generally between 25 and 45) that include denaturation of the DNA followed by hybridization and a period of extension. The result is the amplification of the DNA duplicating the quantity of target DNA in each cycle. The amplification conditions, especially the hybridization temperature, also differ. The amplification profiles are visualized in agarose gels at 1.4% with strain-specific profiles that enable us to identify and differentiate them.

We will go on to talk about each of these techniques in detail and Fig. 3.9 shows examples of the amplification profiles obtained for some of them.

3.3.3.1 Randomly Amplified Polymorphic DNA

The RAPD technique (Williams et al. 1990), is characterized by the fact it uses just one primer, which has the special characteristics of being particularly short (approximately ten nucleotides) and having an arbitrary sequence. The RAPDs-PCR reaction is also characterized by the low hybridization temperature used (37°C). Thus, the pairings between the oligonucleotide and the DNA are determined by the short and arbitrary sequence this has, and favoured by the low temperature used, setting off the amplification of diverse fragments of DNA distributed all the way along the genome. The

result is a pattern of amplified products of different molecular weight that can be characteristic of the species or of the different strains or isolates within the same species (Bruns et al. 1991; Paffetti et al. 1995).

The main advantage of the method is that one does not need previous information about the sequence to design the primer. Moreover, the technique enables one to analyse the variability along the whole genome, thus revealing more polymorphism than other techniques that analyse specific regions. However, owing to the low hybridization temperature used (37°C) the amplification profiles obtained are unstable and difficult to reproduce and it is necessary to carry out several repetitions for each sample, starting off with different DNA extractions. Only the bands present in all the repetitions will be taken into account. This fact together with the need to combine the amplification results with several oligonucleotides to obtain a good resolution power means that the technique is not apt for routine application at an industrial level. Consequently, the technique has not been used much for the characterization of strains.

The efficiency of the technique to differentiate at strain level has been demonstrated by analysing reference strains belonging to different species (Quesada and Cenis 1995; Baleiras Couto et al. 1996; Romano et al. 1996; Tornai-Lehoczki and Dlauchy 2000; Pérez et al. 2001a; Cadez et al. 2002). Recent applications of the technique to *S. cerevisiae* and other yeast species associated with different foods and beverages are shown in Table 3.3.

3.3.3.2 PCR of Repetitive Regions of the Genome (Microsatellites and Minisatellites)

There are repeated regions in the genome that represent potential targets for molecular identification at strain level, as they show great variability. These areas are the microsatellites and the minisatellites that constitute motifs of very varied length, repeated in tandem abundantly and at random along the genome. The microsatellites are usually less than 10 bp in length, while the minisatellites are between 10- and 100-bp long. The variability found in these regions can be shown by means of PCR amplification using specific oligonucleotides, such as $(GTG)_5$, $(GAG)_5$, $(GACA)_4$ or M13. The ability of these oligonucleotides to reveal polymorphism among strains of *S. cerevisiae* was demonstrated by Lieckfeldt et al. (1993) using hybridization techniques. The same authors were the first to use these sequences as primers in a PCR reaction, showing the usefulness of this technique for characterization at strain level. The technique was used by other authors later for the study of reference strains (Baleiras Couto et al. 1996; González Techera et al. 2001; Hennequin et al. 2001; Pérez et al. 2001a, b; Marinangeli et al. 2004) and recent applications are shown in Table 3.3. The amplified products obtained are approximately 700 and 3,500 bp in size; therefore, they can be visualized in agarose gels. Visualization of the amplified products obtained is usually carried out in acrylamide gels, although it can also be done in automatic sequencers. This means that the technique is not very useful for routine application, in spite of its high resolution and its high reproducibility. The resolution power of this technique is comparable to δ elements and restriction analysis of mtDNA.

3.3.3.3 Amplification of δ Sequences

δ sequences are elements measuring 0.3 kb that flank the retrotransposons Ty1 (Cameron et al. 1979). Around 100 δ copies are present in the yeast genome as part of the retrotransposons Ty1 or as isolated elements. However, these δ sequences are concentrated in genomic regions adjacent to the transfer RNA genes (Eigel and Feldmann 1982). The number and the localization of these elements demonstrate certain intraspecific variability that Ness et al. (1993) took advantage of to develop specific primers (δ_1 and δ_2) that are useful to differentiate strains of *S. cerevisiae*. These authors showed that the δ elements are stable enough for this technique to be used as an identification method of *S. cerevisiae* strains at an industrial level, as demonstrated by other authors later (Table 3.3). Some of these studies show the great variability this technique reveals between isolates of the *S. cerevisiae* species compared with other highly resolving techniques, such as restriction analysis of the mtDNA and electrophoresis of chromosomes (Pramateftaki et al. 2000; Fernández-Espinar et al. 2001).

Recently, Legras and Karst (2003) optimized the technique by designing two new primers (δ_{12} and δ_{21}) that are located very near to δ_1 and δ_2. The use of δ_{12} and δ_{21} or of δ_{12} with δ_2 reveals greater polymorphism, which is reflected by the appearance of a greater number of bands. Consequently, the new primers are able to differentiate more strains: 53 commercial strains were differentiated unequivocally (Legras and Karst 2003). Shuller et al. (2004) confirmed it later, showing that the combination of δ_2 and δ_{12} identified twice as many strains as the set of primers designed by Ness et al. (1993).

An important drawback of this technique is the influence that the concentration of DNA can have on the profile obtained, as shown by Fernández-Espinar et al. (2001) and commented on by Shuller et al. (2004). Although this problem is avoided by standardizing the concentration of DNA, the comparison of results between laboratories is complicated. Another problem of this technique is the appearance of "ghost" bands due to the low annealing temperature (42°C) used during the amplification reaction. Recently, Ciani et al. (2004) used an annealing temperature of 55°C to characterize wine strains of *S. cerevisiae*. In this way, the amplification profiles obtained are much stabler, although fewer bands are obtained.

3.3.4 Amplified Fragment Length Polymorphism

Although this technique is fundamentally based on PCR amplification, we will consider it in a different section, owing to its complex methodology, which implies the use of other methodologies, as we will see later.

Amplified fragment length polymorphism (AFLP) is a technique that involves the restriction of genomic DNA followed by the binding of adapters to the fragments obtained and their selective amplification by PCR. The adapter sequence and the restriction sites are used as the primers' target for PCR amplification. The fragments are separated in DNA sequencing gels and visualized by auto-X-ray or in automatic sequencing (Vos et al. 1995). Figure 3.10 outlines this technique.

As in the case of RAPDs, previous information about the sequence is not needed to design the primer, it is easily reproduced and it offers a great deal of information.

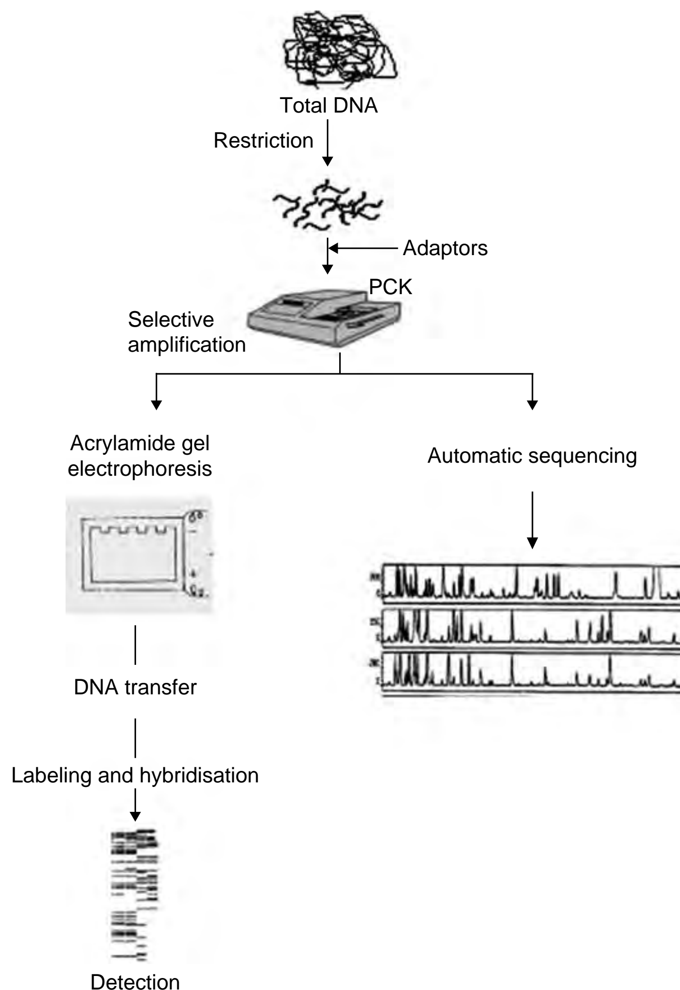

Fig. 3.10. Amplified fragment length polymorphism

AFLP is a useful technique to discriminate between yeasts at strain level, as shown by de Barros Lopes et al. (1999); however, it has the drawback of being a very laborious technique, since it requires automatic sequencers, which are very sophisticated for use in industry, and also the data are difficult to interpret. Although the technique has been very widely used to study bacteria, plants and animals, in the case of yeasts, there are few works in this respect (de Barros Lopes et al. 1999; Azumi and Goto-Yamamoto 2001; Boekhout et al. 2001; Theelen et al. 2001; Borst et al. 2003; Dassanayake and Samaranayake 2003; Trilles et al. 2003).

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Chapter 4

Yeast Ecological Interactions. Yeast–Yeast, Yeast–Bacteria, Yeast–Fungi Interactions and Yeasts as Biocontrol Agents

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4.1 Introduction

When the domains of individual microorganisms overlap, it is likely that interactions will occur (Boddy and Wimpenny 1992). The outcome of these interactions is evaluated on the basis of the effect they have on population size (Odum 1953) regardless of whether the interactions are detrimental, neutral or beneficial. The types of interaction found in mixed populations of microorganisms are classified on the basis of these effects as direct or indirect interactions (Bull and Slater 1982). Indirect interaction refers to competition, commensalism, mutualism, amensalism or antagonism and neutralism (Linton and Drozd 1982), and direct interaction to predation and parasitism (Frederickson 1977; Bull and Slater 1982). However, fermented foods and beverages develop their nutritional and organoleptic qualities as a result of the metabolic activity of a succession of different microorganisms and it is unlikely that the interactions will separate into these discrete groups since more than one type of interaction occurs simultaneously (Verachtert et al. 1990).

Present understanding of the positive, negative or neutral role of interactions between yeasts, bacteria and fungi has its origins the first time fermentation was employed. The fermentation of many products includes interaction both within and between different microbial groups (e.g. yeast–yeast, yeast–bacteria, yeast–moulds), the physiological activity of which brings about desirable changes which decisively determine the character of a product and stabilise the population in a specific ecological niche (Wood and Hodge 1985; Leroi and Pidoux 1993; Geisen et al. 1992; Rossi 1978; Challinor and Rose 1954). However, interaction does not necessarily only imply the positive or negative attributes within fermentation but it also involves the antagonistic activity of yeasts against other microorganisms by means of the production of microcins (Baquero and Moreno 1984; Golubev and Boekhout 1992), secretion of antibacterial and antifungal compounds, co-fermentation, and their role as in biological control.

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4.2 Ecological Interaction Between Microorganisms

4.2.1 The Secretion of Antifungal or Antibacterial Compounds

It is well known that certain fungi (Punja and Utkhede 2003) and members of the bacterial groups (Williams and Vickers 1986) possess the ability to synthesise and secrete secondary metabolites that exhibit antagonistic activities against other microorganisms. However, little attention has been given to yeasts as possible producers of similar substances despite positive indications already published early in the twentieth century (Hayduck 1909; Fernbach 1909).

Hayduck (1909) obtained a volatile thermolabile toxic extract from yeast which was confirmed by Fernbach (1909) to be an amine that inhibited the growth of *Escherichia coli* and staphylococci. Schiller (1924) demonstrated the presence of an inhibitory enzyme active against the staphylococci, while Bachmann and Ogait (1935) argued that the main reason for the inhibitory action of baker's yeast was due to the production of acetaldehyde. Barglowski (1938) found that *Saccharomyces cerevisiae* and *Mycotorula albicans* strains suppressed the growth of *Mycobacterium tuberculosis*, while Cook et al. (1941) prepared an antibiotic from baker's yeast which inhibited the growth of *Aspergillus niger* and *Penicillium glabrum*. Baker's yeast grown in rye decoction is also reported to exhibit strongly bactericidal activities against *Aerobacter aerogenes* owing to thermolabile enzymes (Tikka and Itkonen 1941). Owing to the development of acid, *Torulopsis utilis* showed antibiotic action against *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas pyocyaneus* (Carpentier 1945), while Sartory and Meyer (1946) obtained an inhibitor from baker's yeast active against *Escherichia coli* and *Proteus vulgaris*. Florey et al. (1949) noted that unsaturated fatty acids from baker's and brewer's yeasts, *Debaryomyces mucosus* and *T. utilis*, as well as succinic acid from *T. utilis* var. *major* possess antibacterial properties inhibiting a variety of bacterial organisms. Complete inhibition of *Penicillium glaucum* and *Salmonella typhosa* by yeasts was reported by Toda (1950), while similar inhibiting effects were noted by Motzel (1956) due to cyclic peptides. Despite the inhibition of *Bacillus subtilis* and pediococci obtained by substances produced by the yeasts *Brettanomyces bruxellensis*, *Schizosaccharomyces pombe*, *Saccharomyces cerevisiae* and *Saccharomyces carlsbergensis*, no attempts were made to isolate or identify the substances. Parfentjef (1953) isolated a fraction containing protein possessing anti-infectious properties, malucidin, from baker's and brewer's yeast. This protein protected animals against infection by a number of microorganisms, which included the yeast *Candida albicans*, pathogens like *Proteus* and *Shigella endotoxins* and many species of gram-negative and gram-positive bacteria (Parfentjev 1958).

Robinson et al. (1958) in studies on the decrease of the bacterial population in preferments, isolated two antibiotic substances designated as I1 and I2 from yeasts which possessed inhibitory properties for *Micrococcus pyogenes* and *Escherichia coli*. In a survey of the antibiotic powers of yeasts, MacWilliam (1959) examined 150 yeast strains for their antibiotic powers against bacteria and moulds. Strong inhibition against *Fusarium*, *Mucor* and *Penicillium* was achieved with the yeast strain *Candida pulcherrima* producing pulcherriminic acid, a derivative of the red pigment

pulcherrimin. Robinson et al. (1962) continued their research on the two antibiotic substances they had previously isolated from *Saccharomyces cerevisiae* which they identified as polypeptides, capable of surviving baking and showing antibacterial activity against *Staphyllococcus aureus*.

Despite ongoing arguments that the possible role of yeasts as a source for antimicrobial compounds is merely attributed to the natural effect of competition for nutrients; Fatichenti et al. (1983), in a study on the antagonistic activity of *D. hansenii* against bacteria, found that the yeast species produced extracellular and intracellular antimicrobial compounds that inhibited the growth of *Clostridium tyrobutyricum* and *Clostridium butyricum*. Antibacterial activity was also detected in *Kloeckera apiculata* and *Kluyveromyces thermotolerans*, secreting substances that inhibited the growth of beer-spoilage bacteria (Bilinski et al. 1985). The expression of antibacterial activity by these two yeasts against the gram positive bacteria *Bacillus megaterium* and *Lactobacillus plantarum* involves transformation of methylene blue into a pharmacologically active form. Antibacterial activity against *Staphyllococcus aureus* was noted by the production of extracellular glycolipids, called sophorosides, by *T. bombicola* (Cavalero and Cooper 2003). The sophorosides also proved to be active against *Candida albicans*.

Probably the most significant and well-known antagonistic action by yeasts in recent years comprises the production of killer toxins (Young 1987; Rosini and Cantini 1987; Shimizu 1993; Walker et al. 1995; Suzuki et al. 2001; Marquina et al. 2002). These toxins are extracellular proteins or glycoproteins that disrupt cell membrane function in susceptible yeasts. Although these killer toxins were originally considered species-specific, clear evidence indicated that they occur across species in different yeast genera (Palpacelli et al. 1991; Llorente et al. 1997; Suzuki et al. 2001), and they can kill various filamentous fungi (Walker et al. 1995).

4.2.2 Yeast Co-Interrelationships with Other Microorganisms

Other than the antagonism exhibited by yeasts as just described, ecological theory describes a wide variety of interactions between yeasts and other microorganisms. Yeasts are added to foods and feeds as a source of proteins and vitamins, are represented in waste-treatment facilities, and are used for industrial purposes. These processes frequently rely on a variety of microorganisms (Linton and Drozd 1982; Kuenen and Harder 1982; Frederickson 1977; Hesseltine 1965).

The use of mixed cultures resulted in a higher growth rate, better biotransformations and higher yields in products (Verachtert et al. 1990). Although it has been stressed that the main interaction between the different microorganisms relied on microbial competition for the growth-limiting substrate (Bull and Slater 1982; Alexander 1971), various additional interactions occur simultaneously (Meyer et al. 1975; Yoon et al. 1977; Bungay and Bungay 1968). The consequence of other interactions often results in the interrelationship or co-existence of different species growing on a single growth-limiting substrate (Kuenen and Harder 1982). If physiochemical intrinsic and extrinsic conditions are within specified limits and the environment contains sufficient available energy and required nutrient sources for microbial growth, microbial communities will develop (Meers 1973). Interrelationships between and

within the communities develop, and as a result the stability of the environment is altered (Nakamura and Hartman 1961) by one species to stimulate the growth of other species because of changes in pH, growth factors, oxygen depletion, etc. For example, the growth of lactic acid bacteria reduces the pH value of media to encourage yeast growth, the removal of substances (osmophilic yeasts metabolise high sugar concentrations) that would otherwise prevent the growth of a second species (Mossel and Ingram 1955) or the excretion of relevant enzymes for the breakdown of complex carbohydrates (Antuna and Martinez-Anaya 1993). Owing to the change in the abiotic environmental conditions, the nature of the interactions between the populations may also change (Megee et al. 1972).

4.2.2.1 Yeast–Bacteria Interactions

When bacterial strains grow, environmental alterations may inhibit the growth of other species owing to the removal of essential nutrients or by the production of organic and inorganic toxic compounds (Meers 1973). Bacteria, predominantly lactic acid bacteria, commonly excrete organic acids which lead to a lowering in the pH, which either inhibits the growth of undesired pathogens or promotes yeast growth. Therefore, the interrelationship between lactic acid bacteria and yeasts, as applied in many fermented foods and beverages, plays an essential role in product preservation. In these ecosystems, they may compete for the same substrates (Bull and Slater 1982; Fleet 1990) or synergistically promote the growth of each other. Moreover, the antagonistic and synergistic effects exhibited by using the microorganisms in co-culture, may also be applied in converting wastes into feeds and in industrial processes.

Yeasts (*Trichosporon cutaneum*, *Candida krusei*, *C. valida* and *Pichia membranaefaciens*) and lactic acid bacteria (*Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus buchneri* and *Lactobacillus delbrueckii*), grown in co-culture during the fermentation of animal waste and corn were responsible for an increase in the total amino acid content, total nitrogen and protein content of the final product (Hrubant 1985). Moreover, indigenous enteric bacteria, coliforms and faecal streptococci were destroyed and even selected faecal coliforms and *Mycobacterium paratuberculosis* strains added to the media died within 9 th. In addition, the yeast *Saccharomyces boulardii* may be applied as a probioticum in feeds, preventing the development of the toxigenic *Clostridium difficile* (Elmer and McFarland 1987; Castex et al. 1990; Kimmey et al. 1990) and the consequent diarrhoea, leading to an improvement in the performance of steers (Mir and Mir 1994), lactating dairy cows (Swartz et al. 1994), sheep (Jouany et al. 1998) and poultry (Bradley et al. 1994). The pharmacological protective action of yeasts against pathogenic organisms has even been applied in aquaculture. Other than serving as sources of vitamins and proteins, yeasts increase the non-specific local immunity by changing the production and activity of bacterial toxins (Isayev and Nagornaya 1992). The interaction between bacteria and yeasts in aquaculture, however, remains very vague and needs attention.

Megee et al. (1972) described the symbiosis between *Saccharomyces cerevisiae* and *Lactobacillus casei* and indicated that by varying the concentration of the substrate's different types of symbioses like commensalism + competition, competition, and

mutualism and competition were present. When no riboflavin was present in the medium, the bacteria were dependent upon the yeast for supplying the riboflavin, but competed for limited supplies of glucose in the medium when sufficient riboflavin was present. "True commensalism" was reported by Shindala et al. (1965) on the symbiosis between *Saccharomyces cerevisiae* and *Proteus vulgaris* based on an essential niacin-like factor, and between *Saccharomyces cerevisiae* and *Proteus vulgaris* based on riboflavin deficiency both elaborated by the yeasts and required by the bacterium. Challinor and Rose (1954) observed 13 interrelationships between yeasts, mainly *Saccharomyces cerevisiae*, and *Lactobacillus* spp., and in each of them the yeast appears to be the active organism, synthesising the missing substances, like vitamins, amino acids or purines, essential for the growth of *Lactobacillus*. Symbiotic growth in a chemostat between *Acetobacter suboxydans* and *Saccharomyces carlsbergensis* was reported by Chao and Reilly (1972) based on the inability of the yeast to utilise mannitol which was added as the only carbon source, but actively ferments the fructose once it has been oxidised by the bacterium. On the other hand, during alcoholic fermentation of molasses worts, increasing yeast inocula enhanced the lactobacilli growth and contributed to the consumption of monosaccharides liberated during hydrolysis of sucrose by yeasts (Ngang et al. 1992). In a similar way, when *Lactobacillus plantarum* and *Saccharomyces cerevisiae* were grown in co-culture in a glucose–citrate medium under acid conditions, *Saccharomyces* reduced the lactic acid produced by *Lactobacillus* and thereby stabilised the pH, encouraging the fermentation of citrate by the *Lactobacillus* (Kennes et al. 1991a).

4.2.2.2 Yeast–Yeast Interactions

Mixed microbial populations are intentionally applied in industry to improve flavour and yield (Verachtert et al. 1990), to lower pH to inhibit undesired species and to create stability or to obtain desired physiological properties (Harrison 1978). Yeasts are an integral part of these populations and help to secure quality by a range of mechanisms and activities. Detailed yeast–yeast interactions, however, are not studied systematically as observed with bacterial interactions. Other than the most commonly found interrelationship between yeasts, namely the competition for nutrients to survive (Nissen et al. 2004), significant contributions similar to those for bacteria based on symbiosis between yeasts comprise typical mutualism, commensalism, amensalism and predation. These interrelationships have been successfully applied in industry.

Yeast–yeast co-fermentation of glucose and xylose, as obtained after the breakdown of polymers in agricultural waste streams, with immobilised *Pichia stipitis* and *Saccharomyces cerevisiae* resulted in higher ethanol yields from the mixed substrates (Grootjen et al. 1991). The treatment of the effluent of waste starch with *Endomycopsis fibuliger* and *Candida utilis* yielded high concentrations of single-cell protein (Jarl 1969, 1971) when the former hydrolysed the starch to dextrins and low molecular weight sugars, enabling *Candida utilis* to assimilate the soluble products released. The use of mixed yeast cultures for single-cell protein production from *n*-alkanes was used to overcome vitamin requirements. By culturing the biotin-requiring yeasts *Candida novellus*, *Candida tropicalis* or *Pichia sake* with B_1-requiring yeast species, such as *Trichosporon pululans* or *Candida lipolytica*, good growth was

obtained without any added vitamins as the yeasts supply each other's vitamin requirements. Another application of yeasts (*Candida utilis* and a *Mycotorula* sp.) in co-culture, grown on sulphite waste liquor for the production of single-cell protein, contributed to high yields when *Candida utilis* enhanced the growth of the *Mycotorula* sp. The invaluable role of autolysis of yeasts, as a means of indirect interaction between yeasts, should not be overlooked, as the amino acids and vitamins released may encourage the growth of other yeasts (Fleet 2001).

Direct interaction between yeasts mainly relies on the antagonistic interaction involving yeasts capable of producing soluble killer toxins. The secreted proteinaceous killer toxins are lethal to a wide variety of susceptible yeasts and have many potential applications in environmental, medical and industrial biotechnology (Young 1987; Rosini and Cantini 1987; Suzuki et al. 2001; Marquina et al. 2002). Recently, it was observed that zygocin, a protein toxin produced and secreted by the yeast *Zygosaccharomyces bailii* effectively kills pathogenic yeasts like *Candida albicans*, *Candida krusei and Candida glabrata* (Weiler and Schmitt 2003). In the late 1990s, predacious yeasts based on haustorium-mediated predation were also observed (Lachance and Pang 1997) as another means of direct interaction between yeasts. More information on predation between yeasts other than in laboratory situations, however, is needed.

4.2.2.3 Yeast–Filamentous Fungi Interactions

The most prominent interactive relationships between yeasts and filamentous fungi definitely comprise the antagonistic application of yeasts as biocontrol agents against fungi, and the mutualistic relationship with fungi during the processing of predominantly Asian fermented foods. Both topics will be dealt with later in this chapter. Commensalism and mutualism rely on the co-culture of yeasts and filamentous fungi and the latter provide the necessary enzymes to break down complicated substrates like cellulose. A typical example is when *Candida utilis* species contribute to high single-cell protein content when grown in co-culture with the cellulotic *Aspergillus niger* on apple promace (Bhalla and Joshi 1994). The higher yield of protein from the yeast–fungi co-culture relies on the hydrolysis of lignocelluloses by the fungi releasing hexoses and pentoses which the *Candida utilis* can efficiently metabolise.

On the other hand, yeasts exudates may also stimulate hyphal growth like *Rhodotorula mucilaginosa* enhancing the growth of the arbuscular mycorrhizal fungi *Glomus mosseae* and *Gigaspora rosea* (Fracchia et al. 2003).

4.3 Yeast Interactions in Foods and Beverages

Microbial communities with their combined physiology, interactions and enzymatic activities are responsible for the major biochemical and nutritional changes that occur in the substrates of fermented foods and beverages (Steinkraus 1982; Hesseltine and Wang 1967; Wood and Hodge 1985; Wood 1981). Antimicrobial effects present in fermented foods and beverages are attributed to organic acids, antibiotic factors, volatile acids, hydrogen peroxide and to a number of substrates

excreted in the products. These antimicrobial effects are the result of the presence of several kinds of microorganisms involved in the fermentation and putrefaction of foods which inevitably lead to beneficial or detrimental interaction among the populations (Noda et al. 1980; Frederickson 1977; Bull and Slater 1982; Slater and Bull 1978).

Microbial interactions involving yeasts, bacteria and/or fungi have been indicated from a number of examinations of food products like bread (Lues et al. 1993), meat, fish, fruit, vegetables, protein foods, dairy products and cereals. The metabolic interactions are governed by the inherent technological characteristics and biochemical activities of yeasts providing essential growth metabolites, such as amino acids, vitamins, removing toxic end products of metabolism, inhibiting the growth of undesired microorganisms by lowering the pH, secreting alcohol, producing CO_2, or encouraging the growth of the starter cultures by increasing the pH owing to the utilisation of organic acids.

These properties have been applied successfully in the processing of foods and beverages as a means of biological control to enhance food safety and shelf life by destroying, retarding or preventing the growth of pathogenic and spoilage microorganisms (Ray and Daeschel 1992; Campbell-Platt 1994). The most successful application of interactions in foods and beverages comprises the presence of yeasts and lactic acid bacteria in a product. The interactions rely on several modes of action; however, despite the many references to the occurrences of yeasts in co-culture with lactic acid bacteria (Wood and Hodge 1985; Steinkraus 1982), only a few researchers have studied the interactions systematically in defined media (Gobbetti et al. 1994a, b; Kennes et al. 1991b). Except for the studies in wine and to an extent in bread making, none of the other fermented foods or beverages have been studied in detail.

Yeast–bacteria associations are by far the most prominent interactions occurring in food and beverage production determining the flavour and other qualities by a range of mechanisms and activities. While lactic acid bacteria, comprising *Pediococcus*, *Leuconostoc*, *Lactobacillus*, *Lactococcus* and *Bacillus*, are the main species associated with fermented products, other species have significant roles. Yeast–yeast associations, on the other hand, are frequently indicated in foods and beverages, but few studies have reported the interactions between the yeasts in detail other than referring to the presence of them and their association with bacteria or moulds. Interactions between the different species occur at various stages throughout production, sometimes as multicultures, unimulticultures or as polycultures. In addition, these interactions may be initiated spontaneously, when the organisms originate from the environment or are inoculated as mixed cultures. These interactions again may appear simultaneously or sequentially to achieve a specific goal. A few typical yeast–bacteria interactions as encountered in foods and beverages are highlighted. As these interactions vary between different products, only the major groups will be discussed.

4.3.1 Microbial Interactions in Fermented Starch-rich Materials

Various fermentations of starch-rich raw materials utilising yeast–lactic acid bacteria associations or mixed cultures including fungi are evident in the literature. The processing involves acid fermentation or alcohol fermentation. Both exhibit distinct

advantages like prevention of spoilage, flavour development, preservation and creation of stability within the products. The fermenting processes relying on alcoholic production will be dealt with later under the heading alcoholic fermentation.

It is important to maintain an equilibrium between the yeasts and the lactic acid bacteria during acid fermentation (Wood 1985; Nout 1991). Excessive acid production by the lactic acid bacteria will result in a decline in the number of surviving yeasts, which consequently leads to a deficiency of growth factors. As a result of such deficiencies, the lactic acid bacteria would produce less acid, and in turn allow an increase in yeast numbers (Nout 1991; Nout et al. 1989). The interactive behaviour between yeasts and lactic acid bacteria creates environmental conditions that protect the products from spoilage by fungi and pathogens owing to the low pH and high compositions of acetic and lactic acids.

4.3.1.1 Cereal Fermentations

Sourdough bread leaven relies on various associative interactions whereby the lactic acid bacteria (*Lactobacillus sanfrancisco*) and yeasts (*Saccharomyces cerevisiae* and *Saccharomyces exiguus*) survive in co-existence (Gobbetti and Corsetii 1997; Gobbetti et al. 1995). The *Lactobacillus* sp. utilises the carbohydrate, maltose, made available owing to amylase action, providing the yeasts with glucose, a stage that may be best described as commensalism, since *Saccharomyces exiguus* strains lack the ability to utilise maltose. However, *Saccharomyces cerevisiae* strains may consume maltose competitively, leading to a decrease in bacterial metabolism (Gobbetti et al. 1994a). Under normal fermentation conditions the yeasts utilise the glucose liberated from the breakdown of maltose and in return produce CO_2 for leavening (Sugihara 1985; Steinkraus 1979). *Lactobacillus sanfrancisco* has a positive influence on yeast leavening and gas production (Gobbetti et al. 1995). A similar positive tendency in yeast fermentation and gas production was observed in the Corleywood baking process by Viljoen and Lues (1993) and Lues et al. (1993). The yeasts secrete compounds like amino acids (Gobbetti et al. 1994b; Spicher and Schroder 1979), peptides (Berg et al. 1981) and vitamins (Spicher and Schroder 1979; Spicher and Nierle 1984) that stimulate the growth of the lactic acid bacteria (Spicher et al. 1981, 1982). Moreover, the yeasts produce phenolic compounds, glycerol (Yong and Wood 1976), etc., which are specific for the aroma (Noda et al. 1980), while the synthesis of antimicrobial compounds by the lactic acid bacteria inhibits spoilage organisms like *Bacillus subtilis*, coliforms and others (Corsetti et al. 1994). The increased protective association in co-culture is expanded in bread by the inclusion of *Propionibacterium freudenreichii* in order to prevent ropy bread induced by *Bacillus subtilis* (Odame-Darkwah and Marshall 1993). Killer activity, however, may cause a serious decrease in the quality of the product if the inoculated yeasts are killed, as indicated in the Turkish baking industry.

This associative interaction between lactic acid bacteria and yeasts, as applied in the processing of sourdough bread (Sugihara et al. 1971; Kline and Sugihara 1971; Wood et al. 1975; Martinez-Anaya et al. 1990; Boraam et al. 1993; Gobbetti et al. 1994a, b, 1995; Oura et al. 1982), is also applied during the production of Pannettone, rye sourdough (Spicher et al. 1981) and soda crackers. For more details, Sugihara (1985) reviewed these processing methods.

Various mixed-culture fermentations are initiated spontaneously in cereal ferments from organisms present in the natural environment, equipment, substrates or through the repeated use of inocula originating from a previous fermentation (Hesseltine 1965, 1983; Verachtert et al. 1990). These mixed inocula may be added simultaneously or sequentially. Unfortunately, most of the cereal fermented foods have been inadequately studied, and contribute little to the modes of interaction between yeasts, bacteria and fungi. It is therefore very difficult to refer to precise interactions as they occur. Most of the literature only refers to the microorganisms present or the biochemical changes, with no indications of interaction.

References to the aspects of the microbiology of ogi preparation are abundant (Akinrele 1970; Odunfa 1999; Banigo and Muller 1972; Odunfa and Adeyele 1987; Banigo et al. 1974). Ogi is a natural fermentation, the microbial flora originate from the maize, sorghum or millet grains (Odunfa 1999; Steinkraus 1982). The grain fungal flora *Aspergillus*, *Penicillium*, *Cephalosporium* and *Fusarium* spp. are eliminated early during the steeping period (Akinrele 1970) and their contribution to the product or other organisms is not clear. The cause for their early elimination is probably due to their inability to compete under the acidity and low oxygen conditions prevailing in the fermenting dough-like mass. *Corynebacterium* hydrolyses the starch and initiates acidification owing to the production of organic acids. *Lactobacillus plantarum* and *Aerobacter cloacae* are also involved in the acidification. The *Lactobacillus* utilises the dextrins from the corn following depletion of the fermentable sugars and contributes most to the acidification by producing lactic acid, while *Aerobacter* increases the niacin and riboflavin content of the mash (Akinrele 1970). The lowering of the pH encourages the yeasts *Saccharomyces cerevisiae* and *Candida mycoderma* to grow, contributing to the flavour and enrichment of vitamins. The lactic acid is a good growth substrate for *Candida*, and the species is therefore considered to play an important role in the preparation of ogi involving the partial destruction of organic acids (Akinrele 1970). Consequently, this will increase the pH and may allow the growth of undesired bacteria. The associative action between the yeasts and the bacteria may therefore be explained as mutualism, since the bacteria create growing conditions for the yeasts by hydrolysing the starch and lowering the pH. The yeasts, in return, provide growth stimulants such as vitamins needed by *Lactobacillus plantarum* (Akinrele 1970) and increase the pH. This was shown earlier with lactic acid bacteria from sourdoughs which required vitamins (Spicher and Schroder 1979) and amino acids to be supplied by yeasts.

Similar associative interactions were observed by Nout (1991) studying the ecology of natural lactic acid fermentation of sorghum-based infant food formulas during repetitive fermentation cycles. During the early fermentation stages, *Leuconostoc* and *Lactococcus* spp. dominated, inhibiting yeast growth owing to excessive production of organic acids. When the nutrients became deficient, *Lactobacillus plantarum* and *Candida* spp. succeeded, which consequently led to an interactive equilibrium. The pH was regulated by the lactic acid bacteria producing organic acids, which allowed adequate yeast growth, and the yeasts supplying the micronutrients enabled the growth of the lactic acid bacteria. Moreover, the fermented mixtures of cereals exhibited a strong antimicrobial effect towards a range of pathogenic bacteria (Nout et al. 1989).

Other cereal fermented foods relying on spontaneous fermentation such as kenkey, koko, banku, panjabi waries, papadams, jalebies, pozol, etc., are prepared in much the same way as described for ogi, although with a different microbial composition. Despite inadequate information on the associative interaction among the microorganisms, the same mutualistic relationships as discussed earlier might be possible. Kenkey fermentation is dominated by *Aspergillus*, *Rhizopus* and *Penicillium* in the initial fermenting stages. The acid-producing *Leuconostoc* spp. soon decrease in numbers during the fermentation, succeeded by *Lactobacillus brevis* and *Acetobacter* spp. in the fermenting dough. Wild types of yeasts, including *Saccharomyces cerevisiae*, are present at all stages of the fermentation, contributing to the flavour by producing esters and ethanol (Muller and Nyarko-Mensah 1972). Koko fermentation comprises the lactic acid bacteria (*Pediococcus cerevisiae*, *Leuconostoc mesenteroides* and *Leuconostoc fermenti*) and yeasts. Panjabi waries and papadams include the yeasts *Saccharomyces cerevisiae* and *Candida* spp., while jalebies are prepared with *Saccharomyces bayanus* (Batra and Millner 1974). Pozol includes fungi (*Geotrichum* and *Mucor*), *Trichosporon* and *Agrobacterium* (Verachtert and Dawoud 1990).

4.3.1.2 Cassava

Cassava is considered a major source of starch-rich food, but with low levels of protein (Steinkraus 1982; Odunfa 1999; Akinrele et al. 1975). The fermentation of gari, the most important fermented cassava product, is anaerobic and follows a two-stage process. In the first stage, *Corynebacterium manihot* and *Bacillus* spp. break down the starch owing to the production of pectinolytic enzymes (Okafor et al. 1984) and release organic acids, which consequently lowers the pH (Collard and Levi 1959; Akinrele 1970). *Bacillus* spp. cause hydrolysis of starch by disintegrating the cell components (Ejiofor and Okafor 1981). According to Okafor (1977), the lactic acid bacteria (*Leuconostoc* and *Lactobacillus*) and *Alcaligenes* are also present during the first stages of fermentation, utilising the free fermentable sugars originating from the tuber and adding to the acidity. Abe and Lindsay (1978) supported by Ngaba and Lee (1979) reported the presence of *Streptococcus faecalis* and claimed that the species is the primary fermentative organism in acidic cassava fermentation. In the second stage, the acid condition stimulates the growth of the fungus *Geotrichum candidum* and presumably also the yeast *Candida*. Collard and Levi (1959) and Akinrele (1970) reported that the fungus added to the acidification, and for the production of aldehydes and esters that are responsible for the taste and aroma. The yeast species quickly proliferates and appeared to be essential as part of the microbiota present during gari fermentation (Okafor 1977). However, no indication of the contributions of the yeasts was reported by any of the authors, despite their growing to numbers as high as 10^6 cfu/g. Moreover, no reference to the associative interaction between the yeasts and the other microorganisms was reported.

During the fermentation of foo-foo, similar microbial populations and interactions were evident, with the exception of *Klebsiella* and the absence of the fungi (Okafor et al. 1984). The *Bacillus*, *Klebsiella* and *Corynebacterium* spp. develop early and contribute to acid formation and the hydrolysis of starch, but are overgrown

by the lactic acid bacteria that further increase the acidic conditions. At the same time, the yeast *Candida* develops in large numbers, and contributes to the lowering of the pH. The prevailing acidified environment permits only these organisms to grow.

4.3.1.3 Fermented Flavouring Products

The production of soy sauce represents a typical sequential inoculation method making use of a two-stage process. The first stage is an aerobic process growing *Aspergillus oryzae* or *Aspergillus sojae* on soybeans and wheat which amylolytically hydrolyses the starch (Yokotsuka 1985; Hesseltine and Wang 1967; Verachtert and Dawoud 1990). As predominant in most mixed-culture fermentations, the fermentation relies on the development of yeasts and lactic acid bacteria. This association is only visible during the second stage. After relying on simple sugars liberated from the first stage, an anaerobic fermentation with *Lactobacillus delbrueckii*, *Pediococcus halophilus* and *Zygosaccharomyces rouxii* takes place. The lactic acid bacteria proceed to grow and produce lactic acid, which decreases the pH, encouraging the growth of *Zygosaccharomyces rouxii*, which results in vigorous alcoholic fermentation (Yong and Wood 1976). Excessive lactic acid fermentation by *Pediococcus halophilus*, however, results in the depression of alcoholic fermentation (Noda et al. 1980). Other osmophilic yeasts such as *Candida etchelsii* and *Candida versatilis* present produce phenolic compounds and furfural, which are desirable flavour enhancers (Morimoto and Matsutani 1969; Yokotsuka 1985; Noda et al. 1980; Wood and Hodge 1985). Similar processes occur in the preparation of miso, except for the use of barley or rice and soybeans, kaffir beer, merissa brewing in Sudan, etc.

4.3.2 Microbial Interactions in Dairy Products

4.3.2.1 Milk-Based Beverages

The commensalistic interaction between *Lactobacillus acidophilus* and the lactose fermenting yeast *Kluyveromyces fragilis* in acidophilus-yeast milk (Subramanian and Shankar 1985) relies on the co-existence of both organisms to secure a good product. Although the lactic acid fermentation originally relied on the fermentation of *Lactobacillus acidophilus* either alone or in mixed cultures with other lactic acid bacteria, the overgrowth of these organisms resulted in fewer viable cells of *Lactobacillus acidophilus*, which consequently reduced the species contribution to gastrointestinal disorders (Lang and Lang 1975). The co-culture of *Lactobacillus acidophilus* with lactose-fermenting yeasts reduces the time of coagulation of the milk owing to acid production by the yeasts, elevates the number of viable lactic acid bacteria cells attributed to stimulating influences of yeasts, and inhibits the growth of *Escherichia coli* and *Bacillus cereus* (Subramanian and Shankar 1985).

Mutualism (synergism) occurs between yeasts and lactic acid bacteria during the fermentation of milky kefir (Rossi 1978) and sugary kefir (Leroi and Pidoux 1993). The predominant species isolated from milky kefir are *Saccharomyces kefir*, *Candida kefyr*, *Lactobacillus caucasicus*, *Lactobacillus casei* and *Leuconostoc* spp. (Oberman 1985; Loretan et al. 2003). The yeasts provide growth factors like amino acids,

vitamins and other compounds for bacterial growth, which consequently lead to elevated acid production, while the bacterial end products are used by the yeasts as an energy source (Challinor and Rose 1954; Wood and Hodge 1985). This phenomenon creates stability in the products. However, a decrease in alcohol production by the yeasts might occur owing to excessive lactic and acetic acid production by osmophilic lactic acid bacteria (Noda et al. 1980; Essia Ngang et al. 1990; Tani et al. 1963), competition for the carbon source or lysis of the yeast cell walls by bacterial enzymes (Lonvaud-Funel et al. 1988; Borregaard and Arneborg 1998).

Similar symbiotic relationships based on acid or alcohol fermentation occur when lactic acid bacteria are responsible for lowering of the pH as a result of the secretion of organic acids (Wood 1981) allowing the yeast population to be competitive in the immediate environment, followed by yeast fermentation as in various milk-based fermentations like Leben, Dahi, Koumiss, etc. (Wood 1981; Bankole and Okagbue 1992; Steinkraus 1982). Oberman (1985) and Vedamuthu (1982) reviewed the fermented milks, whereas Narvhus and Gadaga (2003) reviewed the role of interactions in African fermented milks. The combination of conditions (acidic, saturated with CO_2 and alcohol), is inhibitory to many spoilage bacteria and filamentous fungi and thereby substantially increases the shelf life and safety of the products (Wood and Hodge 1985).

4.3.2.2 Cheese

The production of cheeses involves a maturation stage characterised by a complex ecology of yeasts, bacteria and filamentous fungi (Devoyod and Desmazeaud 1971; Fleet 1990; Jakobsen and Narvhus 1996; Viljoen 2001). The microbial interaction between this microbiota determines the quality, safety and acceptability of the final product.

Several yeasts assist the starter cultures in cheeses by proteolytic activity (Besançon et al. 1992), lipolytic activity (Siewert 1986), the formation of aroma components and participation in the maturation (Welthagen and Viljoen 1999). The positive interaction of yeasts with the starter cultures in surface-ripened cheeses has been well reviewed (Fleet 1990; Jakobsen and Narvhus 1996; Corsetti et al. 2001; Addis et al. 2001). The yeasts, by utilising the accumulated lactic acid in the cheeses, increase the pH and secrete growth factors which promote the growth of *Brevibacterium linens*, which is essential for cheese ripening (Marth 1978). Yeasts also assist the development of fungi in blue-veined and Camembert cheeses (Kaminarides and Laskos 1992; Schlesser et al. 1992) by gas production leading to curd openness (Coghill 1979). In contrast, however, strain-specific interactions between *Yarrowia lipolytica* and *Penicillium roqueforti* may result in the inhibition of mycelial growth and sporulation of the mould mainly owing to competition for nutrients (Van den Tempel and Jakobsen 1998).

Similar yeast–lactic acid bacteria associations were detected in harder cheeses like cheddar (Fleet and Mian 1987; Welthagen and Viljoen 1998, 1999), Parmesan (Romano et al. 1989) and Gouda (Welthagen and Viljoen 1999). On the basis of these associations, Guerzoni et al. (1996) proposed the inclusion of *D. hansenii* and *Y. lipolytica* as adjunct starter cultures during the making of cheese to support the

starter cultures during ripening based on proteolytic and lipolytic activity. In addition, the ability of *D. hansenii* to inhibit *Clostridium* species further adds to the justification (Deiana et al. 1984). Ferreira and Viljoen (2003) applied these yeast species as adjunct starter cultures in cheddar cheese and clearly indicated the mutualistic interaction not only between the yeasts and the lactic acid bacteria, but also between the two yeast strains. When the yeast strains were inoculated individually, a much lower survival was evidenced. The exact mutualistic association between the yeast strains, however, was not clarified other than the indication that both strains survived better when co-inoculated and enhanced flavour development was, detected. Addis et al. (2001), however indicated the yeast–yeast interaction between the two strains in blue-veined cheeses, evidenced by an enhancement in the growth of *Y. lipolytica* caused by *D. hansenii*.

4.3.3 Microbial Interactions in Meat Products

The low initial numbers and reduced growth rates at low temperatures of yeasts in meat products are constraints that prevent them from effectively competing with psychrotrophic bacteria (Walker and Ayres 1970; Dillon and Board 1991; Fleet 1990). However, storage and processing conditions that reduce bacterial competition favour the growth of yeasts (Fleet 1990) and they may cause spoilage or add to the flavour. In fermented meat sausages, when *D. hansenii* species is added as an adjunct starter culture, the species adds a yeast flavour and stabilises the reddening reaction (Hammes and Knauf 1994; Geisen et al. 1992). The sulphite-tolerant species (Banks 1983) *D. hansenii* and *Candida* spp. are responsible for encouraging the growth of pseudomonads and members of the *Entobacteriaceae*, which is usually inhibited by sulphite (Banks et al. 1985). Owing to acetaldehyde production and thereby sulphite binding (Dillon and Board 1991) the yeast species reduce the antibacterial activity of the preservative. Similarly, yeasts utilise organic acids playing a preservative role in processing, and thereby increase the pH, favouring the growth of spoilage bacteria (Walker 1977). No specialised studies on the interaction between these microorganisms, however, have been attempted, and therefore data regarding microbial associations remain very vague.

4.3.4 Microbial Interactions During Vegetable Fermentations

The fermentation and the subsequent storage of olives rely on various interactions between a developing yeast flora and bacteria. During the first phase, when active lactic acid fermentation occurs, fermentable sugars are present in the brine under anaerobic conditions. The strong fermentative yeasts predominate when bacteria are inhibited, outcompeting the other yeasts for the available sugars or they may disrupt the lactic acid fermentation under normal conditions causing "stuck" fermentations (Vaughn et al. 1972). When the available sugars are depleted, oxidative yeasts like *Pichia membranaefaciens* and *Candida mycoderma* develop, utilising the desirable organic acids in the brines and thereby increasing the pH, which allows spoilage bacteria to grow (Mrak et al. 1956). The commonest spoilage incurred by fermenting pectolytic or cellulolytic yeasts during this period is gas formation and softening

(Garcia et al. 1992; Vaughn et al. 1972). Similar results were reported for cucumber fermentation (Vaughn 1983). In contrast to the detrimental effects of yeasts in olive brines, Marquina et al. (1992) reported on significant contributions of yeasts which utilise the lipids present in olives or produce lipases, which resulted in the formation of compounds that stimulate the growth of desirable lactic acid bacteria. Moreover, yeasts utilise the bitter oleuropein, an olive component with antibacterial action, which consequently stimulates bacterial growth (Marquina et al. 1992). Halotolerant yeasts contribute to the flavour (Suzuki et al. 1989), and the occurrence of killer activity might be used to avoid the growth of undesired yeast contaminants.

4.3.5 Microbial Interactions During Alcoholic Fermentations

Several types of fermented beverages which include alcoholic production are evident in the literature derived from fruit, sorghum, rice, barley, plants, etc. Many of the beverages rely on mixed-culture fermentations, reviewed by Wood and Hodge (1985), Steinkraus (1982), Verachtert and Dawoud (1990), Wood (1981), and others.

4.3.5.1 Wine

The interaction between the microorganisms associated with wine fermentation relies on a series of inter-relationships: yeasts–fungi, yeasts–yeasts, yeasts–acetic acid bacteria and yeasts–lactic acid bacteria (Fleet 1992). The existing interactions have been reviewed in detail by Fleet (2003). A typical yeast–filamentous fungi interaction occurs when infection is incurred by *Botrytis cinerea* favouring the presence of non-*Saccharomyces* yeasts and causes a slower fermentation and an increase in glycerol and acetic acid production. The extracts of grapes infected with *Botrytis cinerea* will inhibit (Ribéreau-Gayon 1985) or activate (Reed and Nagodawithana 1988) alcoholic fermentation.

Except for killer yeast activity (Young 1987; Shimizu 1993; Guriérrez et al. 2001), the recognition of non-*Saccharomyces* yeasts as important contributors to wine fermentation (Fleet et al. 1984; Heard and Fleet 1987; Martinez-Anaya et al. 1990; Mora et al. 1992; Schutz and Gafner 1993) results in various yeast–yeast associations that can be exploited. Other than the production of ethanol, organic acids, sulphur, etc. (Fleet 1990, 2001; Bisson 1999, Soden et al. 2000; Mills et al. 2002) by some yeasts, inhibitory to the growth of competing yeasts, the medium-chain fatty acids, decanoic and octanoic acids (Lambrechts and Pretorius 2000), their corresponding ethyl esters (Lafon-Lafourcade et al. 1984; Ribéreau-Gayon 1985) and yeast ghosts (Edwards et al. 1990) produced all contribute to yeast–yeast interactions. Inhibitory effects by *Kloeckera apiculata* against *Saccharomyces cerevisiae* (Mortimer 2000) and *Metschnikowia pulcherrima* against a range of other yeasts have been reported (Nguyen and Panon 1998). The interaction between the non-*Saccharomyces* and *Saccharomyces* species based upon competition for carbohydrates, nitrogen, other compounds and dominance during the fermentation remains largely unexplored.

Yeast–bacteria interaction in wine production relies predominantly on the yeast association with the lactic acid bacteria and acetic acid bacteria. Detailed studies on

the combined growth of acetic acid bacteria (*Acetobacter aceti*, *Acetobacter pateurianus* and *Gluconobacter oxydans*) and wine yeasts (*Saccharomyces cerevisiae*, *Kloeckera apiculata* and *Candida* spp.) were performed (Lafon-Lafourcade et al. 1984; Drysdale and Fleet 1988, 1989). Antagonistic effects by *Acetobacter*, due to acetic acid excretion, result in decreased fermentation by *Saccharomyces cerevisiae* and may cause stuck fermentation (Ludovico et al. 2001).

Wine yeasts vary in their interaction with lactic acid bacteria (Fornachon 1968; Thornton 1991; Suzzi et al. 1995) as they may inhibit or stimulate the growth of lactic acid bacteria. The naturally present lactic acid bacteria occur at low numbers, and die during alcoholic fermentation (Fleet 1993, 2003; Fleet and Heard 1993) and exert little or no effect on yeast growth. If the alcoholic fermentation is restricted or retarded, multiple yeast–lactic acid bacteria interrelationships occur that play a substantial role during malolactic fermentation (King and Beelman 1986; Lemaresquier 1987; Markides 1993; Fleet 1990; Martineau and Henick-Kling 1995), which commenced after alcohol fermentation. The antagonism of the yeasts is related to alcohol production (Wibowo et al. 1985), SO_2 (Wibowo et al. 1988), proteins (Dick et al. 1992), fatty acids (Edwards et al. 1990; Edwards and Beelman 1987; Lonvaud-Funnel et al. 1988; Lafon-Lafourcade et al. 1984), antibacterial factors (Fornachon 1968) and the removal of substances important to bacterial growth (King and Beelman 1986). Growth stimulation of the lactic acid bacteria is encouraged by yeast autolysis (Fleet 1992; Charpentier and Feuillat 1993; Crouigneau et al. 2000), removal of inhibitory fatty acids (Edwards and Beelman 1987), yeast ghosts (Lafon-Lafourcade et al. 1984), amino acids (Lonvaud-Funel et al. 1988), vitamins (Lemaresquier 1987), sucrose hydrolysis (Ngang et al. 1992) and ethanol at low concentrations (King and Beelman 1986).

Although amensalism is indicated during wine fermentation, whereby the *Saccharomyces cerevisiae* strains prevent the growth of initially present non-conventional wine yeasts owing to elevated concentrations of ethanol, useful commensal relationships between yeasts occur when wine is allowed to become partially aerobic, leading to the formation of sherry (Amerine and Kunkee 1968; Carr et al. 1969). According to these authors, the interaction between the flor-filming yeasts relies on competitive, amensal and commensal relationships. In addition, neutralism is reported as *Saccharomyces diasticus* strains possess glucoamilase, which enables them to ferment polysaccharides which cannot be metabolised by other yeasts found during beer and wine fermentations.

4.3.5.2 Fruit Juices and Cider

The yeast–bacteria interrelationship between *Saccharomyces cerevisiae* and *Leuconostoc oenos*/*Lactobacillus plantarum* plays an important role in the degradation of glucose, malate and citrate, the major carbon sources in fruits and fruit juices like orange and cider, during fruit fermentations under acidic and anaerobic conditions (Kennes et al. 1991a, b). The microbial ecology follows the principles of wine fermentations. The microflora of the apples includes yeasts (*Saccharomyces cerevisiae*, *Kloeckera apiculata* and *Candida* spp.), lactic acid bacteria (*Lactobacillus brevis*, *Pediococcus* spp., *Leuconostoc mesenteroides* and *Leuconostoc oenos*) and

acetic acid bacteria (*Acetobacter* and *Gluconobacter* spp.). The non-proliferating population of yeasts initiates the fermentation, but is inhibited by a lack of nutritional growth factors and the toxic effect of ethanol owing to competition and amensalism from the ethanol-tolerant species *Saccharomyces cerevisiae*. Similarly, competition for nutrients between lactic acid bacteria and the yeast also exists as well as positive or negative contributions from the indigenous microflora present. The yeast outcompetes the lactic acid bacteria for the utilisation of the sugars, which results in the production of ethanol without changing the pH. The ethanol present in the media favours the subsequent conversion of citric acid (with oranges) or malic acid (with apple juice) to acetic acid by the lactic acid bacteria *Lactobacillus plantarum* and *Leuconostoc oenos*, respectively (Kennes et al. 1991a). *Leuconostoc oenos* usually fulfils a similar role during wine fermentation (Fleet et al. 1984), although it has been proposed that *Schizosaccharomyces pombe* can remove excess organic acids with inferior results. Although the lactic acid bacteria compete for carbohydrates, they also depend on essential stimulants excreted by the yeasts as reported earlier. Similar competitive/commensal interrelationships may occur between *Pediococcus cerevisiae* and the yeasts during beer production when the bacteria form diacetyl, which spoils the taste of the beer, or polysaccharides, which cause ropiness.

4.3.5.3 Beer

Beer is a product derived from malted barley, hydrolysed under controlled conditions by amylases to maltose and glucose to make it available to yeasts which produce ethanol (Rainbow 1981; Priest and Campbell 1996). Under normal conditions, the inoculated brewer's yeast *Saccharomyces cerevisiae* quickly dominates during fermentation and suppresses the growth of spoilage *Enterobacteriaceae*, lactic acid bacteria and other competitive microorganisms by elevated alcohol concentrations, low pH, CO_2 production, SO_2, co-sedimentation and organic acid secretion, while the anaerobic conditions that prevailed prevent the growth of aerobic acetic acid bacteria (Jespersen and Jakobsen 1996). Brewer's yeast inhibition of lactic acid bacteria is also attributed to competition for nutrients (Pfenninger et al. 1979), heat-labile yeast metabolites (Dolezil and Kirshop 1980), alanyl dipeptides and co-sedimentation of certain bacteria with brewing yeasts (White and Kidney 1979, 1981). The hop bitters present also inhibit the growth of lactic acid bacteria. Competition for nutrients, low pH conditions and hops, moreover, inhibits *Pediococcus damnoses* and related *Pediococcus* spp. However, if insufficient cleaning, heating, hops-resistant bacteria (Fernandez and Simpson 1995) and delays in pitching of the wort occur, beer contaminants derived from barley, wort and the equipment may cause spoilage (Flannigan 1996). Filamentous fungi affect the flavour of the beer and mycotoxins have a concentration-dependent effect on yeasts, resulting in reduced CO_2 evolution and ethanol production. Similarly, *Pediococcus* spp. and *Lactobacillus* spp. contribute to the flavour (Priest 1996) and may compete for the available nutrients. The role of "wild yeasts" such as *Pichia membranaefaciens*, *Pichiasubpelliculosa* and species of *Schizosaccharomyces*, *Brettanomyces*, *Kloeckera*, *Debaryomyces*, *Candida* and *Torulaspora* is well reviewed by Rainbow (1981) and Campbell (1996). Although these yeast contaminants are able to grow under anaerobic conditions (Campbell

and Msongo 1991), they do not compete well under the acid and ethanol concentrations of the beer. Access of air stimulates their growth, and competition for the nutrients. Killer strains of *Saccharomyces cerevisiae* may cause the severest competition. The killer strain kills sensitive culture yeast strains, and establish itself as the dominant yeast of the fermentation. Yeast autolysis, which occurs after lengthy secondary fermentation, and nitrogen released by *Saccharomyces carlsbergensis* may also encourage microbial growth in providing essential growth stimulants.

Verachtert et al. (1990) in unravelling the complex mixed-culture process during Lambic and Gueze beer fermentation identified a succession of different microbial species during four fermentation phases. The spontaneous fermentation starts with the development of *Enterobacteria* and low numbers of maltose non-fermenters such as *Kloeckera apiculata*, *Saccharomyces globosus* and *Saccharomyces diarensis*. When these yeasts disappear, a second group of fermenting yeasts, *Saccharomyces cerevisiae* and related *Saccharomyces* spp., are responsible for ethanol fermentation, followed by actidione-resistant yeasts belonging to *Brettanomyces* that metabolise the sugars not assimilated by *Saccharomyces*. A fourth group of oxidative yeasts remain in the yeast layer found on top of the fermenting wort with no significance to the fermentation. After the main ethanol fermentation, lactic acid bacteria, usually *Pediococcus* spp., are responsible for the synthesis of lactic acid, adding to the acid–vinous character of the beer. Despite clear indications of numerous yeast–yeast interactions present during the production of these beers, the data remain vague and need attention.

4.3.5.4 Distilled Beverages

Similar interesting interrelationships may occur in distilled alcoholic beverages, such as whiskey and rum. But, as with beer, the interactions between yeasts, fungi and bacteria have been inadequately studied, despite numerous references to spoilage (Barbour and Priest 1988) and advances in brewing and distilling yeasts. Whiskey fermentation relies on a mixed fermentation of added yeast (*Saccharomyces cerevisiae*) and indigenous bacteria (Barbour and Priest 1988). The fermentation comprises the bacterial species *Lactobacillus*, *Enterobacteriaceae*, *Peddiococcus* and *Leuconostoc*, which originate from the malted barley and equipment. *Enterobacteriaceae* spp. are eliminated in the early stages of the fermentation by the low pH and alcohol concentration, while the lactic acid bacteria proliferate and compete with the yeast, which reduces the ethanol yield. The yeast, however, encourages the growth of lactic acid bacteria by the excretion of glycerol and products due to yeast autolysis (Barbour and Priest 1988).

4.4 Yeast Antagonism Applied as Biocontrol Agents in Preventing Plant-Spoilage Fungi

Numerous yeasts capable of playing a significant role in interactions have been isolated from fruit, fermented products, soil and other natural environments over the last few decades (Fleet 2003). However, our knowledge of the ecological distribution of such yeasts is still very limited. The interest of biological control in the

ecology of representative yeasts species arises from the necessity to control their metabolic activity by factors that can be influenced by technological means. Ideally it should be possible to predict that a yeast which possesses certain characteristics regarding biocontrol occurs most frequently in a certain environment within a defined habitat.

There is little doubt that various yeasts afford some protection to post-harvest spoilage (Chalutz and Wilson 1990; Chalutz and Droby 1998); consequently there is renewed interest in the possibilities of harnessing and accentuating the mechanisms of biological control as awareness of the dangers and disadvantages and public resistance to chemical control by fungicides posing potential oncogenic risks increase (Wilson and Wisniewski 1989). Moreover, the stresses of modern concepts of quality assurance require products of a high standard in quality, and biological control contributes to an improvement in hygienic safety, constant levels of quality and shelf life.

During the last decade a steady flow of reports claimed that particular post-harvest diseases and temperate fruit can be controlled to some extent by the antagonistic interaction between yeasts and mycotoxic fungi (Guinebretiere et al. 2000). Some of these yeasts like *Candida oleophila* and *Pseudozyma flocculosa* have been commercialised, known as Aspire and Sporodex, respectively (Droby et al. 1998; Punja and Utkhede 2003). Yeast antagonistic efficiency is also successfully reproduced in the inhibition of spoilage or toxin-producing fungi in high-moisture wheat stored under airtight conditions (Petersson and Schnurer 1995; Bjornberg and Schnurer 1993).

The high efficiency of yeasts applied as biocontrol agents is related to their indigenous adaptation to the immediate environment, the nutritional conditions prevailing at the wound site (Chalutz and Wilson 1990), their resistance to fungicides, survival at varying temperatures and ability to colonise (Roberts 1990). To evaluate the usage of yeasts as biocontrol agents, an understanding of the antagonistic interaction between the yeasts and fungal pathogens is needed.

Since limited evidence related to the production of antimicrobial compounds is evident, alternative ways of inducing biocontrol activity have been claimed. According to Avis and Belanger (2001) the antifungal metabolites produced by *Pseudozyma flocculosa* are a mixture of fatty acid containing derivatives that affect membrane permeability of the target organisms, thereby inhibiting the growth of powdery mildews. Nutrient competition, however at the wound site is regarded as the principal mode of antagonism (Droby et al. 1989; Punja and Utkhede 2003), although other modes of action like induced resistance, pathogen and antagonist density, age of the cells (Droby et al. 2002), cell wall degradation by β-(1-3)-glucanase enzymes (Wisniewski et al. 1991), killer toxins produced by yeasts (Walker et al. 1995), antifungal toxins like zygocin (Weiler and Schmitt 2003), and attachment of the yeasts by cell-surface proteins or lectin (Wisniewski et al. 1991).

Naturally occurring yeast antagonists like *Metschnikowia pulcherrima*, *D. hansenii*, *Pichia anomala* and *Pichia guilliermondii* are continuously isolated and reapplied to the fruit or silaged grain as effective biocontrol agents. Scientists will continue to collect more species, as our current knowledge regarding yeasts as biocontrol agents has only just begun.

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Chapter 5

Physiological and Molecular Responses of Yeasts to the Environment

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5.1 Introduction

Understanding the ways by which yeasts respond to changes in their physico-chemical environment is very important in the food and beverage industries. For example, it is important for the maintenance of yeast viability and vitality in the production and utilisation of yeasts for food and fermentation processes, and it is additionally important for the control of yeasts that act as spoilage agents of foods and beverages. In the former situation, yeasts are confronted with several environmental stresses including insults caused by changes in temperature, pH, osmotic pressure, ethanol concentration and nutrient availability that individually or collectively can deleteriously affect yeast physiology. These changes may result in lowered yeast growth yield and impaired fermentation performance. In the case of food spoilage yeasts, such organisms have adapted to survive stress caused by low temperature and oxygen levels, anhydrobiosis and high salt/sugar concentrations and their effective elimination is often based on measures to counteract the inherent stress tolerance of these yeasts. Chapter 11 covers food spoilage yeasts in more detail.

The present chapter describes both physiological and molecular aspects of stress on yeast cells and will focus on yeasts' responses to changes in their environment which are pertinent in situations where survival of the yeast is both desirable (e.g. industrial fermentations) and undesirable (e.g. foods and beverages spoilage). The stresses of particular relevance for the food industry are thermostress, pH shock, osmostress, nutrient starvation, ethanol toxicity, oxidative stress, prolonged anaerobiosis, and exposure to chemical preservatives. This chapter will not review biologically related stress factors in yeasts such as cellular ageing, genotypic changes and competition from other organisms, the last of these having been dealt with in Chap. 4.

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5.2 Yeast Nutrition and Growth

5.2.1 General Comments About Cell Physiology of Important Food Yeasts

The premier industrial yeast *Saccharomyces cerevisiae* is widely employed in the production of foods and fermented beverages. As such, it is by far the most economically important microorganism known to mankind. The metabolic activities of *S. cerevisiae* have been exploited for millennia in the leavening of bread and in the fermentation of cereal wort and grape must – these activities will continue to be exploited for future millennia. Why has *S. cerevisiae* found such dominance in baking and alcoholic beverage production? The reasons lie both in the ability of numerous "industrial" strains of *S. cerevisiae* to effectively transform sugars into ethanol, carbon dioxide and numerous secondary flavour compounds and it's ability to withstand stress caused primarily by temperature, osmotic pressure, ethanol toxicity and competitive bacteria and wild yeasts. Figure 5.1 summarises major stresses encountered by industrial fermentation (brewing) yeast strains. Of course, most yeasts are similarly able to ferment sugars, but they may not be able to tolerate the rigours of a large-scale industrial fermentation plant. *S. cerevisiae* is clearly able to do so and has found niches well-suited to it's physiological behaviour in wineries and fermentation plants (Martini 1993; Vaughan-Martini and Martini 1995). In short, *S. cerevisiae* is arguably the most resilient industrial yeast that we currently have at our disposal. Nevertheless, new approaches to improve stress-tolerance of *S. cerevisiae* have been developed with potential benefits for food and beverage production processes (Chap. 13).

Stress-tolerance attributes in other yeast species also impact significantly in foods and beverages. Several non-*Saccharomyces* yeasts have also found beneficial production applications, whilst some species are detrimental after production in storage situations, especially with regard to yeast spoilage of high-sugar and high-salt foods. Some examples of stress-tolerant yeasts important in both food production and spoilage are listed in Table 5.1.

5.2.2 Major Nutrients Encountered in Foods/Fermentation Media for Yeast Growth

Foods and beverages designed for human nutrition also represent rich sources of nutrients for yeasts. In the production of alcoholic beverages, substrates such as malt wort or wine must are complete nutrient sources and contain fermentable sugars (mainly maltose in the case of wort, and glucose and fructose in wine must), assimilable nitrogen (amino acids), minerals, vitamins and oxygen (necessary in initial stages of fermentation), together with minor growth factors. Occasionally, the availability of certain key nutrients in fermentation media may be limiting and this can adversely affect efficiency of yeast fermentation processes. For example, if there is insufficient oxygen available in the initial stages of fermentation, *S. cerevisiae* cells will be unable to synthesise ergosterol and unsaturated fatty acids, which are essential plasma membrane components. In turn, this can affect the ability of cells to with-

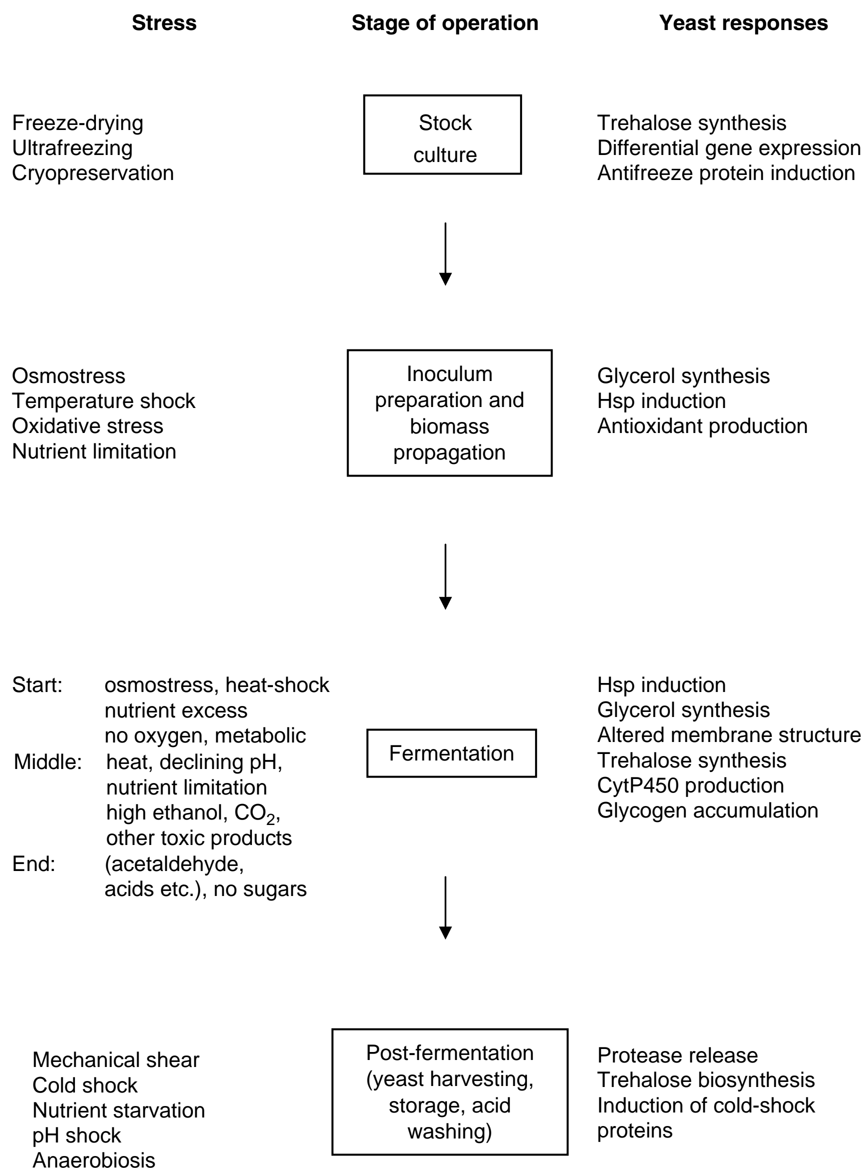

Fig. 5.1. Stresses encountered by industrial fermentation (e.g. brewing) yeasts

Table 5.1 Some important food yeasts

Yeast genus	Importance in foods
Candida spp.	Production of microbial biomass protein, vitamins, citric acid. Some food spoilers (e.g. *C. zeylanoides* in frozen poultry)
Cryptococcus spp.	Some strains are used as biocontrol agents to combat fungal spoilage of post-harvest fruits. *C. laurentii* is a spoilage yeast (poultry)
Debaryomyces spp.	*D. hansenii* is a salt-tolerant food spoiler. It is also used in biocontrol of fungal fruit diseases
Kluyveromyces spp.	Fermentation of lactose in cheese whey for potable alcohol; source of food enzymes (pectinase, microbial rennet, lipase); cocoa fermentations. Some species are spoilage yeasts in dairy
Metschnikowia spp.	*M. pulcherrimia* is used in biocontrol of fungal fruit diseases. Osmotolerant yeasts
Phaffia spp.	*P. rhodozyma* is a source of astaxanthin food colorant
Pichia spp.	Production of microbial biomass protein, riboflavin (*P. pastoris*). *P. membranifaciens* is an important (surface-film) spoiler of wine and beer
Rhodotorula spp.	*R. glutinis* is used as a source of food enzymes (lipases). Some species are food spoilers of dairy products
Saccahromyces spp.	*S. cerevisiae* is used in traditional food and beverage fermentations (baking, brewing, winemaking, etc.); source of savoury food extracts and food enzymes (e.g. invertase). It is also used as a livestock growth factor. *S. bayanus* is used in sparkling wine fermentations. *S. diastaticus* is a wild yeast spoiler of beer. *S. boulardii* is used as a probiotic yeast
Schizosaccharomyces spp.	*S. pombe* is used in traditional African beverages, rum and for wine deacidification. It is regarded as an osmotolerant yeast
Schwanniomyces spp.	Microbial biomass protein (from starch – *S. castellii*)
Yarrowia spp.	*Y. lipolytica* is used in production of microbial biomass protein, citric acid and lipases
Zygosaccharomyces spp.	*Z. rouxii* and *Z. bailii*, being osmotolerant, are important food and beverage spoilage yeasts. *Z. rouxii* is also used in soy sauce production

stand the toxic effects of ethanol produced later in fermentation. Limited availability of metal ions can also influence fermentation performance of yeasts (Birch et al. 2003). For example, in brewing, zinc deficiency may result in slow or incomplete fermentations (Walker 2004). In addition, during fermentation, the concentrations of various nutrients change and yeasts must respond dynamically to such changes. Knowledge of physiological and molecular responses of yeasts to the concentration and availability of specific nutrients is of particular interest for the alcoholic beverage industries because these responses will impact on the progress and efficiency of fermentation. In winemaking, yeast starter culture strain selection focuses on good nutrient utilisation as well as on resilience to unfavourable growth environments (Bauer and Pretorius 2000). These two characteristics are inextricably linked.

For food spoilage yeasts, in addition to nutrient availability, physico-chemical conditions play important roles in dictating the extent of spoilage caused. For example, high osmotic pressure caused by high-sugar- or high-salt-containing foods represents hostile conditions for the survival and growth of most yeasts, but not for osmotolerant strains of the spoilers *Zygosaccharomyces rouxii* and *Debaryomyces hansenii*, respectively. Such yeasts have clearly adapted to such adverse environments by sensing the external stressor(s) and responding accordingly to maintain their survival. Again, knowledge of these responses can assist in control of yeast food spoilage.

5.2.3 Nutrient Sensing and Translocation

Nutrients exert a myriad of effects on living cells as a consequence of their function in both supplying energy and providing substrates for biosynthesis and catabolism. In addition, nutrients exert many regulatory effects that are mediated by nutrient-sensing systems, sometimes largely or even completely independent of their metabolism. Such systems have extensively been studied in yeasts during recent years (see reviews by Boles and André 2004; Holsbeeks et al. 2004). At the level of nutrient sensing, one can distinguish genuine receptor proteins and transporter-like receptor proteins. The first category includes the G-protein-coupled receptor Gpr1 and the second category includes glucose and amino acid transporter-like receptors. Whereas these proteins lost their capacity to transport the ligand and only function as sensors, some active transporters are also able to function as a sensor.

For yeast cells, glucose is the most important nutrient and therefore it is not surprising that yeast cells have developed a number of glucose-sensing pathways. The glucose-repression pathway, the Rgt2-Snf3 glucose-sensing pathway and the glucose-induced cyclic AMP (cAMP)–protein kinase A (PKA) pathway have recently been reviewed (Geladé et al. 2003; Rolland et al. 2002).

The *S. cerevisiae* G-protein-coupled receptor Gpr1 belongs to a small subfamily of the large GPCR family (Graul and Sadée 2001). Sequence database searches have revealed other closely related members of this GPCR subfamily in other fungi like *Schizosaccharomyces pombe*, *Candida albicans*, *Aspergillus* sp., *Neurospora crassa* and other *Saccharomyces* species (Versele et al. 2001 and unpublished results). Whether all the Gpr1 homologues have glucose as their ligand remains to be seen. Recently evidence against glucose as the ligand of the CaGpr1 has been obtained. Moreover, there is evidence that in *C. albicans* this receptor may be a methionine sensor (Maidan et al. 2005). The *S. cerevisiae* G-protein-coupled receptor Gpr1 senses sucrose and glucose in the millimolar range, whereas mannose acts as an antagonist (Lemaire et al. 2004). Binding of glucose or sucrose to the receptor activates the Gα protein Gpa2 (Colombo et al. 1998). Apart from the receptor, Gpa2 also interacts with Gbp1/Krh2 and Gbp2/Krh1, two proteins that have been proposed to act as Gβ-mimicking subunits on the basis of structural resemblance with classical Gβ proteins (Battle et al. 2003; Harashima and Heitman 2002) and also with Plc1 (Ansari et al. 1999). The activity of Gpa2 is controlled by the RGS protein Rgs2 (Versele et al. 1999). Hence, a GPCR system composed of Gpr1, Gpa2 and Rgs2 has been proposed to act as a glucose-sensing system for control of the cAMP pathway (Thevelein and de Winde 1999; Versele et al. 2001). An unusual feature of this GPCR system is that the rapid stimulation of cAMP synthesis requires a low level

of glucose phopshorylation by any one of the three glucose kinases encoded by *GLK1*, *HXK1* and *HXK2*. This means that partial metabolism of the ligand by phosphorylation is required to sustain stimulation of the adenylate cyclase encoded by *CYR1* (Rolland et al. 2002). Activation of Gpa2 stimulates adenylate cyclase and the cAMP that is produced results in the activation of PKA, resulting in a plethora of changes (Thevelein and de Winde 1999).

Important downstream targets of the PKA pathway are the redundant Msn2 and Msn4 transcription factors that interact with the stress response element (STRE) and the Gis1 transcription factor that interacts with the post-diauxic shift (PDS) element. These transcription factors are under negative control of the PKA pathway. Conditions that result in a high PKA phenotype (no stress, cells growing on glucose) result in phosphorylated Msn2 and Msn4 which are localised to the cytoplasm. The cytoplasmic localisation is also regulated by the target of rapamycin (TOR) kinase pathway. Glucose activates TOR kinases that inhibit the dephosphorylation of Msn2 (Beck and Hall 1999). Upon various stress conditions or under nutrient limitation, the PKA activity is low, the transcription factors are dephosphorylated and are translocated into the nucleus, where they regulate gene expression. Inside the nucleus, the Msn2 protein is rapidly degraded despite a constant MSN2 messenger RNA (mRNA) level (Bose et al. 2005; Durchschlag et al. 2004; Lallet et al. 2004). The degradation of Msn2 is dependent on the cyclin-dependent protein kinase Srb10, a member of the transcription machinery. The degradation of Msn2 upon heat shock is mediated via the 26S proteasome (Lallet et al. 2004). Among the targets regulated by the Msn2/4 transcription factors are heat shock protein (Hsp) encoding genes, such as *HSP12* and *HSP104*, which are rapidly repressed upon activation of the PKA pathway (Marchler et al. 1993; Varela et al. 1995). These proteins play important roles in various processes that help yeast cells cope with a broad array of stresses, including heat and ethanol stress (Piper 1995, 1997; Sanchez et al. 1992). Furthermore, high PKA activity also causes repression of the trehalose synthase-encoding genes *TPS1* and *TPS2* (Winderickx et al. 1996). Trehalose plays a prominent role in cellular stress resistance by protecting membranes from desiccation and by preventing protein denaturation (reviewed by Bonini et al. 2004).

A similar phosphorylation-dependent nuclear localisation has recently been shown for the Crz1p/Tcn1p transcription factor. Under normal growth conditions, Crz1p is negatively regulated by PKA phosphorylation and localises to the cytosol (Kafadar and Cyert 2004). Under specific stress conditions, such as high salt, alkaline pH or cell wall damage, this transcription factor is dephosphorylated by calcineurin, a Ca^{2+}/calmodulin-dependent protein phosphatase, translocating it into the nucleus, where it activates the expression of genes whose products promote adaptation to stress (Yoshimoto et al. 2002). In the nucleus, the Czr1 transcription factor binds to the calcineurin-dependent response element (CDRE). All known calci-neurin-dependent transcriptional changes (ion homeostasis, cell wall maintenance, vesicle transport, lipid biosynthesis and small molecule transport) are believed to be mediated through Crz1.

The role of glucose and sucrose signalling through the cAMP–PKA pathway during industrial applications has recently been reviewed (Verstrepen et al. 2004). As mentioned before, the nutrient mixture during the production of wine and beer is very different from the nutrient mixture on which yeast cells are grown in the labora-

tory. The main sugars in grape must are glucose and fructose; beer wort contains glucose, fructose, sucrose, maltose and maltotriose, and the fermentation medium for ethanol production is usually a mixture of any of these sugars in variable concentrations, depending on the origin of the substrate (fermentation feedstock) (Bamforth 2003; Yoon et al. 2003). In most cases, the initial concentration of glucose and/or sucrose in the growth medium is well above the threshold concentrations (20–40 mM) for induction of the sugar signalling cascades (Meijer et al. 1998; Meneses et al. 2002). Hence, both the main glucose-repression pathway and the Ras–cAMP–PKA pathway are triggered at the start of the process. This has three major consequences: the repression of respiration, the arrest of the consumption of other carbohydrates and the loss of cellular stress resistance (Thevelein and de Winde 1999; Van Dijck et al. 1995). While these effects help *S. cerevisiae* survive in its natural habitat, sugar signalling causes several problems in various yeast-based industrial processes.

The low stress resistance during active fermentation of yeasts is disadvantageous for their use in industrial applications (Attfield 1997). A striking example will be discussed later when we describe the process of frozen dough preparation.

In the last few years, genome-wide expression analysis after the addition of glucose or other nutrients to yeast cells has been performed. The results obtained with these arrays have confirmed the strong interconnections between different pathways (Schneper et al. 2004). To determine the function of some of the components in the Ras–cAMP–PKA pathway, various research groups have performed experiments by addition of glucose to mutants in this pathway (Jones et al. 2003, 2004; Lin et al. 2003; Roosen et al. 2005; Wang et al. 2004). These arrays identified novel targets of the cAMP–PKA pathway, including targets that play a role in cell wall biogenesis. The experiments also identified new transcription factors and response elements in regulated promoters. Many of the targets are redundantly activated by a Ras–cAMP–PKA-dependent pathway and by one or more PKA-independent pathways.

Other examples of nutrient-sensing plasma membrane proteins have been discovered in yeasts. Snf3 and Rgt2 are two non-transporting homologues of glucose carriers which are proposed to have a function in glucose sensing (Özcan et al. 1998) and Ssy1 is a non-transporting homologue of amino acid carriers which is proposed to have a function in amino acid sensing (Didion et al. 1998; Iraqui et al. 1999; Klasson et al. 1999).

Snf3 and Rgt2 have a role in the glucose-induced expression of specific glucose transporter genes. The Snf3 protein senses low levels of glucose and is responsible for inducing several *HXT* genes in response to low glucose. The Rgt2 protein senses high levels of glucose and is responsible for inducing *HXT1* in response to high glucose. Both sensor proteins have a long C-terminal cytoplasmic tail that is required for signalling. Recently, the plasma membrane-associated protein kinase casein kinase I was identified as a signal transmitter in Rgt2-mediated glucose signalling. It is now proposed that glucose binding to Rgt2 activates casein kinase I, which then phosphorylates Mth1 and Std1, two proteins that were previously shown to interact with the C-terminal tail of Snf3 and Rgt2. This phosphorylation leads to their degradation and finally to the induction of *HXT* gene expression (Moriya and Johnston 2004; Özcan et al. 1998). Mth1 inhibits the interaction between two different domains of the Rgt1 transcription factor. This intramolecular interaction inhibits DNA binding and proper

expression of the *HXT* genes. The current hypothesis is that Mth1 promotes transcriptional repression by Rgt1 by binding to it and preventing the intramolecular interaction, thereby enabling Rgt1 to bind to DNA, resulting in Rgt1-dependent repression of *HXT* genes in the absence of glucose and Rgt1-dependent activation of *HXT1* gene expression in the presence of high levels of glucose (Polish et al. 2005).

A similar sensing system has been described for amino acid transporter induced expression. The amino acid sensing protein Ssy1 forms a sensor complex with Ptr3 and Ssy5 called the SPS complex. Activation of the SPS complex by amino acids triggers the proteolytic activation of two latent transcription factor precursors, Stp1 and Stp2, that are located in the cytosol, and the active proteins are then targeted to the nucleus, where they induce expression of specific amino acid transporter genes (Andreasson and Ljungdahl 2002). An extensive review on both glucose and amino acid transporter-like sensors has recently been published (Boles and André 2004).

Apart from the non-transporting sensors, there also exist active transporting nutrient permeases that also function as sensors (reviewed by Holsbeeks et al. 2004). These include Mep2 (ammonium permease; Lorenz and Heitman 1998), Gap1 (general amino acid permease; Donaton et al. 2003) and Pho84 (phosphate transporter; Giots et al. 2003). In these proteins, specific mutations have been identified that distinguish transport and sensing capacity. These three permeases are important for activation of PKA targets upon sensing their respective ligands. The downstream targets that are activated by these sensors largely overlap with those of the cAMP–PKA pathway (Roosen et al. 2005). So far, the only component that has been identified in the signal transduction pathway activating PKA is the Sch9 protein kinase which is essential for the nitrogen-induced activation through Gap1.

5.2.4 The Physicochemical Environment and Yeast Growth

Yeasts generally grow well in warm, moist, sugary, acidic and aerobic environments. Those few species which prefer exceptional physical or chemical conditions are, nonetheless, very important in industry, often as spoilage organisms.

With regard to temperature, most laboratory and industrial yeasts are mesophilic and generally grow best between 20 and 30°C, although some species associated with warm-blooded animals will not grow well below 24–30°C, whilst some psychrophilic yeasts grow optimally between 12 and 15°C (Table 5.2). Psychrophilic yeasts that adapt to low temperatures are very important spoilage organisms in frozen foods. Yeasts exhibit characteristic, or “cardinal”, minimum, optimum and maximum growth temperatures (T_{min}, T_{opt} and T_{max}, respectively). For *S. cerevisiae*, T_{max} values range from 35 to 43°C, whereas strains of *S. bayanus* and *S. pastorianus* fail to grow above 35°C.

Most yeasts are mildly acidophilic, being able to grow well between pH 4.5 and 6.5, with several species being are able to grow in more acidic or alkaline conditions. During alcoholic beverage fermentations, the pH declines owing to a number of factors including dissolution of CO_2, proton extrusion and organic acid secretion. Brewing and winemaking strains are able to tolerate low fermentation pH values

Table 5.2 Yeast growth temperature limits

Thermal domain	Broad definition	Examples of yeasts
Psychrophile	A yeast capable of growing between 5 and 18°C. Obligate psychrophiles have an upper growth limit at or below 20°C	*Leucospiridium* spp. (e.g *L. frigidum*). *Torulopsis* spp. (e.g. *T. psychrophila*)
Mesophile	Yeasts with growth limits at 0°C and up to 48°C	Vast majority of yeast species
Thermophile	Minimum temperature for growth at or above 20°C	*Candida sloofii*, *Cyniclomyces gluttulatus*, *Saccharomyces telluris*, *Torulopsis bovina*

Information from Watson (1987)

and the former are additionally tolerant of acid-washing, a practice designed to eliminate contaminant bacteria.

With regard to water requirements, yeasts need water in high concentrations for growth and enzymatic activity. As is the case with temperature, yeasts have cardinal water potentials for growth; namely, ψ_{min}, ψ_{opt}, and ψ_{max} (where ψ_w, water potential, is expressed in megapascals). Most yeasts can grow reasonably well at low water potentials, with those able to grow well in conditions of low water potential (i.e. high sugar or salt concentrations) referred to as osmotolerant or xerotolerant. Such yeasts include *Candida mogii*, *D. hansenii*, *Metschnikowia bicuspida*, *Schizosaccharomyces octosporus* and *Z. rouxii* and are very important economically as food spoilage yeasts. The last species is generally unaffected by ψ_w values between –1.0 and –5.6 MPa.

Concerning requirements for oxygen, yeasts need this nutrient not only as a terminal electron acceptor for respiratory metabolism, but also as a growth factor for the biosynthesis of sterols and unsaturated fatty acids (Walker 1998). In fact, few yeasts can tolerate complete lack of oxygen and anaerobiosis is an effective means of preventing food spoilage (e.g. vacuum packing under nitrogen). For *S. cerevisiae*, the propagation of this species for yeast biomass for food and fermentation applications necessitates vigorous oxygenation (typically using molasses and fed-batch processes), whilst the subsequent employment of this yeast in baking or brewing necessitates very low levels of oxygenation (only at the start of fermentation). *S. cerevisiae* is able to tolerate these two fundamentally different situations of oxygen availability by using either respiratory or fermentative metabolism as appropriate to the prevailing growth conditions.

5.2.5 General Survival Mechanisms of Yeasts

During yeast propagation and fermentation processes in food and beverage production, yeast cell survival is paramount to ensure efficient bioconversion of substrate to biomass or metabolite. In food or beverage storage, however, the opposite is true in that yeast survival is undesired. In essence, yeast cell death needs to be minimised during industrial fermentations to maintain culture viabil-

ities at high levels; whilst on the other hand, yeast cell death needs to be maximised to eradicate undesired yeasts in foods and beverages. Yeasts will obviously die if confronted with excessive heat, extreme cold, high-voltage electricity, ionising radiation, reactive oxygen species, and high hydrostatic and osmotic pressures. Several physical treatments can be used to eradicate food and beverage spoilage yeasts. Some food spoilage yeasts are listed in Table 5.1 and include *Cryptococcus laurentii* and *Candida zeylandoides* (frozen poultry), *Z. bailii* and *Z. rouxii* (fruit and vegetables) *Kluyveromyces* spp., *Rhodotorula* spp. and *Candida* spp. (dairy products) and those spoiling fermented beverages include wild strains of *Saccharomyces* (e.g. *S. diastaticus*), and species of *Candida*, *Debaryomyces*, *Torulaspora*, *Pichia*, *Hanseniaspora*, *Metschnikowia*, *Szhizosaccharomyces* and *Zygosaccharomyces*. Such yeasts may be eradicated by heat (e.g. beer is commonly pasteurised at 70°C for 20 s) or by high-pressure–low-temperature treatments that have potential applications as an alternative to heat in the sterilisation of foods. Spoilage yeasts may also be prevented using chemical preservatives such as the weak acids and sulphur dioxide (Sect. 5.4.5).

Yeast cell death results when cellular damage caused by severe physical or chemical stress overcomes yeast cells' protection responses. These responses, which will now be discussed, are multifarious and involve physiological and molecular genetic level adaptations to ensure yeast survival in the face of environmental insults.

5.3 Yeast Responses to Physical Stresses

5.3.1 Temperature Stress

Table 5.3 summarises some of the general adverse influences of high-temperature stress on yeast cell physiology. Yeasts cannot regulate their internal temperature and thermal stress causes cellular damage by disrupting hydrogen bonding and denaturing proteins and nucleic acids, leading to rapid loss in yeast cell viability. Intrinsically thermotolerant yeasts possess T_{opt} values above 40°C and are able to survive sudden heat shock temperatures (e.g. to 50°C), whereas induced thermotolerance occurs when cells are preconditioned by exposure to a mild heat shock (e.g. 30 min at 37°C) prior to a more severe heat shock. pH also influences yeast thermotolerance and Coote et al. (1991) have provided evidence which implicates alterations in intracellular pH as the trigger for acquisition of thermotolerance in *S. cerevisiae*. Cells growing quickly in a glucose-rich medium are more heat-sensitive than stationary-phase cells, perhaps owing to glucose repressing the synthesis of stress-defence proteins.

Yeast cells exhibit a heat shock response when exposed to transient, sublethal temperatures. This involves induction of synthesis of a specific set of proteins, the highly conserved Hsps. Table 5.4 summarises the major Hsps of *S. cerevisiae*. Note that these proteins are also induced when yeasts are exposed to stresses other than heat shock (Piper 1997), meaning that they should more correctly be referred to as stress proteins.

Table 5.3 General effects of high temperature on yeast cell physiology

Physiological function	Comments
Cell viability	At the highest growth temperature of many yeasts, there is also appreciable cell death. At supramaximal growth temperatures, thermal death rate is exponential
General cell morphology	Atypical budding, irregular cell wall growth and increased cell size
Cell division and growth	Growth of non-thermotolerant yeasts are inhibited at temperatures above 40°C. Actively dividing cells in the S phase are more thermosensitive compared with resting cells. Heat shock transiently arrests cells in the G1 phase of the cell cycle
Plasma membrane structure/function	Increased fluidity and reduced permeability to essential nutrients. Ergosterol is known to increase thermotolerance. Decrease in unsaturated membrane fatty acids. Stimulation of ATPase and RAS-adenylate cyclase activity. Decline in intacellular pH
Cytoskeletal integrity	Extensive disruption of filaments and microtubular network
Mitochondrial structure/function	Decrease in respiratory activity and induction of respiratory-deficient petite mutants. Aberrant mitochondrial morphology
Intermediary metabolism	Inhibition of respiration and fermentation above T_{max}. Immediate increase in cell trehalose and MnSOD following heat shock
Protein synthesis	Repression of synthesis of many proteins, but specific induction of certain heat shock proteins. Mitochondrial protein synthesis more thermolabile than cytoplasmic
Chromosomal structure/function	Increased frequency of mutation of mitotic cross-over and gene conversion. Increased mitotic chromosomal non-disjunction. Inefficient repair of heat-damaged DNA

Several Hsps contribute to yeast thermotolerance by acting as molecular "chaperones" to prevent protein aggregation (Morano et al. 1998). They also promote protelolysis of aberrant stress-damaged proteins. The molecular basis of the heat shock response in *S. cerevisiae* fundamentally involves increased transcription of heat shock element (HSE) genes induced by a sublethal heat shock, but not by other stresses (e.g. osmostress, oxidative stress, DNA damage). The activation of the heat shock transcription factor (HSF) in *S. cerevisiae* does not appear to be required to induce tolerance against severe stress, but is required for high-temperature growth. Although Hsps may not play direct roles in conferment of yeast thermotolerance, Hsp104 does appear to ameliorate effects of thermal stress in respiring cultures of *S. cerevisiae* (Lindquist and Kim 1996) but in brewing strains under industrial fermentation conditions the role of Hsp104 in conferring stress resistance has been questioned (Brosnan et al. 2000). Other Hsps and stress proteins appear

Table 5.4 Major heat shock proteins of *S. cerevisiae*

Heat shock protein	Proposed physiological function
Hsp104	Acquisition of stress tolerance. Constitutively expressed in respiring, not fermenting cells and on entry into stationary phase
Hsp83	Chaperone function
Hsp70 family	Interact with denatured, aggregated proteins and assists in solubilising them with simultaneous refolding (i.e. chaperone function). Also involved in post-translational import pathways
Hsp60	Similar to Hsp70. This chaperone family facilitates post-translational assembly of proteins
Small Hsps – Hsp30, Hsp26, Hsp12	Cellular role still elusive, but may be involved in entry into the stationary phase and the induction of sporulation. Hsp30 may regulate plasma membrane ATPase
Others	
Ubiquitin	Responsible for much of the turnover of stress-damaged proteins
Some glycolytic enzymes	Enolase (Hsp48), glyceraldehyde 3-phosphate dehydrogenase (Hsp35) and phosphoglycerate kinase
Catalase	Antioxidant defence
GP400 and P150	Secretory heat shock proteins (unknown function)

Information from Watson (1990), Mager and Ferreira (1993), Parsell and Lindquist (1994), Mager and De Kruijff (1995) and Tsiomemko and Tuymetova (1995)

constitutively expressed in ale-brewing strains of *S. cerevisiae* in production-scale fermentation conditions (Kobi et al. 2004).

Estruch (2000) and Trott and Morano (2003) have reviewed molecular-level responses of yeasts to heat shock and other stresses. The exact sensing mechanism to activate the heat shock response is not clear. There is evidence that cells may sense heat shock via the accumulation of thermally misfolded proteins. Addition of the imino acid analogue azetidine-2-carboxylic acid (AZC) to yeast cells causes reduced protein stability and strongly induces heat shock factor regulated genes. This induction is dependent on the heat shock factor, indicating that this factor is activated by the presence of the misfolded proteins (Trotter et al. 2002). Addition of azidothymidine (AZT) also resulted in a strong reduction of the expression of ribosomal protein genes, which is also a HSF-dependent process. The sensing of the misfolded proteins seems to be specific for the HSF since the STRE-regulated genes are not strongly induced. Apart from the misfolded proteins other pathways, not activated by misfolded proteins, have been suggested as being important for the heat shock induction (Kamada et al. 1995). The best studied is the cell integrity pathway which senses thermal stress through the plasma membrane sensors Hcs77 and Mid2 (Gray et al. 1997; Verna et al. 1997).

Well-characterised components of the heat shock response are the transcription factors that are required to induce or repress heat shock regulated genes. These include transcription factors that are specific for the heat shock response, such as the HSF (Hsf1), or transcription factors that respond to a variety of cellular and environmental stress conditions, such as the Msn2 and Msn4 transcription factors. The Hsf1 activates gene expression through binding of the HSE, nGAAn, in the promoter of regulated genes, whereas the Msn2/Msn4 transcription factors bind the STRE, CCCCT (Bienz and Pelham 1986; Marchler et al. 1993). The binding of HSF to the HSE is cooperative, and some deviations from the canonical NGAAN sequence are tolerated in functional HSEs. The minimum requirement for recognition by HSF seems to be three canonical inverted repeats of this sequence, since it forms a homotrimer. Chromatin immunoprecipitation combined with microarray analysis identified approximately 165 *in vivo* targets for HSF. Thirty percent of these genes are also induced by the diauxic shift (Hahn and Thiele 2004).

Recently new components important for the signal transduction pathway inducing HSE-mediated gene expression have been identified. These include the ubiquitin ligase Rsp5 and Bul1/Bul2, two homologous proteins interacting with Rsp5 (Kaida et al. 2003). Mutagenesis analysis of the interacting part indicated that these three proteins function as a complex and there is also evidence that this complex functions independently from the Hsf1. As the bul1bul2 double mutant is sensitive to various stresses, such as high temperature, high salt and non-fermentable carbon sources, these proteins may be involved in the general stress response. How this Rsp5–Bul1/2 complex exerts its effect at the level of gene expression remains to be determined.

The question remains how partially denaturated proteins or changes at the level of the cell wall can activate the HSF. It has long been known that Hsf1-regulated response is a transient response, despite the continuous presence of stress. This indicates that Hsf1 activity must be controlled in a very precise manner. In contrast to the situation in metazoan HSF activation, the yeast Hsf1 transcription factor is constitutively bound to the promoters of regulated genes. Many of the regulated genes are also expressed under non-stress conditions and that is why *HSF1* is an essential gene (whereas Msn2 and Msn4 are non-essential genes). In contrast to Msn2 and Msn4, of which activity is regulated by nuclear translocation and DNA binding, the Hsf1 transcription factor can only be regulated by derepression or by activation. Under high-temperature conditions, Hsf1 is activated by phosphorylation (Sorger and Pelham 1988; Wiederrecht et al. 1988). In addition heat shock and the superoxide-generating agent menadione induce distinct patterns of HSF phosphorylation (Liu and Thiele 1996), suggesting that different stresses may cause differential phosphorylation.

Apart from heat stress, Hsf1 activity is also regulated by glucose starvation (Amoros and Estruch 2001). Recently it was shown that Hsf1 and Snf1 interact *in vivo* and that Hsf1 is phosphorylated by Snf1 *in vitro* (Hahn and Thiele 2004). In response to glucose starvation, HSF undergoes phosphorylation in an Snf1-dependent manner. This may indicate that different kinds of stress may activate different kinases that then activate HSF. Differential phosphorylation of HSF by stress-specific kinases might allow sophisticated levels of control of HSF activity to induce a subset of targets in response to specific stress signals.

One form of Hsf1 activation by heat stress is through a conformational change. The HSF contains a DNA binding and trimerisation domain, a repression element (CE2), a C-terminal modulator (CTM) and two transcription activation domains (AR1 and AR2), located at the N and C termini, respectively. Under normal conditions these two activation domains are repressed by interaction with the central regulatory domains and upon heat shock, this interaction is broken and the activation domains become functional (Chen and Parker 2002).

A further level of fine-tuning the response to heat shock is at the level of the HSE. The C-terminal modulator domain of the yeast HSF is required for the activation of genes containing atypical HSE but not typical HSE. This CTM domain is responsible for the hyperphosphorylation of HSF upon heat shock. It is required for activation of genes that have atypical HSE elements in their promoter (Hashikawa and Sakurai 2004). CTM is thought to modulate the activator function of ScHsf1 depending on the architecture of the HSE.

Yeast cells also respond to heat shock by accumulating other stress-protective compounds such as trehalose (Ertugay and Hamamci 1997; Neves and François 1992). Trehalose functions as a thermoprotectant and a cryoprotectant by stabilising cell membranes and accumulates markedly in cells exposed to a non-lethal heat shock (reviewed by Bonini et al. 2004). Together with Hsp104, trehalose acts synergistically to confer thermoprotection in *S. cerevisiae*. Recently a new mechanism of interaction between trehalose and Hsf1 was described. The α-helical content (and therefore the transactivating capacity) of the C-terminal activation domain of Hsf1 can be increased by the addition of trehalose but not by the addition of sucrose. During a heat shock both trehalose and Hsf1 are induced and it seems that in addition trehalose modifies the structure of the C-terminal activation domain (Bulman and Nelson 2005).

Heat shock may also enhance oxidative damage caused by oxygen free radicals, resulting in stimulation of the antioxidant enzymes catalase and superoxide dismutase. The polyamines spermine and spermidine also play important roles in thermal protection of *S. cerevisiae* cells by stabilising the structural integrity of yeast membranes during thermostress.

There is functional overlap between thermal, oxidative and ethanol stress responses of yeasts (but not necessarily between thermal and osmotic stress responses). It should also be noted that combined stresses, commonly encountered in industry, may act synergistically to affect yeast survival (Piper 1995). For example, high ethanol concentration together with high temperature may negatively affect yeast growth and metabolism during beverage fermentations. This would be particularly problematic if large-scale fermentation vessels were not properly cooled.

Cold stress in yeasts affects yeast cells in different ways (Table 5.5) and understanding this is important for controlling the psychrophilic yeast spoilage of foods, cryopreserving yeast stock cultures and maintaining viability of stored yeasts. The last of these is important in brewing when yeasts are cold-stored for later pitching into fermenters – a practice that can adversely affect subsequent fermentation performance (Boulton et al. 1989). Brewer's yeast with elevated levels of trehalose is able to maintain cell viability in cold storage conditions (4°C for several days in 5% v/v ethanol). Cryopreservation of yeasts in liquid nitrogen (−196°C) is a suitable

Table 5.5 Influence of low temperature on yeast cell physiology

Low-temperature stress	Yeast cells' response
Low-temperature exposure (e.g. 15°C)	Reduced sterol synthesis and increased polyunsaturated fatty acids in the cell membrane
Cold shock (e.g. 4–10°C)	Cell division arrest. Differential protein biosynthesis
Freezing	Uniform cell shrinkage. Vacuolar membrane damage
Freeze–thaw stress	Resistance of stationary phase cells. Antioxidant responses (due to reactive oxygen species generated during thawing)
Cryo-stress (liquid nitrogen, –196°C)	Significant induction of genes encoding proteins involved in cellular defence, energy transduction and metabolism

method for long-term maintenance of stock cultures but freezing and thawing does constitute a considerable stress and also causes severe mechanical injury to cells. Yeast cells do respond to such stress at a molecular level and it has been shown using differential hybridisation (Kondo and Inouye 1991) and more recently by DNA microarrays (Odani et al. 2003) that specific genes are induced following cold shock and cryopreservation treatments of *S. cerevisiae*, respectively. Concerning the latter, the genes in question encode a variety of "cell rescue" proteins that may be involved in repairing damage to the yeast cell envelope and to cellular organelles.

Similar to an adaptation to high temperatures, yeast cells can also adapt to low temperatures. Exposure of yeast cells to a temperature of 10°C reveals that there are two groups of transcriptional responses. A cold-adaptation-specific early response and a late cold response, which largely overlaps with the general environmental stress response as it is also dependent on Msn2 and Msn4 (Schade et al. 2004). Genes such as *NSR1* (nucleolin-like protein), *TIP1*, *TIR1* and *TIR2* (serine- and alanine-rich cell wall proteins) are all part of the early response. Genes that are expressed in the late response largely overlap with the genes expressed during the near-freezing response. The near-freezing response is controlled by the Msn2/Msn4 transcription factors and is similar to the late cold response (cells are conditioned for an even colder temperature). The long-term cold adaptation enhances survival at lower or even freezing temperatures. This is mainly because of the strong accumulation of trehalose and molecular chaperones such as Hsp104, Hsp42, Hsp12 and Ssa4 (Kandror et al. 2004). The mRNAs for these proteins are also stabilised more at these low temperatures.

Specific freeze-stress protection mechanisms include the production of antifreeze proteins and ice nucleators. Antifreeze proteins (AFPs) cause thermal hysteresis where they lower the freezing point without affecting the melting point. They also inhibit ice recrystallisation, a process that is the main cause of cell damage. Expression of natural fish AFPs as well as a chemically synthesised DNA fragment encoding an artificial antifreeze protein in *E. coli* has been demonstrated to improve both salt and freeze tolerance (Holmberg et al. 1994; Meijer et al. 1996). Ice nucleators (INAs) limit supercooling and induce freezing at high subzero

temperatures by mimicking the structure of an ice crystal surface. They establish protective extracellular freezing instead of lethal intracellular freezing (Zachariassen and Kristiansen 2000).

Frozen dough technology is a typical example of an industrial application where the freeze tolerance of the microorganism used is of paramount importance. When the flour is mixed with the yeast for the preparation of the dough, the yeast rapidly initiates fermentation and at the same time the freeze resistance of the yeast rapidly drops (Nagodawithana and Trivedi 1990; Rose and Vijayalakshmi 1993). To minimise the problem, freeze doughs are prepared by rapid mixing of the dough at low temperature. However, a minimal prefermentation of the dough appears to be necessary for a good quality of the bread (Hsu et al. 1979a, b; Richard-Molard et al. 1979). The rapid, striking transition from high freeze tolerance to high freeze sensitivity during the start-up of yeast fermentation constitutes a major obstacle for optimal use of the frozen dough technology. Attempts to obtain strains better suitable for use in frozen doughs have been based on the few microbial freeze-tolerance mechanisms identified so far, and have stimulated further research to unravel new underlying mechanisms. The rapid drop in freeze tolerance during the start-up of yeast fermentation also provides an unique model system to identify novel mechanisms involved in freeze tolerance because of the rapid and dramatic change in freeze tolerance over a short time period within the same organism. Screens to identify mutants that do not show the fermentation-induced loss of freeze resistance have been developed (Teunissen et al. 2002; Van Dijck et al. 2000). Recent genome-wide gene expression analyses and Northern blot analyses of freeze-tolerant and freeze-sensitive yeast strains have revealed a correlation between freeze tolerance and expression of the aquaporin encoding genes *AQY1* and *AQY2* (Tanghe et al. 2002). This relationship was confirmed by deletion and overexpression of *AQY1* and *AQY2*, clearly reducing and enhancing yeast freeze tolerance, respectively (Tanghe et al. 2002). Recently, similar results were obtained for the *Candida albicans* and *Schizosaccharomyces pombe* aquaporin genes (Tanghe et al. 2005a, b). Whereas there is a clear correlation between the level of aquaporin expression and freeze tolerance, this correlation is only present under rapid freezing conditions (Tanghe et al. 2004). Normal 800-g frozen dough prepared with a yeast strain overexpressing aquaporin genes did not show improved leavening capacity after thawing, whereas this was clearly the case in 0.5-g doughs. By using various fast and slow freezing conditions, Tanghe and coworkers could nicely show that more aquaporins in the membrane are only beneficial during rapid freezing. Most probably under slow freezing conditions, there is enough time for dehydration of the yeast cells in order to prevent intracellular ice crystal formation (Tanghe et al. 2004).

Yeast cells that have a higher intracellular content of glycerol, either by growing them in glycerol medium or by deletion of the Fps1 glycerol transporter, acquire tolerance to freeze stress and retain high leavening ability in dough after frozen-storage (Izawa et al. 2004, 2005).

Freeze tolerance has also been shown to correlate with the accumulation of specific amino acids. A dominant mutation in the *PRO1* gene, encoding γ-glutamyl kinase, which catalyses the first step in L-proline biosynthesis from L-glutamate, results in L-proline accumulation and a mutation in the *CAR1* gene, encoding

arginase, results in arginine and/or glutamate accumulation (Morita et al. 2003; Shima et al. 2003). Strains harbouring these mutations are more freeze-tolerant.

Other yeast species that are more tolerant to freeze stress have been used to study the cellular and biochemical basis of freeze tolerance. All cells of *Torulospora delbrueckii* strain PYCC5323, isolated from traditional corn and rye bread dough, survive long periods of freezing, whereas under similar conditions only 20% of *S. cerevisiae* cells survive (Hernandez-Lopez et al. 2003). The biochemical basis for the higher stress tolerance was its preservation of the plasma membrane integrity by a lower increase in lipid peroxidation and a higher resistance to H_2O_2. Interestingly, the higher freeze stress tolerance was not based on the trehalose level (Alves-Araujo et al. 2004). The high freezing resistance depends on a period of slow freezing during which the cells adapt their metabolism. This is also the reason why de novo protein synthesis is required. This phenotype, together with the fact that the growth and fermentation capacity of *T. delbreuckii* similar to that of *S. cerevisiae*, holds promise that this strain may become the best strain for freeze-tolerant yeasts for the frozen dough market. Previously, other groups had isolated *Torulaspora* strains from nature and showed that they were freeze-tolerant (Hahn and Kawai 1990).

5.3.2 Reduced Water Availability

Severe water stress (i.e. reduced water availability) occurs when yeast cells are dehydrated, for example, using spray and drum or fluidized-bed driers for use in production of dried yeast for the food and fermentation industries. Dehydration causes a rapid efflux of water through the cell membrane, resulting in collapse of the cytoskeleton. To compensate for this, cells adapt by recruiting intracellular water from the vacuole into the cytoplasm and by synthesising compatible solutes such as glycerol. This anhydrobiosis may deleteriously affect yeast cell physiology by altering the structure and function of vacuolar, nuclear and cell membranes. Remarkably, growth arrest resulting from such changes is reversible and the aim of dried yeast manufacturers is to maintain cell viability as high as possible following rehydration. This causes water influx into cells, increasing turgor pressure, and yeasts with high levels of the osmolytes trehaolse (Eleutherio et al. 1995) or glycerol (Hohmann 1997) appear more resilient to dehydration–rehydration stresses. Bauer and Pretorius (2000) have reviewed these stresses with regard to wine yeasts.

Hyperosmotic shock results in a loss of cell turgor pressure and a rapid decrease in cytoplasmic water content and cell volume (i.e. cells shrink). Conversely, hypoosmotically shocked cells increase in volume owing to the high water permeability of the plasma membrane (they also lower intracellular levels of potassium and glycerol).

Mild water stress in yeasts occurs during osmostress caused either by hypersomotic shock by increasing the solute concentration, or hypoosmotic shock by reducing the solute concentration. Note that both ethanol and heat shock may cause water stress in yeasts by reducing intracellular water (Halsworth 1998; Piper 1995). Generally speaking, *S. cerevisiae* cells are moderately resilient to short-term osmostress with regard to cell survival and growth tolerance. Osmotolerant yeasts are able to adapt to conditions of very low water potentials and are found in natural solute-rich habitats (e.g. honey, tree exudates) and as spoilers of salt-rich or sugar-rich foods.

There are various physiological responses of yeasts to osmostress, including efflux of intracellular water, rapid reduction in cell (and vacuolar) volume, transient increase in glycolytic intermediates, and accumulation of osmolytes in the cytosol. Loss of intracellular water induces the synthesis of osmolytes (or compatible solutes) that are able to restore cell volume, stabilise membrane proteins and maintain enzymatic activity. Glycerol is the major compatible solute in yeasts and can effectively counterbalance the loss of water due to osmotic stress. Increased levels of intracellular glycerol can be the result of increased uptake, increased biosynthesis, increased retention and decreased dissimilation, as summarised in Fig. 5.2. Glycerol is synthesised during glycolysis by reduction of dihydroxyacetone phosphate to glycerol 3-phosphate by glycerol 3-phosphate dehydrogenase (GPD), the activity of which is increased under osmotic stress. When cells are osmotically stressed, there is a requirement for cytosolic NADH that is met by decreased reduction of acetaldehyde to ethanol together with increased oxidation to acetate.

Osmotolerant yeasts like *Z. rouxii* are able to retain their cell volume in media of low water potential and in this yeast an active glycerol transport mechanism enables retention of glycerol as part of an osmotic stress response. As shown in Table 5.6, other polyols (arabitol, sorbitol, mannitol and erythritol), ions (e.g. K^+) and the disaccharide trehalose may accumulate in yeast cells in response to water stress caused by specific solutes. Note that stress induced by high salt (NaCl) conditions creates two different phenomena: ion toxicity and osmotic stress (Serrano et al. 1997). Defence responses to salt stress are based on osmotic adjustments by glycerol synthesis and cation transport systems for sodium exclusion.

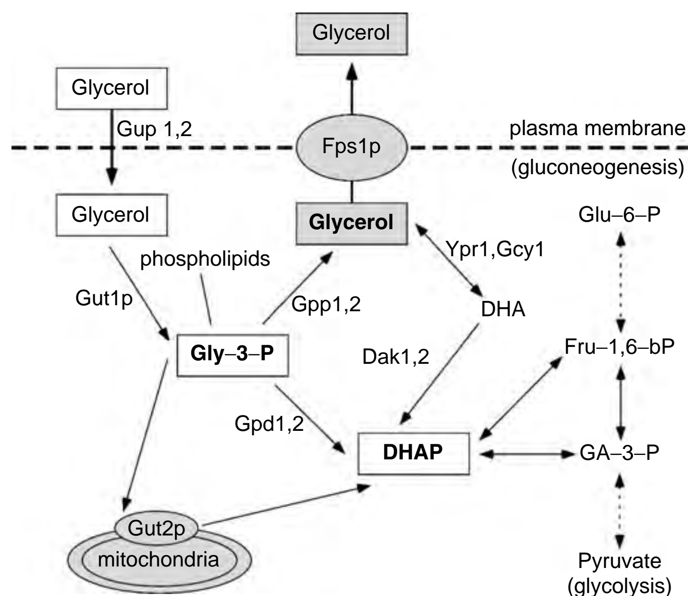

Fig. 5.2. Summary of glycerol uptake and utilisation by yeast

Table 5.6 Responses of yeasts to changes in media osmotic potential

Yeast	Treatment[a]	Intracellular solute
S. cerevisiae	Upshock with NaCl	K^+, Na^+ glycerol and trehalose increased
	Upshock with sorbitol	Glycerol initially, then trehalose increased
Z. rouxii	Upshock with NaCl	Glycerol, arabitol increased
	Upshock with glucose	Arabitol initially, then trehalose increased
D. hansenii	Upshock with various solutes	Glycerol increased
	Downshock by reducing NaCl concentrations	Glycerol decreased

Adapted from Jennings (1995)
[a]Upshock refers to the lowering of osmotic potential in the medium, whereas downshock refers to the raising of osmotic potential due to decreasing solute concentrations

The molecular basis of osmotic stress responses in yeast cells is now well characterised, particularly in *S. cerevisiae*. In this yeast, a variety of stress conditions (heat shock, UV irradiation, osmostress, etc.) induce transcription of genes containing STREs to enable cells to grow under potentially lethal conditions. A signalling system specific to osmostress called the high osmolarity glycerol (HOG) pathway activates a mitogen-activated protein kinase (MAPK) cascade (Fig. 5.3).

The pathway starts with the activation of cell-membrane-bound receptor proteins (Sho1, Msb2 and Sln1) that act to sense the external osmolarity and ends with synthesis of osmoprotectants such as glycerol (Cullen et al. 2004; Hohmann 2002). These osmosensors are encoded by genes (e.g. *SLN1*) which are upstream regulators of the HOG pathway. The Sln1 branch is required to induce the expression of several reporter genes in response to very high solute levels and this indicates that the Sln1 branch operates over a broader range of osmolarities than the Sho1/Msb2 branch. Under osmotic stress, Sln1 leads to phosphorylation of a downstream target protein Ypd1, which continuously transfers a phosphate group to the response regulator protein Ssk1. This pathway activates two partially redundant mitogen-activated protein kinase kinase kinases (MAPKKK) Ssk2 and Ssk22. The Sho1/Msb2 branch requires Cdc42, Ste20 and Ste50 to activate the MAPKKK Ste11. Any of the three MAPKKKs is able to activate Pbs2, which then phosphorylates Hog1. This MAPK regulates the expression of numerous genes by controlling the activity of several transcription factors (activators and repressors) (Saito and Tatebayashi 2004; Tamas and Hohmann 2003; Westfall et al. 2004).

Hog1 dual phosphorylation by the mitogen-activated protein kinase kinase (MAPKK) Pbs2 results in its nuclear localisation and causes regulation of gene expression through several transcription factors, Hot1, Sko1, Smp1, and probably also through Msn1, Mns2 and Msn4 (Gasch et al. 2000; O'Rourke and Herskowitz 2004; Rep et al. 2000). About 580 genes are regulated by Hog1, some of which require the general stress response to be fully activated. Genes such as *CTT1*, *ALD2*

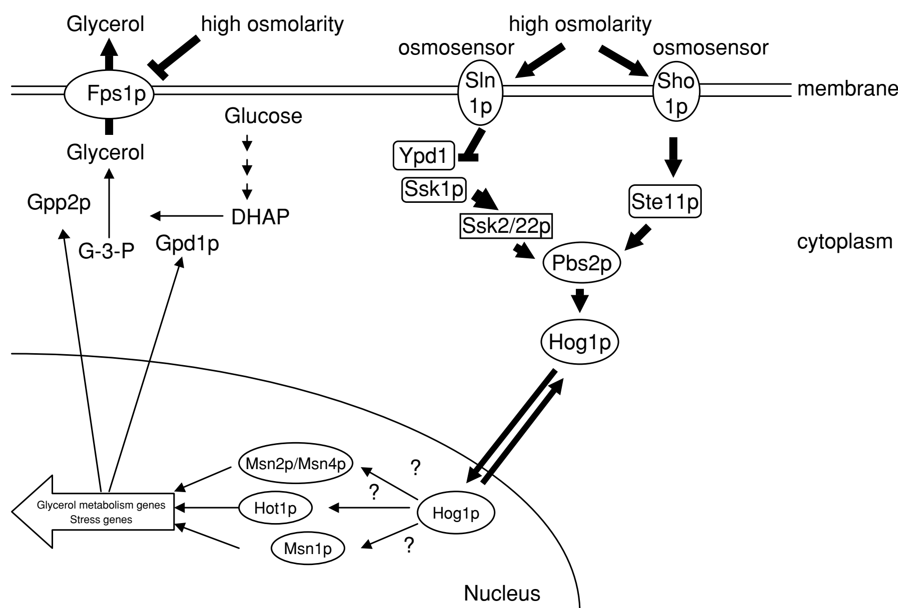

Fig. 5.3. The high osmolarity glycerol (HOG) mitogen-activated protein (MAP) kinase cascade pathway

and *HSP26* require both a HOG-dependent process and the general stress-response pathway. This is in contrast to genes such as *STL1* and *GRE2*, which are directly regulated by Hog1 and the two transcription factors Hot1 and Sko1. Activation of Hog1 also results in a transient cell-cycle arrest in the G1 phase of the cell cycle. Escoté and coworkers have recently shown that the Sic1 cyclin-dependent kinase inhibitor is a direct target of Hog1. Hog1 phosphorylates Sic1, which results in a stabilisation of this protein. This together with the downregulation of cyclin expression results in cell-cycle arrest under stress conditions which allows cells to recover before they progress into the S phase (Escoté et al. 2004).

Hog1 regulates gene expression through interaction with the Rpd3–Sin3 histone deacetylase complex by recruiting this complex to the promoters of regulated genes. Histone deacetylation of the promoters allows proper transcription by the RNA polymerase II under stress conditions (de Nadal et al. 2004).

Recently, a new and physiologically more relevant function for Hog1 was put forward by the group of Peter Coote when they characterised the response of *S. cerevisiae* to citric acid. This organic acid occurs naturally in the juice of lemons and other sour fruits, a habitat populated by different species such as *Candida parapsilosis*, *Candida stellata*, *S. cerevisiae*, *Torulaspora delbrueckii*, and *Z. rouxii* (Lawrence et al. 2004). Functional screening of the yeast genome deletion set, combined with whole genome transcript and proteomics analysis have revealed that the HOG pathway plays a key role in the adaptation to citric acid stress. Only 13% of the genes

whose expression was induced upon citric acid addition displayed a citric acid sensitive phenotype when the corresponding gene was deleted. Addition of citric acid rapidly results in phosphorylation and activation of Hog1 and deletion of *HOG1* (and also of other components of the HOG pathway, such as *PBS2* and *SSK1*) makes the strain sensitive to citric acid. Citric acid does not cause osmotic stress but it does induce a general stress response and glycerol biosynthesis (Lawrence et al. 2004).

Recently, methylglyoxal (MG), a toxic glycolytic metabolite, has been suggested as the putative signal initiator of the Sln1 branch of the HOG pathway. Hog1 is rapidly phosphorylated and activated upon treatment of the cells with low concentrations of MG. Further investigation has also shown that MG activates the Msn2 transcription factor and stimulates the uptake of Ca^{2+} in yeast cells, thereby activating the calcineurin/Crz1-mediated pathway (Maeta et al. 2005).

The MAPK cascades transduce signals in yeasts that are triggered not only by osmotic stress but also by other environmental stresses and external stimuli such as nutrient availability and the presence of growth factors or mating pheromones. There is also considerable crosstalk between different MAPK cascades and recently it was shown that different interaction regions on the Sho1 sensor for either Ste11 or Pbs2 may result in the crosstalk between the osmotic stress sensing pathway and the pheromone pathway (Zarrinpar et al. 2004). In the absence of Pbs2 or Hog1, osmotic stress leads to activation of the MAPKK of the pheromone pathway, and induction of genes normally only expressed in response to the mating pheromone (Rep et al. 2000).

In addition to glycerol, the disaccaride trehalose plays an important role in protecting yeasts against water stress and this has relevance in the baking and brewing industries. For example, baker's yeast with elevated trehalose levels (more than 10% of dry weight) is relatively resistant to the drying process in terms of retention of leavening capabilities. Similarly, baker's yeast with a high trehalose content maintains its viability in frozen doughs (but refer to the previous discussion). Baker's yeast manufacturers therefore employ measures to minimise trehalose loss (e.g. through endogenous fermentation) in baking strains of *S. cerevisiae*.

In brewing yeasts, osmotic stress is encountered when cells are pitched into high-gravity malt wort (e.g. 18°P) and this may result in slow or incomplete fermentation. Intracellular trehalose in brewing yeasts may serve an important role as an osmoprotectant (and stress indicator) during very high gravity fermentations by *S. cerevisiae*. Trehalose acts by stabilising membranes against osmotic shock and trehalose accumulation in stationary phase cells is thought to be partly due to their increased osmotolerance compared with that of actively dividing cells. Trehalose is now widely recognised as a general "stress metabolite" in yeasts since it has been shown to act in yeasts not only as an osmoprotectant, but also as an antidessicant, a cyroprotectant, a thermoprotectant and a chemical detoxicant.

5.3.3 pH Stress

pH_2 stress is encountered by brewing yeasts when they are acid-washed at about pH_2 to eliminate spoilage bacteria prior to fermentation. Phosphoric acid is normally used, rather than organic acids (e.g. acetic acid, lactic acid), which are more inhibitory to yeasts owing to undissociated organic acids lowering intracellular pH following cell

membrane translocation. This is the basis of the action of weak acid preservatives in inhibiting food spoilage yeast growth (Sect. 5.4.5).

5.3.4 Miscellaneous Physical Stresses

In large industrial fermentation vessels, yeasts may encounter stress due to both hydrostatic and gaseous pressure. For example, in brewing cylindro-conical fermenters are employed which create stressful environments for yeast cells owing to hydrostatic pressure and pressure due to endogenous CO_2 produced during fermentation. CO_2-induced pressure effects are exacerbated in the presence of ethanol, the other major fermentation metabolite, and may lead to yeast growth inhibition and production of beer off-flavours. *S. cerevisiae* is not particularly barotolerant and fails to grow at high hydrostatic pressures (above 10 MPa) owing to damage to the cytoskeleton and mitotic apparatus. Barotolerance in yeasts may be linked to trehalose accumulation.

For elimination of spoilage yeasts, the use of high pressure–low temperature as an alternative to heat sterilisation has potential in food preservation.

Yeasts suffer from radiation stress when exposed to UV radiation, γ-rays or X-rays, which cause DNA damage (such as dimerisation, nicks and lesions) and cell-cycle arrest in yeast cells. Ionising radiation effects on yeast cells are indirectly mediated by reactive oxygen species (generated by water radiolysis), which damage DNA and cell membrane function. Both *S. cerevisiae* and the fission yeast *Schizosaccharomyces pombe* have been widely studied as model eukaryotes in fundamental studies of DNA repair following radiation damage. In relation to yeast biotechnology, low doses of γ-radiation may enhanced alcoholic fermentation processes.

Mechanical and gravitational stresses may be encountered by yeast cells during industrial processes such as agitation in stirred-tank bioreactors or high-speed centrifugation. However, owing to the thick cell walls, *S. cerevisiae* is relatively resistant to mild shear stress and quite severe mechanical stress (e.g. glass bead homogenisation or high-pressure extrusion) is needed in order to rupture the cell walls to extract cell components. Similarly, ultrasound, which is capable of rupturing bacterial cells, is generally ineffectual in rupturing *S. cerevisiae*, but may stimulate certain yeast-catalysed transformations (perhaps owing to increased flux of substrates through the cell membrane).

Application of electrical fields affects yeast cell membrane permeability and this is exploited during electroporation or electrofusion to transform yeasts with exogenous DNA. The amplitude, frequency and duration of electrical exposure is crucial for the success of such techniques in yeast recombinant DNA technology. Some experiments have shown stimulatory effects of electrical fields on yeast growth and metabolism, particularly with regard to fermentative activity.

5.4 Yeast Responses to the Chemical Environment

5.4.1 Nutrient Starvation

Yeasts deprived of essential nutrients will be cell division cycle arrested, generally in the unbudded G1 phase. In diploid cells of *S. cerevisiae*, nutrient starvation may

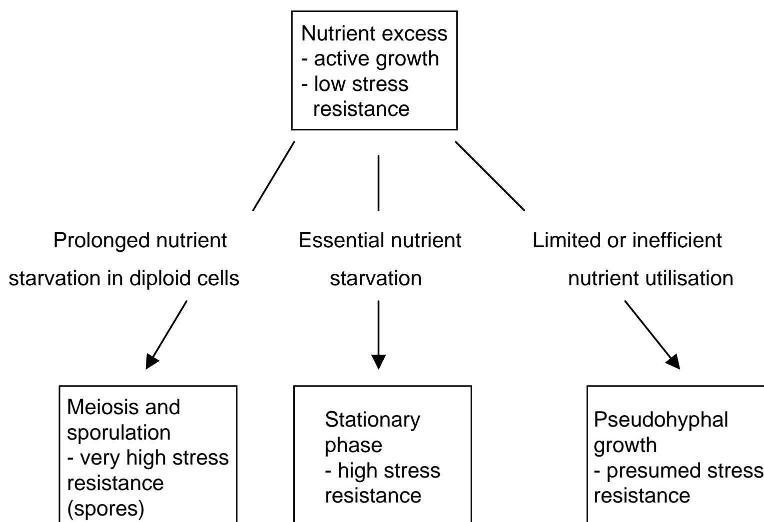

Fig. 5.4. General growth responses of *S. cerevisiae* to availability of nutrients

result in meiosis and sporulation, with formation of an ascus encasing very stress resistant haploid spores (Fig. 5.4). Although some yeasts may be described as oligotrophic and are able to grow in very low nutrient concentrations (Kimura et al. 1998), generally in yeasts nutrient exhaustion represents a stress that requires a cellular response. It is known that when yeast cells enter the stationary phase of growth, following depletion of nutrients, their cell walls thicken, they accumulate reserve carbohydrates (glycogen), they synthesise several stress proteins (Puig and Perez-Ortin 2000) and they generally become more stress-tolerant compared with actively dividing cells (Fuge and Werner-Washburne 1997). Conversely, it means that cells during the exponential phase of growth are less stress-tolerant. This would occur during fed-batch propagation of baker's yeast under conditions of high oxygenation and limited glucose supply (designed to avoid the Crabtree effect (Walker 1998). At the end of the propagation processes of baker's yeast, nutrient (molasses) supply is turned off to induce the biosynthesis of trehalose (Van Dijck et al. 1995) and stress proteins (Sales et al. 2000) that are required to maintain yeast viability and protect cells during subsequent dehydration.

With regard to beverage fermentations, if brewing yeasts suffer from prolonged periods of nutrient starvation in storage tanks, subsequent fermentation performance and flocculation capabilities may be adversely affected. In brewing, nutrient availability is key to efficient yeast metabolism and the best strains are those that adapt quickly to nutrient excess (during fermentation) following periods of nutrient starvation (during storage). Nutrient-related stress responses of wine yeasts have been discussed by Bauer and Pretorius (2000).

In *S. cerevisiae*, starvation of particular amino acids leads to the rapid inhibition of ribosomal RNA (rRNA) synthesis and is signalled when cells sense a rapid reduction in protein biosynthetic rate. In industrial strains of *S. cerevisiae*, if levels of

assimilable amino acids drop below certain threshold levels (e.g. around 150 mg/l), then fermentation may be curtailed prematurely. Also in these strains there is sequential uptake and utilisation of specific amino acids and this may lead to deficiency of certain amino acids at different stages of fermentation, necessitating their de novo biosynthesis. de Winde et al. (1997) have discussed specific molecular-level responses of *S. cerevisiae* to amino acid limitation and starvation.

The molecular response of nutrient limitation has mostly been studied after a switch from a richer to a poorer nutrient source (Winderickx et al. 2003). Yeast cells starved for a single essential nutrient will complete their current cell cycle and arrest in the next G1 phase at "START A". Subsequently, they will progress into an "off-cycle", G0 or stationary phase, in which they can survive nutrient starvation much longer than when arrested elsewhere in the cell cycle. They accumulate elevated levels of the storage carbohydrates glycogen and trehalose and expression of STRE- and PDS-controlled genes is induced. The transcription of ribosomal protein genes is repressed. The same phenotype is observed in cells growing on non-fermentable carbon sources, indicating that these phenotypes are not just a growth-arrest phenotype. Re-addition of nitrogen, sulphate or phosphate to cells starved of these nutrients, respectively, in each case causes within a few minutes a rapid, post-translational activation of the neutral trehalase enzyme (Hirimburegama et al. 1992). Nutrients modulate the critical size threshold, such that cells are large in rich medium and small in poor medium.

Recently, the mechanism by which nutrient availability is sensed and converted into a signal regulating cell cycle progression has been studied in great detail. Nutrient effects are mediated in part by the Ras–PKA pathway and by the TOR pathway. Both pathways seem to regulate the activity and cellular localisation of the transcription factor Sfp1. This transcription factor plays a role in the control of the cell size and regulates ribosomal protein gene expression. Under optimal growth conditions, Sfp1 is localised to the nucleus and regulates ribosomal protein gene expression but upon inhibiting TOR signalling, stress or changes in nutrient availability, Sfp1 is released from the ribosomal protein gene promoters and translocates to the cytoplasm (Jorgensen et al. 2004; Marion et al. 2004). Apart from Sfp1, also the fork-head-like transcription factor Fhl1 and two cofactors Ifh1 (a co-activator) and Crf1 (a co-repressor) are involved in ribosomal protein gene expression under the control of both TOR and PKA. Similar to Sfp1, its activity is regulated by nuclear-cytoplasmatic translocation (Martin et al. 2004). The exact mechanism of Sfp1 regulation and localisation remains to be determined.

The TOR pathway is also involved in other functions. Under nutrient limitation conditions, Tor activity is inhibited and this results in a block in translation initiation through inhibition of eukaryotic initiation factor 4E (eIF4E), in repression of ribosomal genes and rRNA and transfer RNA (tRNA) synthesis and in uptake of amino acids by degradation of high-affinity amino acid permeases. On the other hand, Tor inactivation causes an induction of several genes through nuclear localisation of different transcription factors (Gln3, Rtg1, Rtg3, Msn2 and Msn4) (Schmelzle and Hall 2000).

Apart from a general nutrient limitation response, various other signal transduction pathways exist that sense the nutrient availability. The carbon catabolite

repression pathway is a signalling cascade activated by carbon source depletion. Limitation of sugar availability stimulates the activity of the central protein kinase Snf1. Snf1 will then phosphorylate Mig1 and phosphorylated Mig1 will translocate to the cytoplasm, resulting in the derepression of Mig1-repressed genes in the presence of low levels of glucose. Under amino acid starvation conditions, the sensor kinase Gcn2 detects uncharged tRNAs and phosphorylates and thereby activates the α-subunit of eukaryotic initiation factor 2 (eIF-2) (Kubota et al. 2000). Phosphorylated eIF-2α inhibits general translation but selectively derepresses the synthesis of the transcription factor Gcn4 at the translational level (Grundmann et al. 2001). Gcn4 controls a network of amino acid biosynthetic pathways, genes involved in glycogen homeostasis, genes encoding protein kinases and transcription factors (Natale et al. 1993).

S. cerevisiae prefers inorganic phosphate (Pi) as the phosphorus source. In the presence of sufficient Pi in the medium, all genes required for the utilisation of organic compounds as a source for phosphorus are repressed. Upon Pi depletion, the transcription of some phosphate-starvation response genes such as *PHO5* (encoding a secreted acid phosphatase) is activated by the transcription factor Pho4. In the presence of high concentrations of Pi, Pho4 is negatively regulated through binding of the specific inhibitors Pho80 and Pho85.

An important phenotype observed under nutrient-limitation conditions is the morphogenetic switch from yeast cells to pseudohyphal cells. This occurs during nitrogen limitation on rich medium. Both diploid pseudohyphal development and haploid invasive growth are controlled by the activity of the cAMP–PKA pathway (via Tpk2), the MAPK pathway (via Kss1) and the glucose repression pathway (via Snf1) (Gagiano et al. 2002; Gancedo 2001; Truckses et al. 2004). This morphogenetic switch allows the yeast cells to forage for nutrients.

5.4.2 Ethanol Stress

Ethanol accumulation during fermentation represents a potent chemical stress towards yeast cells. Low ethanol concentrations are inhibitory to yeast growth and cell division, but higher concentrations can be lethal. *S. cerevisiae* (being the predominant fermentative microorganism) is quite ethanol-tolerant, with some strains able to produce over 20% v/v ethanol by glycolytic metabolism. Other yeasts die at around 5% ethanol. Therefore, a fundamental understanding of ethanol-induced toxicity and ethanol tolerance in yeasts is of distinct commercial significance for alcohol producers, especially those involved in bioethanol (fuel alcohol) processes. Ethanol tolerance may be defined as the ability of yeasts to withstand higher levels of ethanol without any deleterious effects on growth and metabolic activities. Table 5.7 summarises some effects of ethanol on yeast physiology.

Although ethanol passively diffuses out of yeast cells, the plasma membrane is the primary target of ethanol toxicity. Whilst ethanol does deleteriously affect many aspects of yeast cell physiology, increased membrane fluidity and disruption of membrane structural integrity represent major consequences of yeast cell exposure to toxic levels of ethanol. Dissipation of cellular pH gradients and inhibition of the proton pumping plasma membrane ATPase are major deleterious effects of ethanol

Table 5.7 Important effects of ethanol on yeast cell physiology

Physiological function	Ethanol influence/cellular response
Cell viability and growth	General inhibition of growth, cell division and cell viability Decrease in cell volume Induction of morphological transitions (e.g. promotion of germ-tube formation in *Candida albicans*) Enhancement of thermal death
Intermediary metabolism and macromolecular biosynthesis	Denaturation of intracellular proteins and glycolytic enzymes Lowered rate of RNA and protein accumulation Reduction of V_{max} of main glycolytic enzymes Enhancement of petite mutation Enhanced mitochondrial superoxide dismutase activity Induction of heat shock-like stress proteins Elevated levels of cellular trehalose Increase in oxygen free radicals Induced synthesis of cytochrome P450
Membrane structure and function	Decrease in membrane saturated fatty acids (e.g. palmitic) Increase in membrane unsaturated fatty acids (e.g. oleic) Acceleration of sterol biosynthesis (squalene, ergosterol) Induced lipolysis of cellular phospholipids Increased phospholipid biosynthesis (e.g. phosphatidyl inositol) Increased ionic permeability Inhibition of nutrient uptake Inhibition of H^+-ATPase and dissipation of proton motive force Uncoupling of electrogenic processes by promoting passive re-entry of protons and consequential lowering of cytoplasmic pH Hyperpolarisation of plasma membrane

on yeasts (Alexandre et al. 1994). Tolerance to ethanol may be induced in yeast cells following a sublethal heat shock and this indicates that the latter confers a degree of cross-protection at the level of membrane stabilisation. Interestingly, both heat shock and ethanol induce the biosynthesis of a set of common stress proteins and is part of the cells' *adaptive stress response*. Numerous physiological adaptations in yeasts confer protection against ethanol and many of these operate at the level of plasma membrane structural maintenance. For example, *S. cerevisiae* responds to ethanol by increasing fatty acyl chain length and the proportion of unsaturated fatty acids and sterols in the plasma membrane. Ethanol-stressed *S. cerevisiae* also accumulates trehalose, which can also stabilise membranes. That ethanol can induce the synthesis of oxygen free radicals is evident by increased activity of the antioxidant enzyme mitochondrial superoxide dismutase.

Several environmental factors, including media composition, play important roles in dictating yeast ethanol tolerance. For example, divalent cations, particularly Mg^{2+} ions, are able to act as stress protectants against both temperature stress and ethanol toxicity, presumably at the level of cell membrane stabilisation (Birch and

Walker 2000; Walker 1999). Lipid supplementation of fermentation media is also known to improve the ability of yeast cells to withstand otherwise toxic levels of ethanol.

The response of yeast cells to ethanol stress is very similar to the response of cells encountering a heat shock (Piper 1995). The main target of ethanol is the plasma membrane. The fluidity alters during ethanol stress, resulting in changes in permeability to ionic species, especially protons. Microarray analysis of yeast cells after treatment with ethanol (7% v/v) confirmed that the cells try to rescue themselves by altering the expression of genes involved in ionic homeostasis and energy metabolism (Alexandre et al. 2001). In addition there is an activation of the plasma membrane $H(^{+})$-ATPase protein (Piper 1995). The microarray analysis resulted in a large number of differentially expressed genes. About 3% of all genes were upregulated and a similar number of genes were downregulated (Alexandre et al. 2001). About half of these genes are regulated by the Msn2 and Msn4 transcription factors and are also regulated by other environmental stresses. Similar to what has been observed during heat stress, so far only for a few genes altered in gene expression, it has been shown that they are required for the tolerance to high concentrations of ethanol. These include Hsp104, the trehalose biosynthesis genes and the mitochondrial superoxide dismutase Sod2 and the alcohol-sensitive ring/plant homeodomain (PHD) finger protein Asr1 (Betz et al. 2004; Costa et al. 1997; Piper 1995). Apart from microarrays, metabolomics (Martini et al. 2004) and proteomics (Zhou et al. 2004) have also been performed after treatment of yeast cells with ethanol.

However, none of these studies resulted in the identification of an ethanol-specific stress response. Recently two important steps in elucidating ethanol-specific signal transduction mechanisms have been described. First, two-hybrid interaction studies using the nucleoporin Nup116 as a bait resulted in the identification of Asr1. The N-terminal region of Asr1 contains two regions with homology to the interesting new gene (Ring) type or PHD-type finger domain (Betz et al. 2004). These authors found that the localisation of Asr1 is specifically and rapidly regulated by the external level of different kinds of alcohols. Treatment of exponentially growing cells with 7.5% ethanol very rapidly (10 min) relocated the Asr1 protein to the nucleus in a reversible way. As mentioned before, Asr1 is required for ethanol tolerance but not for any other stress type.

Second, one of the phenotypes observed after heat or ethanol stress is the selective mRNA export from the nucleus. Whereas the majority of the mRNAs accumulate in the nucleus, Hsp mRNA is exported under such conditions. The mechanism behind this selective export system has recently been determined. In cells treated with 10% ethanol there is a rapid and reversible nuclear localisation of the DEAD box protein Rat8p. This change correlates very well with the blocking of bulk poly(A)(+) mRNA export. The nuclear localisation is caused by a defect in the Crm1p exportin, the same protein that also interacts with Yap1 to keep it in the cytoplasm under non-oxidative stress conditions (see further). Interestingly, the localisation of Rat8p did not change in heat shocked cells, indicating that there is a different response after heat shock than after ethanol stress (Takemura et al. 2004).

Both ethanol-specific responses open the possibility to characterise the specific signal transduction pathway involved. In addition to ethanol, other fermentation

metabolites are toxic to yeasts, including the other major fermentation product, carbon dioxide, together with secondary products like acetaldehyde.

5.4.3 Oxidative Stress

Oxidative stress causes damage to yeast DNA, proteins and lipids owing to reactive oxygen species such as the superoxide anion ($O_2^{-}\bullet$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^{\bullet}$). These species may be formed endogenously by mitochondrial respiration or exogenously by increasing dissolved oxygen tension of yeast growth media. Oxidative stress arises when yeast antioxidant defences are insufficient to maintain the intracellular redox balance. These defences include non-enzymic (e.g. glutathione, metallothioneins) and enzymic (e.g. catalases, superoxide dismutases) mechanisms (Table 5.8). Glutathione is a thiol compound that protects yeasts by scavangeing oxygen radicals and the metallothioniens can detoxify metal ions (e.g. copper) as wells as protect yeast cells against the damaging effects of oxidants. The antioxidant enzymes able to neutralise oxygen free radicals in yeasts include peroxisomal and cytosolic catalases (encoded by *CTA1* and *CTT* genes, respectively); mitochondrial and cytoplasmic superoxide dismutases (Mn Sod and Cu/Zn Sod, respectively encoded by *SOD2* and *SOD1* genes) and peroxidases (e.g. cytochrome c peroxidase). Interestingly, overexpression of catalase and superoxide dismutase genes increases yeasts' thermotolerance, indicating that oxidative stress may form the underlying basis of other environmental insults.

Pretreating yeasts with sublethal levels of oxidants confers protection owing to an adaptive oxidant stress response, in a similar way to conferment of thermotolerance by pre-heat-shocking cells. One of the adaptive mechanisms seems to be a decrease

Table 5.8 Main antioxidant defences of yeasts

Defence system	Function
Enzymes	
CuZn superoxide dismutase	Dismutation of superoxide anion (cytoplasm)
Mn superoxide dismutase	Dismutation of superoxide anion (mitochondria)
Catalase A	Decomposition of hydrogen peroxide (peroxisome)
Catalase T	Decomposition of hydrogen peroxide (cytoplasm)
Cytochrome c peroxidase	Reduction of hydrogen peroxide
Glutathione reductase	Reduction of oxidised glutathione
Chemicals	
Glutathione	Scavenging of oxygen free radicals
Metallothionein radicals	Cu^{2+}-binding, scavenging of superoxide and hydroxyl
Thioredoxin	Reduction of protein disulphides
Glutaredoxin	Similar function to thioredoxins, protection against H_2O_2
Polyamines	Protection of lipids from oxidation

Modified from Moradas Ferreira et al. (1996) and Estruch (2000)

in H_2O_2 plasma membrane permeability during adaptation to H_2O_2 (Branco et al. 2004). Heat shock will also confer protection against oxidative stress, indicating close interrelationships between various physiological stress responses in yeasts. The signal transduction pathways involved in this oxidative stress tolerance have recently been reviewed (Ikner and Shiozaki 2005).

The sensors to activate the pathways that control the oxidative stress adaptive response were first identified in *E. coli* and have resulted in the identification of specific H_2O_2 and O_2^- sensors, OxyR and SoxR, respectively (Zheng and Storz 2000). In contrast to most other sensors that are located at the plasma membrane, these sensor proteins are transcription factors, localised in the cytoplasm. Genome-wide expression analysis upon treatment of yeast cells with sublethal doses of H_2O_2 has resulted in more than 100 induced spots and about 50 repressed spots on two-dimensional maps and more than 900 induced genes and about 600 repressed genes by DNA microarray mRNA profiling (Causton et al. 2001; Godon et al. 1998). Most of these genes seem to be regulated by only three different transcription factors, functioning alone or in combination. These are Yap1, Skn7 and Msn2/4. These three transcription factors are not only activated by oxidative stress but also by various other types of stress.

Yap1 is important for oxidative, cadmium and drug stress responses. The oxidative and chemical stress sensing seems to occur directly at the level of the Yap1 protein and involves protein phosphorylation followed by cellular redistribution. It has recently been shown that Mtl1, an upstream activator of the PKC1–MAPK cell integrity pathway, may be a cell wall sensor for oxidative stress (Vilella et al. 2005). Addition of diamide or H_2O_2 results in actin cytoskeleton depolarisation. Mtl1, Rom2 and Pkc1 functions are all required to restore the correct actin organisation. Pkc1 is also required to overcome the effects of oxidative stress by enhancing the machinery required to repair the altered cell wall and to restore actin cytoskeleton polarity by promoting actin cable formation.

A hypothesis that has been proposed is that the signal transduction pathway that is activated upon oxidative stress may constitute the upper part of the cell wall integrity pathway which then at the level of Pkc1 may branch into the cell integrity MAPK pathway and into a specific oxidative stress induced pathway consisting of the downstream transcription factors Yap1, Skn7 and Msn2/Msn4. Although this may be true for some inducers of oxidative stress, such as diamide, it is not the case with H_2O_2, where no difference in the expression pattern of genes regulated by Yap1, Msn2/Msn4 or Skn7 between wild-type and *mtl1*Δ strains was observed.

Similar to what we have previously mentioned for Msn2 and Msn4, Yap1 cellular localisation is also dependent on the stress situation. Under non-stress conditions, Yap1 is rapidly exported out of the nucleus by interaction with the nuclear export protein Crm1 (Yan et al. 1998). Upon oxidative stress induction, the interaction between Yap1 and Crm1 is lost, probably by Yap1 phosphorylation, and Yap1 is rapidly redistributed to the nucleus, with the help of the nuclear import receptor Pse1 (Isoyama et al. 2001), where it can bind to Yap1 response elements (YRE) in the promoter regions of different genes.

Skn7 as well as Msn2 and Msn4 seem to be stress-response coordinators as they are involved in many types of stress.

5.4.4 Anaerobiosis

Most yeasts are aerobic and few tolerate strictly anaerobic conditions. *S. cerevisiae* has an absolute requirement for oxygen that is necessary for the synthesis of certain fatty acids and sterols This species is auxotrophic for oleic acid and ergosterol under strictly anaerobic conditions. Oxygen is also required as the terminal electron acceptor for yeasts' respiration. Yeasts can be placed in different groups based on their growth responses to oxygen availability (Table 5.9). The molecular basis of anaerobic stress tolerance and the signal transduction pathways involved have not yet been studied at the molecular level.

5.4.5 Yeast Biocides

In addition to the chemical stresses mentioned earlier, yeast cells may also be subjected to chemicals that are purposely designed to control their growth and metabolic activities. This is precisely the case when endeavouring to prevent growth of spoilage yeasts in foods and beverages. Such yeasts may be controlled by temperature, anhydrobiosis or by addition of yeast preservatives such as weak acids. These weak acids include sorbic, benzoic and acetic acids that have wide uses as anti-yeast agents in foods and beverages. These acids are transported into yeasts in their undissociated form and act by dissipating plasma membrane proton gradients and depressing cell pH when they dissociate into ions in the yeast cytoplasm.

To counteract the effects of weak acids, *S. cerevisiae* is endowed with a stress response that acts to reduce the possibility that the weak acid will accumulate within its cells to potentially toxic levels. The high anion accumulation may influence free-radical production, leading to severe oxidative stress. In the presence of oxygen the

Table 5.9 Classification of yeasts based on responses to oxygen

Class	Examples	Comments
Obligately fermentative	*Candida pintolopesii* (*Saccharomyces telluris*)	Naturally occurring respiratory-deficient yeasts. Only ferment, even in presence of oxygen
Facultatively fermentative		
Crabtree-positive	*S. cerevisiae*	Such yeasts predominantly ferment high-sugar-containing media in the presence of oxygen
Crabtree-negative	*Candida utilis*	Such yeasts do not form ethanol under aerobic conditions and cannot grow anaerobically
Non-fermentative	*Rhodotorula rubra*	Such yeasts do not produce ethanol, either in the presence or in the absence of oxygen

Adapted from information by Van Dijken and Scheffers (1986) and Scheffers (1987)

energy crisis is further exacerbated because of the mitochondrial electron transport chain dysfunction. This results in high endogenous levels of superoxide free radicals (Piper 1999).

Different yeast species have developed different strategies to cope with this kind of stress. The spoilage yeast *Z. bailii* seems to limit the diffusion of the acid into the cell. In addition, this yeast can also oxidatively degrade sorbate and benzoate, two of the most commonly used food preservatives. This explains the recent finding that tolerance to weak acid stress in this yeast occurs at no energy cost (Leyva and Peinado 2005).

This is in contrast to the situation in *S. cerevisiae* where weak acid stress results in a strong induction of the expression of the ATP binding cassette (ABC) transporter Pdr12 that is located in the plasma membrane. This transporter extrudes the acid from the cell at a high cost of ATP. In addition, to keep the electrochemical potential difference across the membrane the protons are extruded by the plasma membrane H^+-ATPase (Pma1). The strong upregulation of Pdr12 upon being challenged with 1 mM sorbate at pH 4.5 seems to be very specific as it is not present upon being challenged with other types of stress (Piper et al. 1998). This indicates that there must exist a weak-acid-induced signal transduction pathway that is different from the other stress-induced pathways.

Recently, the combined efforts of functional genomics and microarray analysis resulted in the identification of a transcription factor, War1p, for weak-acid resistance. This factor has been identified in two independent screening assays. In a functional screening assay, strains deleted for putative transcription factors were tested for their capacity to grow in the presence of 1 mM sorbate (Kren et al. 2003). Alternatively, classical mutagenesis in a strain that harbours the *PDR12* promoter–*LacZ* reporter construct was performed and mutants that were not able to induce the *LacZ* reporter gene were identified as carrying loss-of-function alleles of *WAR1* (Bauer et al. 2003). The probable mechanism of action is presented in Fig. 5.5 (Kren et al. 2003). Weak acids can enter the cell by passive diffusion at low pH. The higher intracellular pH dissociates weak acids, generating protons and $RCOO^-$ anions that accumulate intracellularly. Within minutes, the transcription factor War1p, which is constitutively bound to the weak-acid response element (WARE) present in the promoter of *PDR12*, becomes more phosphorylated, resulting in the induction of transcription of the Pdr12 efflux pump. Cells lacking either War1 or Pdr12 are weak-acid-hypersensitive. There is no increased sorbate sensitivity in the *war1*Δ *pdr12*Δ strain, suggesting that Pdr12 is the most important War1 target (Kren et al. 2003). Ectopic expression of *PDR12* from the *GAL1-10* promoter fully restored sorbate resistance in a strain lacking War1p, demonstrating that *PDR12* is the major target of War1p under sorbic acid stress. An important question that remains to be answered is the identification of the upstream parts of this new signalling pathway. What is the sensor? What is the signal transduction pathway upstream of War1? Is War1 activated directly by the organic monocarboxylate anions? To identify novel components of the pathway, genome-wide expression analysis of the response of yeasts to weak-acid stress was performed and this resulted in more than 100 genes that were induced. Three different pathways seem to be responsible for the weak-acid response. These are pathways mediated by War1,

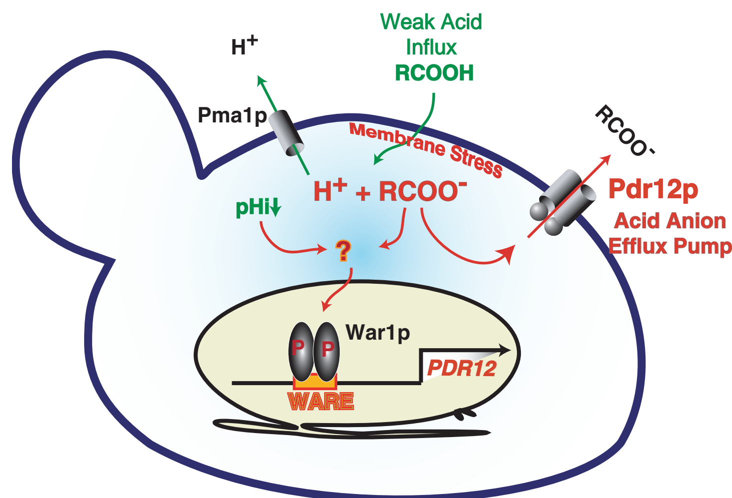

Fig. 5.5. Weak-acid-specific signal transduction pathway in *S. cerevisiae* (Kren et al. 2003)

Msn2/Msn4 and a third pathway. Only one of the targets, *PDR12*, turned out to be both stress inducible and required for weak-acid resistance (Schüller et al. 2004). In an alternative approach, the yeast deletion mutant collection was screened for mutants whose growth is affected in the presence of sorbic acid. Two hundred and thirty-seven mutants were identified as incapable of growing at pH 4.5 in the presence of 2 mM sorbic acid, whereas 34 mutants were more resistant compared with the wild-type strain (Mollapour et al. 2004). The direct role in weak-acid-stress signalling for these various genes awaits further investigation.

Important for the food industry is that similar to heat or cold adaptation, yeast cells can also adapt to weak acids. Challenging *S. cerevisiae* cells at low pH (4.5) with low concentrations of sorbate or benzoate (0.5–2.5 mM) results in a rapid entry into the G0 phase of the cell cycle. After several hours, they resume growth because they are then weak-acid-adapted (Holyoak et al. 1999; Piper et al. 1998). A first clue in the molecular mechanism behind this behaviour came from the *S. cerevisiae* Cmk1 mutant. This mutant did not show the long period of growth arrest. Cmk1 is a Ca^{2+}/calmodulin-dependent protein kinase. These data indicated that the weak-acid-induced cell cycle arrest must be repressed by this kinase (Holyoak et al. 2000). Whether Cmk1 is responsible for the phosphorylation of War1 remains to be investigated.

Sulphur dioxide has long been used as a yeast (and bacterial) preservative in the manufacture of alcoholic beverages, especially wine. It acts by dissociating within the yeast cell to SO_3^{2-} and HSO_3^{-}, decreasing intracellular pH. Unlike *S. cerevisiae*, *Z. bailii* growth is very sensitive to increasing extracellular Ca^{2+} concentrations, which suggests a simple expedient to limit spoilage by *Z. bailii* (Demidchik et al. 2004).

In clinical mycology, infective yeasts like *Candida albicans* are controlled using agents that act by targeting the plasma membrane by inhibiting the biosynthesis of ergosterol and cell wall components (e.g. glucan, mannoprotein and chitin) and the activity of the plasma membrane H^+-ATPase. Chauhan and Calderone (2004) have recently reviewed adaptive stress responses in human pathogenic yeasts.

5.5 Summary and Conclusions

Table 5.10 summarises some of the physiological responses of yeasts to physical and chemical stresses that impair growth and metabolism.

It is apparent that yeasts, particularly industrial strains of *S. cerevisiae*, are actually quite resilient organisms that are able to respond quite well, and often very rapidly, to sudden changes in their physico-chemical environment. Of course, some yeasts are better than others in adapting to stress and there are many examples of yeast species that can be described as osmotolerant, ethanol-tolerant, etc. In an effort to survive multiple environmental stresses, yeast cells may activate certain common molecular-level responses, such as trehalose accumulation, antioxidant production and stress protein biosynthesis. Stress responses are therefore inter-related and yeasts will succumb to stress and die when protective measures (either general or specific) initiated by cells are overcome. A lot of fundamental physiological and molecular knowledge has been accumulated in recent years concerning the stress responses of *S. cerevisiae*. However, we now need to extend this knowledge to production strains of this yeast growing in industrial environments, and to other important food yeast species. Deeper understanding of stress responses in such yeasts is key to their successful exploitation in food fermentations and to control of food spoilage yeasts.

Table 5.10 Major physiological responses of yeast to environmental stress

Stress	Physiological responses
High temperature	Heat shock protein biosynthesis Decreased membrane lipid unsaturation Altered cell pH Polyamine biosynthesis
Low temperature	Enhanced trehalose accumulation Increased membrane lipid polyunsaturation
Osmotic pressure	Accumulation of compatible solutes (glycerol, trehalose) Increased K^+ uptake/Na^+ efflux
Dehydration	Trehalose accumulation
Oxidants	Enzymic: superoxide dismutase, catalase, cytochrome peroxidase Non-enzymic: glutathione, thioredoxin, metallothionein, polyamines, carotenoids
Toxic chemicals	Ethanol: stress proteins, altered membrane transport, mitochondrial superoxide dismutase Xenobiotics: glutathione Heavy metals: stress proteins, metallothioneins

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Chapter 6

Molecular Mechanisms Involved in the Adaptive Evolution of Industrial Yeasts

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6.1 Introduction

Adaptation is obviously a key concept in modern biology, but its precise meaning has often been controversial (Mayr 1982). At the most basic level, the concept of adaptation is related with function. This way, some trait, or integrated suit of traits, of an organism is adaptive if it performs a function that is, in some way, beneficial to the organism to live in an environment. Adaptations can involve aspects of an organism's behavior, physiology, morphology, etc., or the ability of an individual to alter those properties depending on the environment (phenotypic plasticity). The originality of the theory of natural selection proposed by Charles Darwin lay in the fact that it provided a hypothesis to explain the origin of adaptations. Since then, adaptive traits have been considered the result of adaptive evolution, i.e., an evolutionary process directed by natural selection.

The neo-Darwinian theory of evolution by natural selection, also known as the new synthesis, was based on the idea that most natural populations contain enough genetic variation to respond to any sort of selection. Most of this genetic variation is due to the presence of different alleles generated by mutation and homologous recombination. Adaptation may then be explained by the gradual evolution resulting from small changes in the allele frequencies acted upon by natural selection.

However, with the advent of molecular methods, the potential importance of major, new mutations (novelties) in adaptive evolution has been emphasized (Nei 1987; Li 1997). Molecular studies have shown that mutations include not just the generation of new alleles by nucleotide substitution, but such important processes as the generation of new genes, not only by gene duplication (Long et al. 2003), or radically new alleles by unequal crossing over. The complete sequencing of different

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yeast genomes as well as the study of the molecular basis of the physiological properties of yeasts have provided unique tools to study the molecular mechanisms involved in the adaptive evolution of yeast traits of industrial interest.

In the present chapter, we are not going to deal with the procedures to identify, demonstrate or understand the adaptive significance of the traits and properties of industrial yeasts. Rather, we are going to review the different molecular mechanisms involved in the generation of these major genetic novelties that can explain the adaptive evolution of industrial yeasts.

6.2 The *Saccharomyces sensu stricto* Complex Includes the Most Important Industrial Yeasts

Yeasts are defined as unicellular ascomycetous or basidiomycetous fungi whose vegetative growth results predominantly from budding or fission, and which do not form their sexual states within or upon a fruiting body (Kurtzman and Fell 1998). Of the more than 700 known yeast species, several dozen are used in industrial processes, mainly in the production of fermented products and metabolites. Among them, the most useful and widely exploited species are those from the *Saccharomyces* genus, especially *S. cerevisiae* and its relatives, included in the *Saccharomyces sensu stricto* complex.

The *Saccharomyces sensu stricto* complex (Vaughan-Martini and Martini 1998) consists of three species associated with industrial fermentation processes, *S. bayanus*, *S. cerevisiae*, and *S. pastorianus*, and four species isolated from natural habitats, *S. cariocanus*, *S. kudriavzevii*, *S. mikatae*, and *S. paradoxus*.

S. cerevisiae has been found associated to very diverse fermentation processes, including baking, brewing, distilling, winemaking, and cider production, and also in different traditional fermented beverages and foods around the world. The origin of *S. cerevisiae* is controversial. Some authors propose that this species is a “natural” organism present in plant fruits (Mortimer and Polsinelli 1999). Others argue that *S. cerevisiae* is a “domesticated” species found only in association with human activities, because attempts to find this species in regions remote from human activities have been unsuccessful (Naumov 1996). Moreover, some authors suggested that this species could originate from its closest relative *S. paradoxus*, a wild species found all around the world (Vaughan-Martini and Martini 1995). This debate is important in postulating the original genome of *S. cerevisiae* and how the strong selective pressure applied since its first unconscious use in controlled fermentation processes has reshaped it.

The cryophilic *S. bayanus* has been found in nature in cold areas of Europe and also appears associated with different fermentation processes: winemaking, cider production, brewing, and as grape must contaminants. The type strain of this species, originally isolated from beer, has recently been described as a hybrid possessing also a nuclear genome from *S. cerevisiae* (Nguyen et al. 2000; de Barros Lopes et al. 2002; Nguyen and Gaillardin 2005), which led to the proposal of the reinstatement of *S. uvarum*, a former taxon included in *S. bayanus*, as a distinct species (Pulvirenti et al. 2000; Nguyen and Gaillardin 2005) or as a different variety within *S. bayanus* (Naumov 2000).

S. pastorianus (synonym *S. carlsbergensis*) is the bottom-fermenting yeast responsible of the production of lager beer, although it has also been found in musts and

wines. Different studies (Hansen and Kielland-Brandt 1994; Nguyen et al. 2000; Casaregola et al. 2001) have demonstrated that strains of this species correspond to natural hybrids between *S. cerevisiae*, and a *S. bayanus* like yeast. Chromosome sets from both parental species are present in strains of *S. pastorianus* (Tamai et al. 1998; Yamagishi and Ogata 1999), while the mitochondrial DNA (mtDNA) was inherited from the non *S. cerevisiae* parent (Piškur et al. 1998).

The wild yeast *S. paradoxus*, the closest relative to *S. cerevisiae*, according to phylogenetic reconstructions (Rokas et al. 2003), is a natural species distributed worldwide with a fortuitous presence in fermentation processes. However, it has recently been described as the predominant yeast in Croatian vineyards (Redzepović et al. 2002).

Finally, the *Saccharomyces sensu stricto* complex also includes three other wild species, *S. cariocanus*, *S. mikatae*, and *S. kudriavzevii*, whose description (Naumov et al. 2000a) was based on a few strains isolated from natural habitats in Brazil, the first one, and Japan, the other two.

Saccharomyces sensu stricto yeasts possess a series of unique characteristics that are not found in other genera (Vaughan-Martini and Martini 1998). One of these unique characteristics is their high capability to ferment sugars vigorously, both in the presence and in the absence of oxygen, to produce ethanol. This ability allows them to colonize sugar-rich substrates (plant saps and fruits) and compete with other yeasts, which are not so tolerant to alcohol. The aparition of angiosperm plants with sugar-rich saps and fruits introduced a new ecological niche with a different selection regime that likely imposed altered physiological demands on the ancestors of *Saccharomyces* yeasts (Wolfe and Shields 1997). Under such circumstances, adaptive evolution took place in this new ecological context favoring the acquisition of such high fermentative capability.

This capability has unconsciously been used by humans to produce fermented foods and beverages, which introduced new selective pressures on these yeasts. Neolithic human populations likely observed that fruit juice spontaneously ferments producing an alcoholic beverage (Mortimer et al. 1994). Since then, the yeast *S. cerevisiae* and related species have become an essential component of many important human activities, including baking, brewing, distilling, and winemaking.

In general, these industrial *Saccharomyces* strains are highly specialized organisms which have evolved to utilize the different environments or ecological niches that have been provided by human activity. This process can be described as "domestication" and is responsible for the peculiar genetic characteristics of the industrial yeasts. During the last few years, intensive research efforts have been focused on elucidating the molecular mechanisms involved in yeast adaptation to the industrial process, and the reshaping of genomic characteristics of the industrial yeast which have been unconsciously selected over billions of generations (Querol et al. 2003).

6.3 Adaptive Evolution by "Genome Renewal"

Although *Saccharomyces sensu stricto* yeasts are becoming ideal model organisms to test population genetics models (Zeyl 2000) and to study speciation mechanisms (Greig et al. 2002a), very little information is available about the genetic variability of natural *Saccharomyces* populations.

The analysis of natural populations of *S. cerevisiae* from spontaneous wine fermentations (Mortimer et al. 1994) showed that, although genetic diversity was high, almost all strains were homozygous for most of the genes analyzed. This observation, together with the high fertility of the strains and their homothallic character, led the authors of the study to propose a mechanism of evolution for natural wine yeasts, termed genome renewal. This hypothesis is based on the ability of homothallic haploid *S. cerevisiae* cells to switch their mating type and conjugate with cells of the same single-spore colony to produce completely homozygous diploids. Strains of *S. cerevisiae* accumulating heterozygous recessive mutations can change to completely homozygous diploids by sporulation and homothallic switching of individual haploid spores. This process would favor the action of selection, removing recessive deleterious genes and fixing recessive beneficial alleles, thereby enabling yeasts to adapt efficiently to changing environmental conditions. However, Puig et al. (2000) demonstrated that homozygosis could also be achieved by mitotic recombination or gene conversion during vegetative growth.

6.4 Molecular Mechanisms Involved in the Generation of Evolutionary Novelties

Decades of genetics research and the development of large-scale genomic approaches led to the complete sequencing of the genome of *S. cerevisiae* (Goffeau et al. 1996), the first eukaryote to have its genome sequenced. The available molecular techniques and the rapidly expanded genome data with recent publication of new genome sequences from yeasts (Cliften et al. 2003; Kellis et al. 2003, 2004; Dietrich et al. 2004; Dujon et al. 2004), including other *Saccharomyces sensu stricto* species, provided a new approach to decipher the molecular mechanisms involved in the generation of evolutionary novelties in yeasts. Also, molecular evolution and molecular population genetics have provided useful analytical tools for the detection of the processes and mechanisms that underlie the origin of these evolutionary novelties.

Recently, Long et al. (2003) reviewed the different molecular mechanisms that are known to be involved in the creation of new gene structures, the details of which are understood to varying degrees. In the next sections, we will provide evidence of the role of several molecular mechanisms in the adaptive evolution of yeasts.

6.4.1 Gene Duplication

Gene duplication as the most important source of new genes was postulated by Haldane (1933). He proposed that redundant gene copies generated by gene duplications (called paralogues, i.e., genes that are homologous by duplication of an ancestral gene, in contrast to orthologues, genes that are homologous by descent) are not constrained to maintain their original function and, hence, they can accumulate divergent mutations, resulting in new gene functions.

Gene duplications can be produced by different mechanisms resulting in the duplication of a single gene or a group of adjacent genes (Koszul et al. 2004), in the duplication of a chromosome, called aneuploidy (Hughes et al. 2000), or in the duplication of the whole genome content, called polyploidy (Wolfe and Shileds 1997).

In some cases, redundant genes could be retained if there is an evolutionary advantage to having extra dose repetitions. In others, one duplicate will be free to accumulate mutations because only one of the duplicates will be under purifying selection owing to the restrictions to maintain the ancestral gene function. The classical model of acquisition of new genes by duplication proposes that both paralogues could be preserved if one of them acquires a mutation with a new, beneficial function and the other retains the original function (a process called neofunctionalization). However, this process was assumed to be extremely rare (Wagner 1998), because most changes neutrally fixed in the unrestricted duplicate will be loss-of-function mutations, and, hence, this copy will become a pseudogene to be finally lost (a process known as nonfunctionalization). Accordingly, the classical model predicted that few duplicates should be retained in the genome over the long term, but the sequencing of complete genomes showed that retention of ancient duplicates is very common (Wagner 1998).

To explain the preservation of paralogous genes, Force et al. (1999) proposed an alternative process, called subfunctionalization, whereby both members of a pair acquire complementary degenerative mutations in independent subfunctions, originally present in the ancestral gene. This way, both duplicates are required to produce the full patterns of activity of the single ancestral gene, and subsequent adaptive evolution will promote their subfunctional specialization.

The *GAL1* and *GAL3* paralogous genes of the *Saccharomyces sensu stricto* species provide an example of subfunctionalization in yeasts (Hughes 1999). The galactose-inducible *GAL1* gene encodes a galactokinase that catalyzes the production of galactose-1-phosphate from galactose and ATP, whereas the galactose-inducible *GAL3* gene encodes a regulatory protein involved in the activation of both *GAL1* and *GAL3* genes in the presence of galactose and ATP. *Kluyveromyces lactis* contains a single *GAL1* gene encoding a protein with both regulatory and structural functions. The phylogenetic analysis of these genes indicates that *K. lactis GAL1* diverged from the *Saccharomyces sensu stricto GAL1-GAL3* genes before the gene duplication event, indicating that each paralogue specialized by subfunctionalization.

6.4.1.1 Polyploidization: Whole Genome Duplication in Yeasts

The importance of whole genome duplication in the evolution of higher eukaryotes was postulated by Ohno (1970). The complete sequencing of diverse eukaryote genomes revealed that whole genome duplications occurred several times during the evolution of certain eukaryotic lineages (some plants, fishes, amphibians, etc.).

One of the most striking results obtained from the sequencing of the *S. cerevisiae* complete genome was the presence of 376 gene pairs within 55 large duplicated regions. This observation led Wolfe and Shields (1997) to propose that a whole-genome duplication event, polyploidization, occurred in an ancestor of *S. cerevisie* after the split from *K. lactis*, some one hundred to two hundred million years ago. Polyploidization followed by extensive gene loss of most paralogues by pseudogenization and the accumulation of chromosomal rearrangement events explains the observed pattern of dispersed, large segmental duplications present in the *S. cerevisiae* genome (Keogh et al. 1998).

The hypothesis that *S. cerevisiae* is a paleopolyploid was initially very controversial. Other authors suggested that the duplicated segments could arise via independent local duplication events (Souciet et al. 2000), but the comparative analysis of gene order (Wong et al. 2002) in the genomes of different yeast species, partially sequenced by the Genolévures consortium (Souciet et al. 2000), corroborated this hypothesis and also allowed the location of the polyploidization event in the phylogeny of the hemiascomycetous yeasts. The complete, or almost complete, sequencing of genomes from yeast species of the *Saccharomyces* complex diverged before the genome duplication event (*Saccharomyces sensu stricto*, Kellis et al. 2003; Cliften et al. 2003; *S. castellii*, Cliften et al. 2003; *Candida glabrata*, Dujon et al. 2004) and after (*S. kluyveri*, Cliften et al. 2003; *Ashbya gossypii*, Dietrich et al. 2004; *K. waltii*, Kellis et al. 2004; *K. lactis*, Dujon et al. 2004) confirmed that the duplication event encompassed the entire genome, and was produced by polyploidization of an ancestor of *S. cerevisiae* and related species. The comparison of pre- and postduplication genomes allowed the conclusion to be drawn that the whole genome duplication event doubled the number of chromosomes in the *Saccharomyces* lineage, but subsequent gene-loss events, 88% of paralogous genes were lost, led to the current *S. cerevisiae* genome, which contains only about 500 more genes than the preduplication species, but distributed among 16 chromosomes instead of eight. The polyploid genome returned to functional normal ploidy, not by meiosis or chromosomal loss, but instead by a large number of deletion events of small size (average size of two genes), balanced between the two duplicated regions.

Polyploidization in yeasts can theoretically occur by different mechanisms: (1) an error during meiosis can lead to the production of diploid spores and subsequent conjugation between diploid cells, (2) an error during mitosis in unicellular organisms, (3) rare mating between two diploid yeasts of the same species that became mating-competent by interchromosomal mitotic recombination at the MAT locus (de Barros Lopes, 2002), (4) interspecific hybridization by conjugation of spores from different species, and subsequent genome duplication by errors during mitosis or meiosis, or (5) rare mating between two mating-competent diploid strains belonging to different species (de Barros Lopes 2002). In the first three cases, the result is an autotetraploid yeast, whose nucleus contains four allelic copies of each chromosome; however, in the last two cases, the result is a fertile allotetraploid (also called amphidiploid) yeast, containing pairs of "homeologous" chromosomes, i.e., homologous chromosomes coming from two different species. Examples of both types of polyploid yeasts have been described (Naumov et al. 2000b).

Andalis et al. (2004) demonstrated that isogenic autopolyploidy is accompanied by defects affecting viability and subsequent survival of the new organisms, and, hence, postulated that the entire genome duplication event that occurred in an ancestor of *S. cervisiae* was likely generated by allopolyploidization.

But the most important consequence of the whole genome duplication event was the sudden acquisition of extra copies of each gene in the genome. Wolfe (2001) suggested that these duplicated genes formed by polyploidy should be called "ohnologues", after Susumu Ohno, to distinguish them from other kinds of paralogues because they are all the same age.

The complete genome sequences of *K. waltii* (Kellis et al. 2004) and *A. gossypii* (Dietrich et al. 2004), species that diverged before the polyploidization event, were used to map and analyze the fate of the ohnologues during the evolution of the *S. cerevisiae* lineage. The different expected outcomes with respect to the fate of duplicated genes, described in Sect. 6.4.1, were observed. This way, nonfunctionalization was the most frequent process: 88% of paralogous genes generated by polyploidization were lost.

Of the approximately 460 surviving ohnologues, 60 pairs showed decelerated evolution and tend to be highly similar, even at the silent codon positions, suggesting that they may be subject to periodic gene conversion. Moreover, in about half of these cases, the two paralogues in *S. cerevisiae* are closer in sequence to each other than either is to its orthologue in *S. bayanus*, showing that gene conversion occurred after the relatively recent divergence of the two *Saccharomyces* species. These cases often involve proteins known to be highly constrained, such as ribosomal proteins, histone proteins, and translation initiation/elongation factors, indicating that they have likely been retained because of the advantage of having extra dosage of the genes.

The remaining ohnologues have diverged in sequence and often also in function. Kellis et al. (2004) found that more than 100 gene pairs show a higher rate of protein evolution relative to *K. waltii*, with one ohnologue accumulating significantly more amino acid replacements than the other. They also argue that, in many of these cases, accelerated evolution was confined to only one of the two paralogues, which strongly supports a process of neofunctionalization, the slowly evolving paralogue has probably retained the ancestral gene function and the rapidly evolving paralogue probably corresponds to the copy relieved of selective constraints, which is free to evolve more rapidly to acquire a derived function after duplication. Most of these ohnologues correspond to protein kinases and regulatory proteins, generally involved in metabolism and cell growth.

The other approximately 300 ohnologue pairs did not show significant differences in their rates of evolution. In some cases, the functional changes may be similar to those just described but subtler. In other cases, gene pairs may have been retained by subfunctionalization. Specialization to different ancestral subfunctions may explain the similar rates of evolution in both ohnologues. Moreover, this subfunctionalization may have occurred by divergence in regulatory sequences.

The polyploidization event suddenly provided new gene functions that have had a profound impact in the evolution of the *Saccharomyces sensu stricto* lineage (Piškur and Langkjær 2004; Wolfe 2004). The partitioned functions of most ohnologues, retained in the *Saccharomyces sensu stricto* lineage, indicate that the genome duplication provided new genes that played a direct role in the adaptation of these species toward a highly efficient fermentation performance under anaerobic conditions. Wolfe and Shields (1997) indicated that many ohnologue pairs are differentially regulated in the presence and absence of oxygen (DeRisi et al. 1997), including genes of proteins of the electron transport chain complexes (e.g., *CYC1*/*CYC7* encoding cytochrome c isoforms, or *COX5A*/*COX5B* encoding cytochrome c oxidase subunit 5 isoforms) and genes encoding enzymes of the glycolysis/gluconeogenesis pathway (e.g., *PYK1*/*PYK2* coding for pyruvate kinases, *ENO1*/*ENO2* for enolases, etc.).

The polyploidization also allowed the development of efficient glucose-sensing and glucose-repression pathways (Kwast et al. 2002). Ohnologues encoding regulatory proteins are involved in the development of the two glucose-sensing pathways of high affinity and low affinity, the Snf1 pathway of glucose-repression of gluconeogenesis and respiration, and in the glucose-responsive protein kinase A pathway (Wolfe 2004).

In conclusion, the polyploidization event provided the basis for the evolution of new gene functions during the competition to colonize sugar-rich substrates supplied by fruit-bearing plants. The competitive advantage of a fermentative metabolism, fast growth and the production of toxic ethanol put the ancestors of the industrial *Saccharomyces* yeasts in the pole position to become, under the selective pressures unconsciously imposed to improve controlled fermentation processes, the highly efficient mono- and oligosaccharide fermenters that exist today.

6.4.1.2 Aneuploidy: Chromosome Duplication

An alternative mechanism to provide potential new genes is by changing chromosome copy numbers, which is known as aneuploidy. However, the most important consequence of aneuploidy is the increase of gene dose.

Aneuploidy arises by nondisjunction, i.e., inaccurate chromosome segregation, during meiosis or mitosis. The increase in copy numbers for some genes results in an imbalance of the gene products and disruption of the regulatory interactions, which could be deleterious or even lethal for many organisms. Although aneuploidy is tolerated in industrial yeasts, it is one of the causes of the poor sporulation exhibited by some strains.

Wine *Saccharomyces* strains are frequently aneuploid, with disomies (two chromosome copies), trisomies and, less frequently, tetrasomies (Bakalinsky and Snow 1990). This aneuploidy, and also autopolyploidy, has been postulated as a mechanism that may confer advantages for adaptation to variable external environments by increasing the number of copies of beneficial genes or by protecting the yeasts against recessive lethal or deleterious mutations (Bakalinsky and Snow 1990; Guijo et al. 1997; Salmon 1997).

Hughes et al. (2000) observed that the deletion of a gene strongly favors the acquisition of a second copy of a whole chromosome or a chromosomal segment containing a paralogue of the deleted gene. About 8% of 300 yeast deletion mutants examined had acquired a detectable aneuploidy, and in six of the cases they examined, the amplified chromosome contained a close paralogue of the deleted gene, implying that characteristic aneuploidies can act as dominant suppressors and under some circumstances lead to increased fitness.

Kellis et al. (2004) correlated these deletion results with the identification of the ancestral and derived functions of paralogues (Sect. 6.4.1.1). Strikingly, deletion of the ancestral paralogue was lethal in 18% of cases, whereas deletion of the derived paralogue was never lethal. The derived paralogue is thus not essential under these conditions, either because it does not function in a rich medium or because the ancestral paralogue can complement its function. Along with possibly gaining a new function, the derived copy has lost some essential aspect of its function, and cannot typically complement deletion of the ancestral gene.

6.4.1.3 Single Gene and Segmental Duplications

Gene duplication can also involve either a single gene or a group of adjacent genes (segmental duplication). Genome sequencing projects have revealed that multigene families, i.e., groups of identical or similar genes generated by successive single gene or segmental duplications, are common components of all genomes. This way, the *S. cerevisiae* genome contains 265 multigene families with three or more paralogues, including a family with 108 members (Llorente et al. 2000), which indicates that successive gene duplications should have occurred.

Genome comparisons (Souciet et al. 2000; Dujon et al. 2004) showed that tandem repeated gene duplication is very common among yeasts and illustrates the importance of ancestral duplications that occurred before divergence of hemiascomycetous yeasts. Sequence divergence between paralogues in different yeast species shows bimodal distributions, with a fraction of multigene families showing high sequence identities, probably reflecting recent duplications and/or sequence homogenization by gene conversion, and an important fraction with low identities, corresponding to ancient duplications that occurred before species divergence.

Single-gene and segmental duplications mainly correspond to intrachromosomal direct tandem-repeat duplications. Although there are some examples of segmental duplications that are dispersed throughout the genome, most gene families are located in subtelomeric regions (adjacent to chromosome telomeres). Classical examples of redundant genes in subtelomeric regions are the *MEL*, *SUC*, *MGL* and *MAL* genes involved in the assimilation of sugars. Yeast strains differ by the presence or absence of particular sets of these genes, which could be attributed to selective pressure induced by human domestication, as it appears that they are largely dispensable in laboratory strains.

Clusters of duplicated genes have also been found internal to chromosomes. A typical example is the large gene cluster on chromosome VIII near *CUP*1. The *CUP*1 gene encoding copper metallothionein, is contained in a 2-kb repeat that also includes an open reading frame (ORF) of unknown function (Fogel and Welch 1982). The repeated region has been estimated to span 30 kb in laboratory strains, which could encompass 15 repeats, but the number of repeats varies among yeast strains.

Different mechanisms have been postulated to explain the origin of single-gene and segmental tandem duplications. The critical step is the origin of the first tandem duplication, which requires the presence of similar nucleotide sequences flanking the duplicated region. These similar sequences may also be provided by transposable elements. Ectopic recombination between homologous chromosomes or unequal sister chromatide exchange, at the similar sequences, will result in the duplication of the genome region. Subsequent duplications can occur by ectopic recombination between paralogous repeats.

The fate of the duplicated genes is discussed in Sect. 6.4.1. However, many tandemly duplicated genes exhibit identical or nearly identical sequences, indicating that these multigene families evolve in a concerted way to preserve gene function, and, hence, increase gene dosage. Ectopic recombination and gene conversion are the mechanisms postulated to explain the concerted evolution observed in the members of multigene families (Li 1997).

Another process, postulated to preserve identical function in the members of a gene family, is the birth-and-death model of multigene family evolution (Nei et al. 1997), in which repeated gene duplications are counterbalanced by gene degeneration or deletion (nonfunctionalization). A systematic analysis of *S. cerevisiae* intergenic regions revealed the presence of many degenerated pseudogenes, called gene relics, homologous to extant *S. cerevisiae* ORFs (Lafontaine et al. 2004). Gene relic distribution is mainly subtelomeric and related to multigene families. Thus, multigene family evolution by a gene birth-and-death mechanism is also compatible with the presence of new paralogues and relics in several yeast strains and the sequence polymorphism within the tandem *DUP240* family, one of the largest *S. cerevisiae* gene families (Leh-Louis et al. 2004a, b).

Many of the tandemly repeated genes, especially the subtelomeric multigene families, are involved in secondary metabolism. These genes are not essential, but they play an important role in the adaptation to new environmental conditions. For example, subtelomeric gene families in *S. cerevisiae* are often related to cell membrane and cell wall components, such as lectine-like proteins (the *FLO* family), sugar transporters (the *HXT* family), genes related to cell–cell fusion (the *PRM* family), and assimilation and utilization of nutrients (*GAL*, *MAL*, *SUC*, and *PHO* families) (Vega-Palas 2000; Harrison et al. 2002). Some dispersed gene families may also be related to adaptation to environmental conditions, such as the *CUP1* gene tandem repeats present in copper-resistant *S. cerevisiae* strains (Fogel and Welch 1982).

Other species, including those that diverged before the whole genome duplication event, also contain subtelomeric gene families that are probably involved in adaptation to changing environments. For example, the genome of *K. waltii* also contains several families of membrane proteins, hexose transporters, and flocculins (Kellis et al. 2004); and multigenic families encoding multidrug resistance proteins and hexose transporters are specifically more expanded in *Debaryomyces hansenii* than in the other yeasts (Dujon et al. 2004).

Many of these subtelomeric repeats were likely advantageous to industrial strains during selection for thousands of years of human biotechnology practices. Rapid changes in the gene composition of these families may increase the chances of acquiring a selective advantage and improving their industrial fitness. In fact there are several examples of spontaneous gene duplications selected as a response to limiting conditions (Brown et al. 1998).

6.4.2 Lateral Gene Transfer: Acquisition of New Genes from Another Species

Another possible way in which a genome can acquire new genes is to obtain them from another species. This process, known as lateral or horizontal gene transfer, has been proven to be very important in prokaryotes, but not so frequent in eukaryotes. In the case of eukaryotes, allopolyploidy and introgression due to interspecific hybridization could be considered as mechanisms of lateral gene transfer, and they will be treated in Sect. 6.4.3.

Genome sequencing has revealed the presence of a few genes occurring in a single yeast species that have close homologues in bacteria. These genes, most of them

encoding metabolic enzymes, are rare in the yeast genomes less than 1%), but do appear.

A recent study (Gojković et al. 2004) demonstrated that lateral gene transfer has played, together with the whole-genome duplication event, a major role in the evolutionary history of the *Saccharomyces* complex yeasts. These authors proposed that horizontal gene transfer promoted evolution of the ability to propagate under anaerobic conditions in *Saccharomyces* yeasts. In strict aerobic yeasts, the "de novo" pyrimidine biosynthesis, more precisely the fourth enzymic activity catalyzed by a mitochondrial dihydroorotate dehydrogenase (DHODase) is dependent on the active respiratory chain. However, the facultative anaerobic *Saccharomyces sensu stricto* yeasts have a cytoplasmic DHODase independent of the respiratory chain, which is phylogenetically related to a bacterial DHODase from *Lactococcus lactis*. Gojković et al. (2004) demonstrated that *S. kluyveri*, which separated from the *S. cerevisiae* lineage more than one hundred million years ago, represents an evolutionary intermediate, having both anaerobic cytoplasmic and aerobic mitochondrial DHODases. From these observations, they suggested that a *Saccharomyces* yeast ancestor, which originally had a eukaryotic-like mitochondrial DHODase, acquired a bacterial DHODase, which subsequently allowed cell growth gradually to become independent of oxygen.

6.4.3 Interspecific Hybridization and Introgression

In the case of *Saccharomyces sensu stricto*, one of the most interesting mechanisms observed in the adaptation of these yeasts to industrial process is the formation of interspecific hybrids. Allopolyploidy and introgression by interspecific hybridization are the main mechanisms of lateral gene transfer in eukaryotes.

Artificial interspecific hybridization experiments indicated that *Saccharomyces* "*sensu stricto*" interspecific hybrids can easily be formed (Naumov 1996), and, although sterile, they are viable and can be maintained by asexual reproduction. *Saccharomyces sensu stricto* species are present in the same ecological niche and could hence be involved in the formation of hybrids because haploid cells or spores of these species are able to mate with each other and form viable, but sterile, hybrids. Hybrids produce spores with extensive imbalance in chromosome number and low frequencies of genetic exchange. The mismatch repair system plays a major antirecombination role in these yeast hybrids. The ways in which yeast hybrids may escape this postzygotic barrier are achieved either by doubling of the chromosome number, which results in an allotetraploid (Naumov et al. 2000b), or by recovering euploidy by homothallic diploidization of spores, which results in a homoploid (Greig et al. 2002a).

The best described example of hybrid yeasts is the lager yeasts, included in the taxon *S. pastorianus* (synonym *S. carlsbergensis*) (Vaughan-Martini and Kurtzman 1985). This yeast is a partial allotetraploid hybrid between two species of the *Saccharomyces sensu stricto* group, *S. cerevisiae*, and a *S. bayanus* related yeast (Hansen and Kielland-Brandt 1994; Nguyen et al. 2000; Casaregola et al. 2001). Chromosome sets from both parental species are present in strains of *S. pastorianus* (Tamai et al. 1998; Yamagishi and Ogata 1999), while the mtDNA was inherited

from the non *S. cerevisiae* parent (Piskur et al. 1998). Extensive and variable aneuploidy is found in different *S. pastorianus* isolates (Casaregola et al. 2001), and many of them are chimerical, with part from each parent indicating recombination sometime in their history (Bond et al. 2004).

Moreover, the type strain of *S. bayanus*, originally isolated from beer, has recently been described as possessing also a nuclear genome from both *S. cerevisiae* and *S. bayanus* (Nguyen et al. 2000; de Barros Lopes et al. 2002; Nguyen and Gaillardin 2005).

New natural hybrids have been found in environments different from brewing. Masneuf et al. (1998) characterized a *S. bayanus* × *S. cerevisae* hybrid strain (S6U) isolated from Italian wine, and a triple hybrid present in a homemade French cider (CID1). This hybrid contained two copies of the nuclear gene *MET2*, one coming from *S. cerevisiae* and the other from *S. bayanus*, and the mitochondrial genome originated from a third species, which Groth et al. (1999) demonstrated corresponded to the type strain of the species *S. kudriavzevii*. This was the first report indicating that a rare *Saccharomyces sensu stricto* species, for which only two strains isolated from tree exudates in Japan were known (Naumov et al. 2000a), was involved in interspecific hybridization.

New hybrids *S. cerevisiae* × *S. kudriavzevii* isolated from both natural habitats and fermentation processes, and natural *S. cerevisiae* × *S. paradoxus* hybrids have also been postulated on the basis of their patterns of hybridization with repetitive elements (Liti et al. 2005). Natural hybrids are not restricted to the *Saccharomyces sensu stricto* complex: James et al. (2005) have recently described hybrids between species of the genus *Zygosaccharomyces*.

In two recent studies, new hybrids resulting from the hybridization between *S. cerevisiae* and *S. kudriavzevii* have been described among wine strains (González et al. 2005a) and among brewing yeasts (González et al. 2005b). These wine hybrid strains were predominant in spontaneous fermentations from eastern Switzerland (Schütz and Gafner 1994), and different brewing hybrids were isolated from three Belgian Trappist beers, and also from English, German and New Zealand beers. These authors also found a *S. bayanus* × *S. cerevisiae* × *S. kudriavzevii* hybrid strain, also isolated in Switzerland in 1951, that shows a different genome structure than the other triple hybrid CID1. The sequencing analysis of gene regions located at different chromosomes and the comparative genome hybridization to *S. cerevisiae* DNA microarrays showed that *S. kudriavzevii* hybrid strains contain aneuploidy differences and chimerical chromosomes resulting from recombination between “homeologous” chromosomes of different parental origin (S.S. González, A. Querol, J. García-Martínez, J.E. Pérez-Ortín, and E. Barrio, unpublished results).

The diversity of *Saccharomyces sensu stricto* hybrids, their distinct origins and their presence in different habitats indicate that, in spite of the homothallic character of most natural *Saccharomyces* strains and the persistence of their asci, interspecific hybridization is not so infrequent. Pulvirenti et al. (2002) proposed that yeast-feeding invertebrates may provide the appropriate conditions promoting intra- and interspecific hybridization, because these animals produce, in their digestive tracts, enzymes that hydrolyze the ascus wall, releasing free spores able to conjugate.

As an alternative to haploid cell conjugation, de Barros Lopes et al. (2002) proposed that rare mating between diploid strains of the *Saccharomyces sensu stricto* complex could be involved in the generation of interspecific hybrids. They demonstrated that rare mating is possible not only between nonhybrid diploid strains, but also between CID1, S6U, and lager hybrids with *S. paradoxus* and *S. cerevisiae* diploids, indicating that this mechanism may be involved as well in the generation of multiparental hybrids also from allopolyploids, such as S6U (Naumov et al. 2000b).

Natural interspecific hybridization in yeasts is more frequent that suspected and has probably been undervalued as an important mechanism in the evolution of yeasts by providing new gene combinations of adaptive value (Masneuf et al. 1998; Greig et al. 2002b), genetic robustness due to redundancy, new or specialized functions from divergence of redundant genes (Wolfe and Shields 1997), and also new species through allopolyploid (Naumov et al. 2000b) or homoploid (Greig et al. 2002b) speciation.

In fact, interspecies hybridization might have been a key event in evolution of the high fermentation capabilities of the species of the *Saccharomyces sensu stricto* complex. As mentioned in Sect. 6.4.1.1, Andalis et al. (2004) proposed that the whole-genome duplication in the ancestor of the *Saccharomyces sensu stricto* complex was probably generated by allopolyploid hybridization.

6.4.4 Recruited Autonomous Mobile Elements as a Source of New Genes

There are different examples in eukaryotic genomes indicating that an autonomous mobile element could be directly recruited by host genes to generate a new gene function (Long et al. 2003). In fact, 4% of new exons of human protein-coding genes correspond to recruited autonomous mobile elements.

In the case of yeasts, Butler et al. (2004) demonstrated that homothallic mating (self-fertility based on a mating type switch mediated by HO endonuclease) in the *Saccharomyces* complex originated through the acquisition of an intein-like sequence. Inteins are selfish DNA elements inserted in-frame and translated together with their host proteins (Gogarten et al. 2002). This precursor protein undergoes an autocatalytic protein splicing reaction resulting in two products: the host protein and the intein peptide, which exhibits endonuclease activity involved in the intein mobility.

The close resemblance between HO endonuclease and the endonuclease encoded by the VMA1 intein suggests that, shortly before the whole duplication event, an intein from an unknown origin invaded the *VMA1* gene of the ancestor of the *Saccharomyces sensu stricto* yeast, which gave rise to the HO endonuclase encoding gene after subsequent duplication (Butler et al. 2004). The *HO* mating type switching gene facilitated the change from a cell cycle with a major haploid phase to a cycle with a major diploid phase, which increased the level of genetic robustness of the yeast genome, at least owing to dominance, and promoted the evolution of a repair system based on efficient homologous recombination (Piškur and Langkjær 2004).

6.4.5 New Genes Generated by Retroposition

Retroposition may create duplicate genes in new genomic positions through the reverse transcription of expressed parental genes (Long et al. 2003). This way, messenger RNAs (mRNAs) can be retrotranscribed to complementary DNAs (cDNAs) by a retrotransposon reverse transcriptase and inserted in a new genome position. These retrotransposed genes differ from their parental genes in the absence of introns and the presence at the 3′ end of an A–T stretch coming from the retrotranscription of the mRNA poly(A) tail. As a retroposed protein-coding gene copy lacks internal promoter sequences, it has to recruit a new regulatory sequence to be functional or it will become a processed pseudogene.

Schacherer et al. (2004) recently described in yeasts experimental evidence for the recovery of a function involving duplication by retroposition. They used a positive selection screen of *S. cerevisiae URA2* mutants to isolate spontaneous revertants containing a duplication of the terminal part of the *URA2* gene.

The molecular characterization of the duplicated *URA2* regions showed that they were generally punctuated by a poly(A) tract and were always located in Ty1 sequences. Schacherer et al. (2004) demonstrated that the duplication mechanism involves the reverse transcription of *URA2* mRNA packed in Ty1 viruslike particles, and the subsequent integration of the cDNA into a Ty1 resident copy. Reverse transcription was initiated in the poly(A) region via the terminal part of the URA2 gene and switch at the level of the 5′ junction observed on a Ty element template, leading to the formation of the chimerical structure observed: a δ long terminal repeat (LTR) TyA segment in frame with the duplicated terminal part of the *URA2* gene. Integration was mediated by a homologous recombination event resulting from gene conversion between preexisting chromosomal Ty elements and the 5′ end of the cDNA. Finally, in order to be transcribed to mRNA, the chimerical gene was likely using the promoter located in the δ-LTR region.

6.4.6 Domain Shuffling: New Chimerical Genes Generated Unequal Crossing Over

The ectopic recombination either between similar short sequences (microhomology) present in nonhomologous genes or between divergent paralogous genes could generate new chimerical genes with a different function. An ectopic recombinational event that combines a gene with a new promoter may be a way to generate a dramatic change in the pattern of expression and, thus, may be important in adaptive evolution.

Experimental evolution with yeasts has shown that natural selection can rapidly favor new gene functions generated by ectopic recombination between paralogous genes and subsequent duplications. Brown et al. (1998) analyzed a population of *S. cerevisiae* yeasts that underwent 450 generations of glucose-limited growth. Relative to the ancestral strain, the evolved strain grew at significantly lower steady-state glucose concentrations and demonstrated enhanced cell yield per mole of glucose, significantly enhanced high-affinity glucose transport, and greater relative fitness in pairwise competition. The analysis of the evolved strain revealed the existence of more

than three tandem duplications of a chimerical gene, derived from unequal crossing over, containing the upstream promoter of *HXT7* and the coding sequence of *HXT6*, two adjacent highly similar genes encoding high-affinity hexose transporters originating from a recent duplication. Selection under low glucose concentrations favored a strain containing these duplicated *HXT7*/*HXT6* chimaeras, which increase the ability of *S. cerevisiae* to scavenge glucose at low substrate concentrations.

Another example comes form the study of *S. cerevisiae* yeasts present in spontaneous wine fermentations. Pérez-Ortín et al. (2002) found in several wine strains a new allele of *SSU1* (SSU1-R), a gene that mediates sulfite efflux and, hence, confers sulfite resistance. This new allele was the product of a reciprocal translocation between chromosomes VIII and XVI owing to unequal crossing over mediated by microhomology between very short sequences on the 5′ upstream regions of the *SSU1* and *ECM34* genes. This ectopic recombination put the coding sequence of *SSU1* under the control of the promoter upstream region of *ECM34*, which resulted in a significant increase of *SSU1* expression. They also showed that this chimerical gene (and the translocation) is only present in wine yeast strains, suggesting that the use for millennia of sulfite as a preservative in wine production could have favored its selection.

6.4.7 Domain Duplication: Gene Elongation Generated by Tandem Duplications

Internal duplications have occurred frequently in eukaryote evolution. This increase in gene size, or gene elongation, is an important mechanism to generate complex genes from simple ones (Li 1997).

In the case of yeasts, the most important source of gene elongation is the presence of codon repeats, i.e., trinucleotide microsatellite expansions in coding regions. The most abundant codon repeats found in yeasts are those coding for the amino acids glutamine, asparagine, aspartic acid, glutamic acid, and serine (Albà et al. 1999; Malpertuy et al. 2003).

In most cases, codon repeats show a significant bias toward long tracts of one of the possible codons, suggesting that "trinucleotide replication slippage" is the most important mechanism generating these reiterations (Albà et al. 1999). Replication slippage occurs when a template strand containing contiguous short repeats, in this case trinucleotide repeats, and its copy shift their relative positions during replication owing to mispairing between neighboring repeats, so that part of the template is either copied twice or missed out (Hancock 1999).

However, these different codon repeats are concentrated in different classes of proteins. Thus, acidic and polar amino acid repeats, particularly glutamine, are significantly associated with transcription factors and protein kinases (Richard and Dujon 1997). Changes in the length of repeats in such cellular components of the cell signaling system could alter their biochemical properties, and, hence, modify their interactions with DNA, with other DNA binding proteins, or with other transcription factors and contribute to their evolutionary diversification (Albà et al. 1999; Malpertuy et al. 2003). This modified protein can then be selected for its new function, allowing the cell to increase diversity among its transcription factors,

to specialize them, to adapt to a new environment, and eventually to speciate (Malpertuy et al. 2003). Such diversification could be relatively rapid on an evolutionary time scale because of the high mutation rates of microsatellites (Hancock 1999), which is congruent with the overrepresentation among these transcription factors containing trinucleotide repeats of hemiascomycete-specific genes, which were shown to diverge more rapidly during evolution (Malpertuy et al. 2000).

6.5 Gross Chromosomal Rearrangements in Yeast Evolution

It has largely been proposed that speciation frequently occurs when a population becomes fixed for one or more chromosomal rearrangements that reduce fitness when they are heterozygous. This way, chromosomal rearrangements induce the formation of multivalents during meiosis, resulting in a loss of gamete viability (50% reduction for each translocation).

In the case of *Saccharomyces sensu stricto* species, chromosomal rearrangements have been suggested to account for their postzygotic reproductive isolation (Ryu et al. 1998). However, Fischer et al. (2000) characterized the translocation differences in the species of the *sensu stricto* complex, and concluded that these rearrangements are not required for speciation, since translocations are present only in three species and are not shared between species, indicating that occurred after species divergence.

Delneri et al. (2003) used a reverse approach to determine the role of translocations in speciation. They engineered the genome of a *S. cerevisiae* strain to make it collinear with that of two different *S. mikatae* strains differing in one and two translocations, respectively, with respect to *S. cerevisiae*. Interspecific crosses between strains with collinear genomes resulted in hybrids showing an increase in spore viability (up to 30%). These results indicate that although chromosome rearrangements are not a prerequisite for yeast speciation, they may likely contribute to the reduction of gene flow by suppressing recombination.

The comparative analysis of genomes (Kellis et al. 2003) showed that paralogous genes, transposons, and transfer RNAs (tRNAs) are located at the rearrangement breakpoints, which indicates that ectopic recombination may have been involved in the origin of these chromosomal rearrangements. Indeed, Ty elements or δ-LTRs are well known to induce chromosomal deletion, duplication, translocation, and inversion events by allelic or ectopic recombination in yeasts (Kupiec and Petes 1988; Rachidi et al. 1999). Ectopic recombination, between similar sequences present in nonhomologous genes, between divergent paralogous genes, or between transposable elements could generate evolutionary novelties such as new chimerical genes with a different function of adaptive value (discussed in Sect. 6.4.6) or changes in gene regulation caused by transposable elements on nearby genes.

The fact that selected industrial yeast strains display differences in fitness and in phenotypic traits of industrial relevance that are associated with chromosomal variation (Codón and Benítez 1995) suggests that gross chromosomal rearrangements may be involved in the adaptive evolution of yeasts and account for the high

capacity of industrial yeasts to rapidly evolve. There are several studies whose conclusions support the role of chromosomal rearrangements in the adaptive evolution of yeasts.

Dunham et al. (2002) analyzed the karyotypic changes in six yeast strains, evolved after 100–500 generations of growth in glucose-limited chemostats. These strains contained different chromosomal rearrangements mediated by Ty and tRNA recombinations. Moreover, evolved strains from three independent cultures shared a similar translocation in a chromosome XIV region immediately adjacent to *CIT1*, which encodes the citrate synthase involved in the regulation of tricarboxylic acid cycle. The fact that the same genomic rearrangements recur in different strains suggests that they may be adaptive and responsible for the increased fitness of these strains. Dunham et al. (2002) also postulated that some of the approximately 300 transposon-related sequences found in the *S. cerevisiae* genome are in positions that may provide a selective advantage by allowing adaptively useful chromosomal rearrangements.

Colson et al. (2004) used *S. cerevisiae* strains with artificial translocations, introduced to make their genomes collinear with those of *S. mikatae* strains (see earlier; Delneri et al. 2003), in competition experiments under different physiological conditions. Their experiments showed that the translocated strains of *S. cerevisiae* consistently outcompeted the reference strain with no translocation, both in batch and chemostat culture, but especially under glucose limitation. These results also suggest that chromosomal translocations in yeasts may have an adaptive significance.

Another example comes from the analysis of natural strains. Pérez-Ortín et al. (2002; Sect. 6.4.6) demonstrated that the translocation between *S. cerevisiae* chromosomes VII and XVI, found very frequently in wine strains, was generated by ectopic recombination between genes *ECM34* and *SSU1*, resulting in a chimerical gene that confers a higher resistance to sulfite, a preservative used during winemaking.

Finally, Infante et al. (2003) used the method of comparative genome hybridization with DNA chips, to analyze the genomes of two variants of *S. cerevisiae* flor yeasts, which are adapted to grow aerobically on the surface of sherry wines by transforming ethanol into acetaldehyde. This analysis showed that both strains differ in 116 rearranged regions that comprise 38% of their genomes. These authors concluded that the presence of genes that confer specific characteristics to the flor yeast within these regions supports the role of chromosomal rearrangements as a major mechanism of adaptive evolution in *S. cerevisiae*.

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Chapter 7

Principles and Applications of Genomics and Proteomics in the Analysis of Industrial Yeast Strains

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7.1 Introduction

Yeasts are presumably the oldest commercially cultured microorganisms and are widely used in the food and beverage industries. *Saccharomyces cerevisiae* belongs to the phylum *Hemiascomycetes*, and at least 1,000 separate strains of *S. cerevisiae* are currently being used in the baking, brewing, distilling and wine-making industries with at least two million tons of yeasts produced per year in the brewing and baking industries alone. Industrial yeast strains can be obtained from a number of repositories, such as the National Collection of Yeasts Culture (http://www.ncyc.co.uk/), The Culture Collection of Yeasts (http://www.chem.sk/yeast/culture_collection_of_yeasts.htm), The European Culture Collections' Organization (ECCO; http://www.eccosite.org/) and the Collection de Levures d'Interêt Biotechnology (http://www.inra.fr/Internet/), to name but a few. The high fermentative capacity of yeasts, together with their ability to withstand the extreme environmental conditions experienced during industrial fermentations, has led to the selection of strains with unique characteristics. In this chapter we focus on the recent use of genomics and proteomics approaches to gain an understanding of the nature of the genomes of industrial strains of yeasts, the expression of genes within these genomes and their final proteome complement.

7.2 DNA Sequencing of Yeast Genomes

To completely understand the molecular and physiological composition of any organism, it is essential to have the complete DNA sequence of its genome. This systematic approach, at a minimum, allows the researcher to determine the number of genes encoding functional proteins and provides opportunities for the theoretical

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and experimental analysis of all these genes. In this section we outline the background to the sequencing of the haploid genome of the yeast *S. cerevisiae* and show how this endeavour acts as a paradigm for the analysis of the more complex genomes of the industrial strains of yeasts.

The establishment of the *Saccharomyces* Genome Database (SGD; http://www.yeastgenome.org) has been an important hub in the dissemination of information regarding the yeast genome and allows one to access the DNA sequence information of all open reading frames (ORFs). Additionally, the SGD provides links to other web-based databases such as GenBank (DNA) and GenPept (protein) at the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov), Munich Information Centre for Protein Sequences (http://mips.gsf.de/genre/proj/yeast/), Protein Information Resources (http://pir.georgetown.edu/) and SwissProt (http://us.expasy.org/sprot/), to name but a few. Links to gene expression and other functional genomics databases are also available which provide the possibility for direct comparisons of the gene expression profiles for the entire genome under a variety of environmental conditions and in a variety of genetic backgrounds (see later). Presently, the Yeast Genome Sequencing Project and the subsequent annotation of the sequences into a user-friendly database at the SGD is the prototype for the sequencing and annotation of other genomes.

7.2.1 Sequencing of the Genome of *Saccharomyces cerevisiae*

In order to obtain the entire genome sequence of an organism, it is essential to first create a complete representative genomic library. In the early 1980s, the pioneering work of Burke and Olsen allowed the cloning of large genomic fragments in the order of several 100 kb in length into yeast artificial chromosomes (YACs) (Burke et al. 1987). YAC plasmids contain both centromere and telomere sequences, thus allowing the plasmid and the inserted DNA to replicate like a chromosome. Yeast genomic libraries were also prepared in cosmids which are replicating plasmids containing the cos sites of the bacteriophage λ. Genomic fragments as large as 45 kbp can be cloned into cosmids. By sequencing and examining overlapping sequences, of YAC or cosmid clones, a physical map of the yeast genome was created (Cherry et al. 1997). This map with its linked library of DNA clones provided the starting point for the yeast genome sequencing project.

The DNA sequence of the *S. cerevisiae* genome was completed in 1996 (Goffeau et al. 1996). This was achieved through the cooperation of over 600 scientists from Europe, the USA and Japan using automated robotic machines to sequence DNA from random YAC or cosmid clones. Adding to this was DNA sequence information emerging from a "network" of small to medium-sized yeast laboratories which also led the interpretation and verification of the DNA sequences emanating from the DNA sequencing centres. From this information, it was possible to deduce the organisation of the *Saccharomyces* genome, which was shown to be composed of twelve million base pairs arranged on 16 chromosomes (Mewes et al. 1997). Originally, 6,275 theoretical ORFs containing greater than 100 amino acids were identified in the genome sequence. Following subsequent reanalysis of the data and the inclusion of additional information from the scientific literature, this number

was revised downward as of May 2002 to 6,062, of which 3,966 represented ORFs previously identified by genetic analysis or by the presence of structurally and functionally related orthologues in other species and 2,096 genes of unknown function. The estimate for the number of protein coding genes has more recently been further refined and the current estimate at the SGD (as of January 2005) is 5,798. In addition to ORFs, sequences corresponding to non-protein coding genes such as those coding for ribosomal RNA, transfer RNAs (tRNAs), small nuclear RNAs, small nucleolar RNAs, non-coding RNAs, transposable elements (Ty) and long terminal repeats (LTRs) have been identified in the genome.

7.2.2 Genome Sequencing of Other Yeast Species

Since the initial sequencing of the *S. cerevisiae* genome, the DNA sequences of a number of other members of the *Saccharomyces sensu stricto* group have been completed. A comparative analysis of the genomic sequences of *S. bayanus*, *S. mikatae* and *S. paradoxus*, which are separated from *S. cerevisiae* by approximately five million to twenty million years of evolution, confirms the organisation of ORFs onto 16 chromosomes with an average genome size of approximately $11.5 \times 10^6 – 12 \times 10^6$ bp for this genera (Kellis et al. 2003). The four genomes show a high degree of conservation of synteny with only 1.3% of sites of insertions or deletions falling within protein coding regions. The 32 telomeric and subtelomeric regions of the 16 chromosomes represent the regions of greatest sequence diversity. By using a reading frame conservation (RFC) test, together with manual inspection of dubious ORFs for all initially predicted 6,275 theoretical ORFs from the *S. cerevisiae* genome, Kellis et al. (2003) identified 5,458 ORF orthologs in all four species, thus leading to a re-evaluation of the number of true ORFs in the *S. cerevisiae* genome.The comparative genomic analysis from multiples members of the *Saccharomyces sensu stricto* group, in addition to defining the number of true ORFs in these species, has allowed a comprehensive analysis of the rate of evolution of these genomes and the identification of ORFs unique to each species. Additionally, the genome alignments of all four species has allowed the identification of regulatory elements in the intergenic regions of the genomes. The sequencing of more distantly-related yeasts such as *S. kluyveri* (Cliften et al. 2003; Kellis et al. 2003), *Candida glabrata*, *Kluyveromyces lactis*, *Debaryomyces hansenii*, *Yarrowia lipolytica* (Dujon et al. 2004), *Ashbya gossypii* (Dietrich et al. 2004) and *Kluyveromyces waltii* (Kellis et al. 2004) has added to our understanding of the evolution of the genomes of the *Hemiascomycetes*.

7.3 Whole Genome Approaches to the Characterisation of Industrial Strains of Yeasts

The complete genome sequence is an important resource allowing scientists to examine the physiology and evolution of related organisms. It is also the prerequisite for some of the developed genome-wide techniques that have revolutionised the way biological systems are studied. In this section we outline some of the basics of these techniques aiming at the analyses of the complete complement of transcripts (the transcriptome) and proteins (the proteome) in a cell.

7.3.1 Microarray Technology for Genome and Transcriptome Analysis

Microarray technology grew out of the complete DNA sequencing of the haploid *S. cerevisiae* genome and involves robotic application of DNA, representing each gene in the genome, to glass slides, silicone or nylon membranes. The DNAs are arrayed in an orderly fashion to allow easy identification of genes within the array (Chittur 2004; Epstein and Butow 2000; Gerhold et al. 1999; Hardiman 2004). Two types of DNA microarray chips are currently used. Firstly, DNA sequences (500–5,000-bases long) representing individual ORFs are PCR-amplified using DNA oligos specific to each gene. These DNAs are robotically "spotted" onto the glass slides or nylon membranes. The second method generates arrays of oligonucleotides (20–80-mer oligos) representing each gene. These oligonucleotides can be directly synthesised using photolithographic techniques in situ or by conventional synthesis followed by on-chip immobilisation. A variation on this second approach is to include internal controls on the microarrays in which single nucleotide mismatches of each oligonucleotide are included on the chips to allow quantification of the specificity of hybridisation to a given probe. The prototype oligonucleotide microarrays were developed at Affymetrix, which sells its products under the GeneChip trademark.

The arraying of the whole genome, representing each individual gene, on a single matrix, allows the simultaneous analysis of the complete messenger RNA (mRNA) profile (transcriptome) of an organism in a single experiment. Relative steady-state levels of mRNAs are normally examined temporally or spatially under experimental conditions where perturbations from the normal growing conditions are imposed. These perturbation may include changes in genetic background, environmental changes or pharmacological changes, to name but a few. This is achieved through the principles of nucleic acid hybridisation. Briefly, heat denatured double-stranded DNA or single-stranded DNA on the microarray chips is incubated with a labelled probe. For transcriptome analysis, the probes are prepared by first converting RNA to complementary DNAs (cDNAs) by random priming using the enzyme reverse transcriptase. This cDNA probe represents a "snapshot" of the total pool of mRNAs present in the cell under a specific set of experimental conditions. A similar cDNA pool is prepared from RNA extracted from cells grown under "control" conditions. The cDNA probes are differentially labelled by incorporating the fluorescently tagged nucleotides Cy3 (green; 635 nm; control) and Cy5 (red; 532 nm; experimental), respectively, during the reverse transcription reaction. The differentially labelled cDNAs are then mixed and added to the microarray chip, where they compete for hybridisation to the DNA sequences on the chip. The extent of hybridisation is directly proportional to the amount of cDNA, representing a specific mRNA in the sample, that is complementary to a given DNA sequence on the chip and the degree of sequence homology between the two sequences.

Hybridisations are normally carried out in a solution containing 5X SSC (0.6 M sodium chloride, 0.06 M sodium citrate), 7% sodium dodecyl sulfate (SDS) and 50 mM sodium phosphate, pH 7.0. Blocking reagents such as Denhardt's solution [0.02% poly(vinylpyrrollidone), 0.02% bovine serum albumin, 0.002% Ficoll 400] or commercially available blocking reagents are added to the hybridisation solution to

increase the effective concentration of the probe and to reduce non-specific hybridisation. Hybridisations are normally carried out at 68°C or at a temperature below the melting temperature of the probe. Under these hybridisation conditions the labelled probe will bind to its complementary sequence on the DNA chip. The stringency of hybridisation can be varied by changing the temperature of hybridisation or the composition of the hybridisation solution. Following hybridisation, the filters are washed to remove unhybridised probe. Again the stringency of hybridisation can be adjusted here by varying the washing conditions; high-stringency washing (0.5X SSC, 0.1% SDS; 68°C) will allow only completely identical sequences to hybridise, while low-stringency conditions (2X SSC, 0.1% SDS; 68°C or at a lower temperature) will allow hybridisation between DNAs containing mismatches.

The hybridised DNA can then be measured using a fluorescence scanner. The readout from the fluorescence detector is then analysed using programs such as Genepix Pro or ScanAlyze and is expressed as pixels of green or red fluorescent light per square millimetre for each gene. Following normalisation of the data to correct for different efficiencies of labelling and corrections for size of the spot, subtraction of background and removal of spurious readings, the data can be directly fed into spreadsheet programs. The data are presented as a normalised linear ratio or a normalised $\log_2$ ratio of red-to-green fluorescence. The red-to-green fluorescence ratio gives a direct measurement of the relative proportions of RNA (cDNA) in the starting samples. A $\log_2$ ratio of greater than zero indicates a higher level of RNA (cDNA) or DNA in the experimental sample compared with the control sample, while a $\log_2$ ratio of less than zero indicates the opposite.

The red-to-green fluorescence ratios can then be fed directly into clustering programs such as Cluster (http://rana.lbl.gov/eisensoftware.htm) (Eisen et al. 1998). Clustering programs use hierarchical or *K*-means algorithms as a means of identifying and correlating patterns of gene expression and can be used to group together, into expression classes, genes showing similar gene expression patterns. Clustering programs also allow data from multiple microarray experiments to be analysed simultaneously. The cluster output can be viewed in TreeView (http://rana.lbl.gov/eisensoftware.htm) as a colour-coded graphical representation of expression profiles at a glance (Fig. 7.1).

Microarray technology has been exploited to generate a vast amount of data examining the gene expression patterns of *S. cerevisiae* under a variety of experimental conditions. The majority of these can be accessed through the SGD or directly at the site Yeast Microarray Global viewer (yMGV; www.transcriptome.ens.fr/). The latter site contains data from 1,544 experiments mainly showing gene expression patterns for the haploid yeast *S. cerevisiae*. Comparison of gene expression data generated from different sources is often hampered by differing experimental parameters being examined. However, as mentioned before, programs such as Cluster allow the side-by-side clustering of gene expression patterns from any number of differing sources and will reveal overall similarities and differences in the patterns. The yMGV site provides a graphical representation of gene expression variations for each published genome-wide experiment. Additionally, one can examine the effects of experimental conditions on one or a group of genes and identify groups of genes sharing similar transcription profiles in a defined subset of experiments.

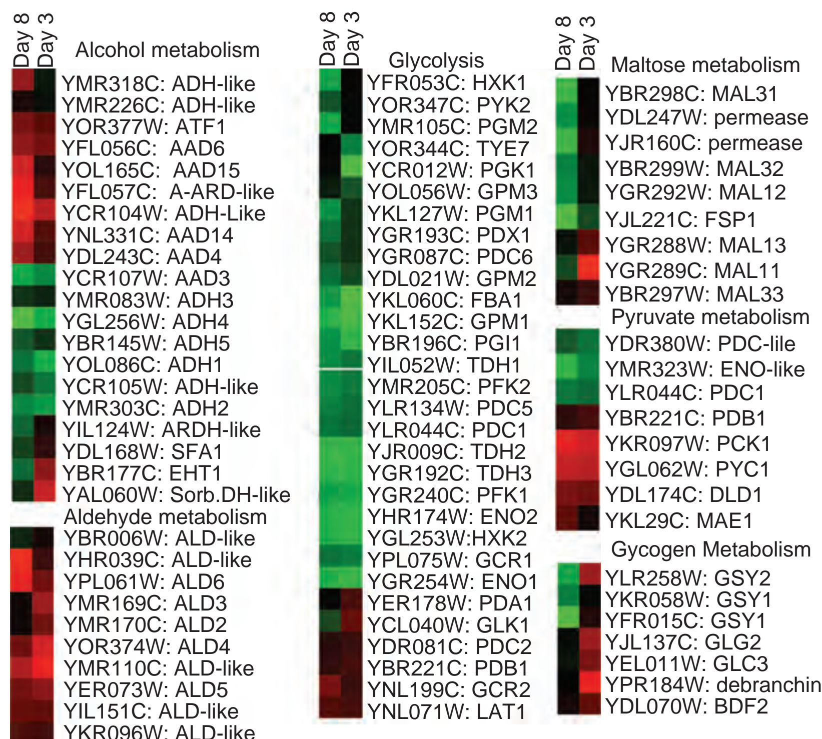

Fig. 7.1. TreeView display showing expression profiles of genes encoding proteins involved in carbohydrate metabolism. Log_2 ratios of transcript levels on days 3 and 8 of a brewing fermentation with a lager strain of a yeast relative to the levels on day 1 were obtained from a microarray analysis (James et al. 2003). Genes were first grouped together by cellular process and then clustered to reveal transcripts showing similar levels of expression (log_2 ratios) using the program Cluster. The range and intensity of colours from *red* to *green* represents a continuum of highest levels of induction (*red*) or repression (*green*)

One of the most useful datasets is that of gene expression patterns of *S. cerevisiae* under a variety of environmental conditions experienced by yeasts, such as heat and cold shock, amino acid starvation, nitrogen depletion, and during the exponential and stationary growth phase (Gasch et al. 2000).

7.3.2 Technologies for Proteome Analysis

The mRNA expression changes estimated by microarray analysis should ideally reflect the change in the amount of protein under the same experimental conditions. However, in many instances this is at its best a good approximation and rather large discrepancies are revealed. Additionally, transcriptome analysis does not reflect the complex myriad of post-translational features, like protein modifications

(e.g. phosphorylation, N-terminal acetylation or ribosylation), protein association with cofactors (e.g. NADH or zinc), protein complex formation (e.g. the ribosome contains almost 100 components), protein localisation (e.g. into mitochondria or the nucleus) and protein degradation (e.g. ubiquitination and breakdown via the proteasome), that eventually define the active proteome component of the cell. Ideally all these levels of complexity should be examined for a full understanding of protein activity in the cell and hence the system under study. This, in short, defines the great challenge in proteomics. In addition, the quantitative range of proteins in the cell is huge; recent estimates indicate that protein abundance in yeasts covers roughly 5 orders of magnitude (Ghaemmaghami et al. 2003), which of course adds to the technological challenge. Proteomics on *S. cerevisiae* currently includes a plethora of techniques, where many of the methodologies rely on the fact that the full genome sequence is available. In the analysis of industrial yeast strains where the full genome has not yet been released, the most fundamental proteomics approach with electrophoretic separation of cell extracts, subsequent image analysis and protein identification has so far been applied. However, the application of some large-scale techniques in proteomics, presently only within reach for the analysis of laboratory *S. cerevisiae* strains, will most likely in the near future also be applied for the analysis of industrial strains (Sect. 7.6).

7.3.2.1 Two-Dimensional Polyacrylamide Gel Electrophoresis

The standard methodology in proteomics has been the combination of two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) separation and quantification with mass spectrometry (MS) based identification of resolved proteins. Separating/arraying all proteins in a yeast cell provides a substantial experimental challenge. The technique applied must deal with the great qualitative and quantitative complexity and should be able to do so with a reasonably high throughput of samples. The technique most frequently used to obtain high-resolution separation of proteins is 2D-PAGE, which resolves proteins in two consecutive steps that separate on the basis of independent protein-specific properties, i.e. the isoelectric point (pI) and the molecular weight (M_r). The principle is simple and elegant and when first applied in the mid-1970s it was a major breakthrough in the molecular global analysis of biological samples (Klose 1975; O'Farrell 1975). The major procedure has not changed much over the years; however, substantial refinements in chemistry and hardware have made the currently applied technique vastly superior to the initial setup.

Isoelectric focusing of native proteins is well established and has been extensively used. To increase the resolving power, separation under denaturing conditions by adding high amounts of urea (about 9 M) and 1–4% of a non-ionic detergent was developed. The denaturing capacity is increased even further by inclusion of thiourea in addition to urea as a chaotroph, and the use of zwitterionic amphiphilic compounds (e.g. 3-[3-(Cholamidopropyl)dimethylammonio]-1-propanesulfonate, CHAPS, or SB 3–10) (Rabilloud et al. 1997). This procedure is particularly useful for the resolution of problematic proteins (Rabilloud 1998), but has become the standard procedure in many laboratories.

Separation based on different protein pIs was initially achieved by the inclusion of carrier ampholytes, molecules that in an electric field will generate a pH gradient in which proteins migrate until they reach the pH where their net charge is zero. However, when applying a wide-range ampholyte mixture, such as one with pH 3–10, the separation will not cover this wide a pH range at the end of the run; the final gradient will cover not more than about pH 4–6.5 (O'Farrell et al. 1977). The consequence is, of course, that proteins with pI values outside the produced pH range of the gradient will not focus. One solution to this problem is to pursue isoelectric focusing for shorter times, leading to a gradient with greater pH coverage (O'Farrell et al. 1977). This technique is then a non-equilibrium one, and proteins will, in general, not reach their true pI value. These non-equilibrium pH gradient gel electrophoresis (NEPHGE) gels have been applied to the separation of yeast samples, especially in the past (Bataillé et al. 1988; Boucherie et al. 1995; Brousse et al. 1985; Iida and Yahara 1984; Shin et al. 1987). The main problem with the NEPHGE procedure is reproducibility and standardisation.

The technical solution to at least some of the problems mentioned before for ampholytes came with the invention and application of immobilines (Bjellqvist et al. 1982; Görg et al. 1988, 1999). Immobilines produce a pH gradient that is generated by the covalent anchoring of the immobilines to the polymer matrix. The consequence is that the gradient will be stable and not collapse during prolonged running time. In addition, shallow (zooming in on narrower pH regions) or very broad gradients can be formed, and even the use of sigmoid-shaped non-linear pH 3–10 gradients for optimal protein pattern spread has been applied for the analysis of yeasts (Muller et al. 1999; Norbeck and Blomberg 1997, 2000). In addition, with the development of more alkaline pH gradients (up to pH 12.5) the 2D system can resolve very alkaline proteins like histones and many ribosomal proteins (Wildgruber et al. 2002). The use of shallower gradients, where better physical separation is obtained between proteins with closely matching pI values, can be very useful, and will minimise the shielding effect on low-abundance proteins from abundant neighbours (Wildgruber et al. 2000).

For the 2D separation there is presently no good system for the parallel separation of proteins in the range 1–600 kDa, which is the size range provided by the yeast proteome. Size differences can be dealt with but only if we apply slightly different techniques for different size classes: i.e. altering the concentration of the polyacrylamide matrix will enable better separation of small proteins (high total percentage of acrylamide, %T) or large proteins (low %T) (Garrels 1979). However, choosing either of these extremes will of course compromise the resolution of the opposite size class. In practice this means that more gels have to be run and fewer samples will be analysed. Most groups have thus adopted some intermediate concentration (10–12%T) as the standard, thus accepting minor losses in resolution of extremes in the process.

7.3.2.2 Post-Separation Analyses – Image Analysis

Visualisation of 2D-PAGE-separated proteins can be performed by different means, and when dealing with the industrial-scale visualisation this is achieved by the use of

some sensitive stain. Silver deposits at protein spots can detect low nanogram quantities of protein; however, different silver staining protocols are more or less sensitive and some are more useful for quantitative purposes (Blomberg 2002; Rabilloud 1992). The signal-response curve for silver staining is linear for all protocols over a rather narrow dynamic range, and proper quantification over a wider range can only be achieved by the use of calibration strips. A recent development of great use in protein quantitation is the use of fluorescent dyes. The currently most frequently used dye is Sypro Ruby, which has been documented to generate a linear response over some orders of magnitude. This staining procedure is, however, a bit less sensitive than silver staining (Rabilloud et al. 2001; Blomberg et al., unpublished data). In Fig. 7.2a the 2D pattern of lager yeast strain CMBS33 from Sypro Ruby staining can be observed from cells grown under laboratory conditions in synthetic defined medium containing glucose. Recently dual labelling with covalent linkage of fluorescent dyes prior to electrophoretic separation was also applied, i.e. fluorescence-based multiplexed proteomics (Patton and Beechem 2002). Different samples are labelled with dyes with different spectral properties, mixed and separated by 2D-PAGE. The resulting gel is scanned in different spectral windows and the individual quantities estimated. The main advantage of this methodology is that between-gel variation is cancelled, which makes sample comparison and spot matching more straightforward. However, if more than two or three samples are to be analysed, which is usually the case in proteome analysis if statistics are to be applied (which it should), the problem still persists with gel-pattern matching. Any of these means for

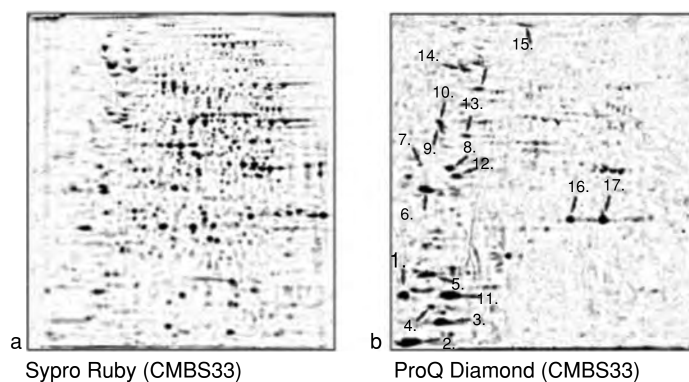

Fig. 7.2. Differential staining with Sypro Ruby and ProQ diamond of 2D-separated proteins from a lager yeast strain. The lager strain CMBS33 was grown in synthetic defined medium with glucose as the carbon and energy source and harvested in the mid-exponential growth phase. (**a**) Total proteins were separated using a wide-range non-linear pH 3–10 gradient in the first dimension and were visualised by the fluorescent dye Sypro Ruby. (**b**) The same gel as in **a** was subsequently stained with the phosphoprotein specific stain ProQ diamond to visualise proteins in the pattern that were modified by phosphorylation (about 25 proteins displayed significant phosphorylation specific staining). The acidic side in the first dimension is to the *left*. (Adopted from R. Caesar, E. Pettersson, J. Gustafsson, A. Hashemi, A. Blomberg, unpublished results)

visualisation can be utilised for protein expression analysis of samples cultivated on an industrial scale. However, the most precise quantitative method is not really applicable to large-scale fermentations – isotopic labelling of proteins during cultivation by addition of a radioactive amino acid, usually ^{35}S-methionine. After separation the gels are dried and exposed to image plates that have a higher sensitivity and a wider linear range of response compared with those of ordinary X-ray film (Blomberg 2002).

Protein stains have also been developed to specifically capture protein features. One good example of this is the recently developed stain ProQ diamond, where gels are fluorescently stained and imaged to reveal phosphorylation levels using this fluorescent phosphosensor dye (Schulenberg et al. 2003). The initial staining step is subsequently followed by staining and imaging to reveal general protein expression levels using a total protein indicator such as Sypro Ruby. ProQ diamond specific proteins can subsequently be identified by peptide mass fingerprinting. Figure 7.2b displays the phosphorylated proteins indicated by ProQ diamond staining in the lager strain CMBS33. Some of the phopsphorylated proteins were identified earlier as phosphoproteins in *S. cerevisiae*, while others are novel phosphorylation targets (R. Caesar, E. Pettersson, J. Gustafsson, A. Hashemi, A. Blomberg, unpublished results).

Generated images can be analysed for quantitative changes in protein spot volume. For this task there are currently a number of different commercial software packages. All of them are based on some procedure for background subtraction, smoothing and subsequent spot identification and quantification (Blomberg 2002). Different software packages apply different strategies in the analysis of spot volumes. Some use mathematical modelling based on spot width and peak height, while others identify the outer borders of the spot and sum all the pixel values; however, the quantitative results are usually more or less similar, at least for well-resolved spots. The final analysis is to compare the quantitative values of individual protein spots between different samples. This matching process is supposed to be automated in many of the software packages, but still much time is spent in manual editing and matching of different images. Currently procedures are being developed that hopefully will lead to more accurate automatic handling of large collections of image data; these are based on image warping prior to matching (Gustafsson et al. 2002; Veeser et al. 2001).

7.3.2.3 Protein Identification: a Historical Survey

The 2D map obtained is of rather limited biological value if resolved protein spots are not identified. This was initially performed by co-migration with purified preparations of enzymes. Applying this procedure, Calvin McLaughlin and colleagues were able to produce the first annotated 2D map of *S. cerevisiae*, which in 1978 contained identity for 17 proteins (Elliott and McLaughlin 1978). The proteins identified were mostly very abundant and involved in glycolysis, like hexokinase, triosephosphate isomerase and enolase.

Another early procedure for the identification of proteins in the 2D patterns was by the use of antibodies. Proteins were separated and subsequently blotted onto

filters where polyclonal antibodies were used for identification. This approach was first attempted for yeasts by the Bordeaux group lead by Helian Boucherie. Antibodies were produced against purified preparations of enolase and glyceraldehyde-3-phosphate dehydrogenase, and these antibodies were used for protein identification on 2D gels (Brousse et al. 1985) and were also applied in the first analysis of some wine yeasts (Brousse et al. 1985). The antibodies detected both Eno1p and Eno2p as well as Tdh2p and Tdh3p (as we know them today). This exemplifies a weak point when using polyclonal antibodies; they are prone to cross-reactivity. The future use of antibodies in protein characterisation would most likely be found in the large scale analysis of diverse protein modifications or in the analysis of low-abundance proteins.

The advent of molecular biology techniques provided novel avenues for protein identification since genes could be cloned and overexpressed by the use of multicopy plasmids. This strategy was explored for the identification of the location of *PGI* and *PGM* encoded polypeptides in the 2D pattern (Bataillé et al. 1987), as greatly enlarged spots. A variation on this theme would be the 2D analysis of a gene knockout, where the gene product in question is scored as a missing 2D spot in the mutant.

A cheap, rapid, yet efficient way of identifying proteins on a laboratory scale is based on the determination of their amino acid ratios (Garrels et al. 1994; Maillet et al. 1996). Specific double-labelling with ^{3}H and ^{14}C or ^{35}S-labelled amino acids, chosen among those that are specifically incorporated into proteins without interconversion, allowed an accurate measurement of different amino acid ratios for roughly 200 proteins. This double-labelling technique is particularly suited for the analysis of proteins that are only transiently expressed; these proteins will be difficult to analyse by other means since in many instances they will be present at very low levels.

The first technique for direct identification of resolved proteins was microsequencing. In principle, microsequencing works extremely well in most cases; however, a number of problems have also been encountered, e.g. proteins frequently carry modifications in their N-terminus, which blocked and stopped the sequencing reaction. Thus, novel procedures for generating material to be analysed in the microsequencer were developed. The most appealing and experimentally straightforward method was in-gel digestion with a specific protease, mostly trypsin, and subsequent elution and fractionation of the peptides generated (Norbeck and Blomberg 1995; Rosenfeld et al. 1992). The in-gel digestion turned out to be a major breakthrough in the analysis of proteins and is still the main method in use today for the generation of peptide cocktails from 2D-resolved proteins. A number of laboratories have successfully adopted this strategy of microsequencing of isolated peptides and used it for the annotation of their yeast map (Garrels et al. 1997; Norbeck and Blomberg 1995, 1997; Sanchez et al. 1996). Microsequencing is a very powerful technique when it comes to stringent identification of proteins, in particular for closely related proteins (Norbeck and Blomberg 1995). However, this technique also has a number of drawbacks: (1) it is rather expensive to run and maintain (e.g. chemicals have to be ultrapure), (2) the analysis time for each amino acid is about 30–45 min, at least for the somewhat older microsequencers, which results in few protein identifications per day, and (3) rather large amounts of protein are required (1–10 pmol). This

excludes the use of microsequencing in any type of really large scale programme when linked to the characterisation of hundreds of proteins resolved by 2D-PAGE.

7.3.2.4 Protein Identification: Mass Spectrometry

Currently the methodology of choice for large-scale protein identification is based on the use of mass spectrometry. Molecules to be analysed by mass spectrometry are ionised in the gas phase and are subsequently resolved in relation to their mass-to-charge ratio. Key in the protein analyses by mass spectrometers was to get large molecules like proteins and peptides into the gas phase as ions. Much effort was spent in the past to develop non-destructive "soft" ionisation methods that are compatible with studies of proteins and peptides. Two different ionisation methods were eventually developed that proved versatile, non-destructive and robust in the ionisation of a wide spectrum of peptides and proteins.

In matrix-assisted laser desorption ionisation (MALDI) a large excess of matrix material is mixed with the analyte molecule and a small volume of the mixture is placed on a metal target where it is allowed to dry and form crystals (Mann et al. 2001). Nanosecond laser pulses from nitrogen lasers are then used to irradiate the crystals under vacuum. The matrix is a small organic molecule like the commonly used compound α-cyano-4-hydroxycinnamic acid, and it is believed that the matrix molecules absorb the laser energy, resulting in desorption, and throw out of a small volume of matrix and embedded analytes. The matrix thus serves the purpose of propelling the non-volatile proteins and peptides into the gas phase. The ionisation process of a peptide competes with ionisation of all other peptides in the mixture, and this suppression phenomenon results in a context-dependent signal intensity for a particular peptide (Larsson et al. 1997). This phenomenon makes MALDI-MS non-quantitative and also explains why some peptides never "fly" in the analysis of complex peptide mixtures.

In the other ionisation method, electrospray ionisation (ESI), a low flow rate of liquid (microlitres per minute or less) is pumped through a very narrow and high electric potential needle. This procedure leads to a dispersion of the liquid into micrometre-sized charged droplets, which is called an electrospray (Mann et al. 2001). When these small droplets evaporate charge is transferred to the analyte molecules. Electrospray is regarded as a very soft ionisation method since it rarely fragments the analyte ions. ESI works well on many types of macromolecules and usually results in multiply charged larger molecules. Since the separation and analysis in the mass spectrometer is based on mass over charge, multiple charges produced by ESI bring even very large proteins into the mass/charge analytical range of most mass spectrometers. This ionisation method can be directly coupled in-line with a high-performance liquid chromatography system. This system design is rather robust since sample cleanup, separation and concentration is performed in a single in-line chromatographic step. Many of the very potent applications of ESI-MS in proteomics are currently based on in-line separation of peptides by 1D or 2D chromatography systems.

Mass spectrometers measure the mass-to-charge ratio of ions. This can be achieved by separation based on time-of-flight (TOF-MS), quadrupole electric fields

generated by metal rods (quadrupole MS), or selective ejection of ions from a 3D trapping field (ion-trap MS) (Mann et al. 2001). When structural information is to be gained, e.g. in peptide sequencing, two different steps of *m*/*z* analysis are performed in tandem MS (MS/MS) with some kind of fragmentation procedure, in a collision-induced dissociation (CID) chamber, placed in between. The MS/MS analysis can in principle be performed by employing the same *m*/*z* separation principle twice (e.g. TOF-TOF) or by combining two different ones (e.g. quadrupole TOF). Both ionisation procedures described, MALDI and ESI, can be coupled to any of the *m*/*z* separation methods; however, some combinations appear to be a better match and are more frequently found in current commercial instruments (e.g. MALDI-TOF; ESI ion trap).

In the TOF-type mass spectrometers ions are accelerated to a fixed amount of kinetic energy by a strong electric field, applied some 100–500 ns after the laser pulse, and the ions travel down a flight tube. This procedure leads to molecules with low *m*/*z* values having higher velocities and getting to the detector before the ones with high *m*/*z* values. The mass accuracy of MALDI-TOF instruments is roughly 10 ppm (0.01 Da for a 1-kDa peptide). The quadrupole is a mass filter that is composed of four metallic rods to which an oscillating electric field is applied. This filter lets through only ions with a certain *m*/*z* value, with the other ones not reaching the detector. The mass-over-charge spectrum is obtained by changing the amplitude of the electric field and recording the ions that reach the detector. Most peptide sequence analyses have been performed on triple quadrupole instruments where the design is divided into three sections. Two sections that provide *m*/*z* filters and one central quadrupole section that contains the ions during fragmentation thus constitute the CID chamber. Quadrupole mass spectrometers are capable of unit mass resolution and mass accuracy of 100–500 ppm. In ion traps the ions are physically trapped in a 3D electric field. Ion traps capture the continuous beam of ions up to the limit of the maximum number of ions that can be introduced into the trap without distorting the electrical field. After capture, the ions are subjected to additional electrical fields that lead to one ion after the other being ejected from the trap. The ejected ions are detected and this produces the *m*/*z* spectrum. In cases where MS/MS analysis is to be performed with the ion trap, all except the desired ion are first ejected. After that the remaining ion is fragmented by collision to a gas and the fragments are analysed by the same procedure as before. This construction allows for multiple rounds of analysis and fragmentations, with the result that a large number of MS/MS analysis can be performed (MS^n). Ion traps are compact, robust and very versatile instruments for which the operation can be highly automated. The mass accuracy of the ion traps is slightly less than for the quadrupole instruments, which is usually not a great problem in the analysis of yeast proteins because of the relatively small genome (only roughly 6,000 proteins).

Algorithms for protein identification based on MS data has been developed and refined (Mann et al. 2001). Originally protein identification was almost exclusively performed using MALDI-TOF-MS data, where the peptide mass fingerprint generated was compared with the theoretically predicted masses of peptides for each entry in the protein database. Trypsin digestion has most frequently been used (cleavage after arginine and lysine residues), but in principle any type of specific protease

could be used in the generation of peptides. The first step in the procedure is that visualised proteins are physically cut out from preparative gels. These gel pieces are destained and washed, dehydrated and then rehydrated with trypsin solution. With the growing number of sequences in the databases, more sophisticated algorithms have been developed that generate lists of protein hits with corresponding probability scores. However, as a rule of thumb for an unambiguous identification, when high mass accuracy in the range 10–50 ppm is achieved, at least five peptide mass MALDI-MS data need to match that should cover at least 15% of the length of the protein. MS/MS data can also be used for database searches. Since sequence information is revealed in the tandem mass spectra, these searches provide higher confidence and greater discrimination. Several alternative algorithms exist, but here it suffices to mention that experimental spectra are matched against calculated fragmentation spectra for all peptides in the database. The power of the MS/MS approach truly comes into play when mixtures of proteins (or whole proteomes) are to be analysed (Washburn et al. 2001), since single peptide fragmentations can be sufficient to identify a particular protein in the sample.

7.3.2.5 Yeast Proteome Databases

Currently the most impressive and complete global analysis of the yeast proteome includes the analysis of 400 proteins corresponding to 279 different genes for the laboratory strain S288c during exponential growth in synthetic defined medium (Perrot et al. 1999). In this analysis, a combination of different techniques was used in the identification of the protein spots; genetic alterations (gene overexpression or deletion) 121 proteins, amino acid composition 114 proteins, and MS 221 proteins. Many of the most abundant proteins in the 2D pattern are involved in energy metabolism, of which proteins encoded by 48 genes have been identified. A large proportion of the proteins identified are involved in biosynthesis of either small molecules (65 different genes) or macromolecules (79 genes). Some of the metabolic pathways are almost completely covered, like purine synthesis, which was also used for the first complete study of all its components (Denis et al. 1998). However, only a small proportion (five proteins) of the subunits in the ribosome were separated and identified. A good proportion of the aminoacyl-tRNA synthetases have been identified, 13 out of the 20 believed to be present in the genome. In addition, a high number of chaperones are present; these are mostly rather dominant and are represented by a large variety of proteins and different families in yeasts. Also identified are components involved in the cellular response to growth perturbations (stress); osmotic adaptation (four proteins) and detoxification (six proteins). This data can be explored at the Bordeaux 2D database (YPM; http://www.ibgc.u-bordeaux2.fr/YPM/).

7.4 Genome Constitution of Industrial Strains of Yeasts

The genomic analysis of industrial strains of yeasts has lagged behind that of the prototype haploid *S. cerevisiae* species. This is partly due to the complex nature of their genomes. Industrial strains of yeasts, in particular those involved in the beer and wine industries, all belong to the *Saccharomyces sensu stricto* group. Currently,

this taxonomic group includes seven yeast species, *S. cerevisiae*, *S. paradoxus*, *S. pastorianus*, *S. cariocanus*, *S. kudriavzevii*, *S. bayanus* and *S. mikatae*.

Yeasts isolated from wine fermentation in which no starting inoculum is used tend to belong to the species *S. cerevisiae* or *S. bayanus*, with the majority being *S. cerevisiae* (Mortimer 2000). A small number of wine yeasts appear to be interspecies hybrids of *S. cerevisiae* and *S. bayanus*, while one particular cider yeast strain (CID1) contains DNA from three separate species (de Barros Lopes et al. 2002; Mortimer 2000). The majority (approximately 70%) of wine yeasts are prototrophic, heterozygous and homothallic and most possess diploid genomes (Mortimer 2000).

Industrial beer fermentations in modern-day breweries use two uniquely different strains of yeasts, the top-fermenting ale strains and the bottom-fermenting lager strains, the names "top" and "bottom" reflecting whether the yeast rises to the top of the fermentation or settles to the bottom at the end of fermentation (Kielland-Brandt et al. 1995; Kodama et al. 2005). Top-fermenting yeasts produce ales and closely resemble *S. cerevisiae*, while the bottom-fermenting lager strains appear to have arisen from a fusion between two yeast species, one closely resembling *S. cerevisiae*, while the other appears similar to *S. bayanus* and/or *S. pastorianus* CBS1503 (*S. monacensis*) (Kodama et al. 2005). The lager yeasts have been grouped with *S. pastorianus* (Vaughan-Martini and Martini 1987) but are commonly referred to as *S. carlsbergensis*.

The lager yeast genome is believed to have undergone a genome duplication following the species fusion, leading to an allotetraploid strain containing varying numbers of *S. cerevisiae* and non-*S. cerevisiae* chromosomes. Generally lager yeast genomes are considered to be aneuploid in nature, possessing unequal numbers of chromosomes, e.g. a strain could have one copy of chromosome III and five copies of chromosome IV. In addition, recombination between the two homeologous sets of chromosomes has occurred, generating mosaic chromosomes (Kodama et al. 2005). These complex genomes appears to confer unique selective characteristics to these strains, e.g. the ability of lager yeasts to grow at low temperatures (7–13°C) and to withstand high osmotic pressure, high hydrostatic pressure, and high ethanol and CO_2 concentrations.

7.4.1 Classical Approaches to the Analysis of Industrial Yeast Genomes

7.4.1.1 Single Chromosome Transfer of the Lager Yeast Genome

The poor sporulation ability of lager yeast strains has hampered classical genetic analysis of these polyploid strains. However, preceding the development of molecular approaches for genome analysis, studies using the technique of single chromosome transfer from lager strains to well-defined laboratory *S. cerevisiae* strains helped to elucidate the polyploid and mosaic nature of lager yeast genomes (Casey 1986; Kielland-Brandt et al. 1995; Nilsson-Tillgren et al. 1981). Using this experimental approach three chromosome types in the lager strains have been defined: (1) *S. cerevisiae* chromosomes capable of recombining with *S. cerevisiae* chromosomes, (2) non-*S. cerevisiae* chromosomes showing no recombination and (3) mosaic chromosomes where regions of the lager yeast chromosome can recombine with an

S. cerevisiae chromosome (Kielland-Brandt et al. 1995). The conclusions reached by these seminal experiments defined the complex makeup of the lager yeast genomes and have been borne out and refined by subsequent analysis using molecular and whole genome approaches (see later).

7.4.1.2 Electrophoretic Analysis of Lager and Wine Strains of Yeasts

The characterisation of the structure and composition of industrial yeast chromosomes has been greatly aided by the development of pulsed-field gel electrophoresis. Classical single (continuous) field gel electrophoresis on agarose gels in tris(hydroymethyl)aminomethane acetate buffers has an upper limit of separation of approximately 30–50 kb. Pulsed-field gel electrophoresis, in which the direction and the duration of the current are varied (Coulson et al. 1988), relieves this upper restriction and allows the separation of DNA fragments in the size range from 10 kb to 10 Mb. The earliest models of pulsed-field gel electrophoresis employed a single field inversion of the electric current: field-inversion gel electrophoresis (FIGE) (Carle et al. 1986). FIGE periodically inverts the polarity of the electrodes subjecting the DNA to a 180° reorientation. The more complex pulsed-field systems such as contour-clamped homogeneous electric field (CHEF) (Chu 1990; Chu et al. 1986) transverse alternating field electrophoresis (TAFE) (Gardiner et al. 1986) and rotating gel electrophoresis (RGE) (Anand and Southern 1990; Gemmill 1991; Southern et al. 1987) all subject the DNA to smaller changes in orientation, generally between 96 and 120°. This results in the DNA moving forward in a zigzag manner allowing for separation over a wider range of sizes.

The first yeast chromosome fingerprint of the haploid strain of *S. cerevisiae*, using FIGE (Carle and Olson 1985), confirmed the presence of 16 chromosome bands. Separation of industrial yeast chromosomes on CHEF or TAFE gels revealed a wide heterogeneity in the chromosome banding pattern; however, in each case chromosomes equivalent in size to the *S. cerevisiae* chromosomes are apparent. (Casey 1996). Strains of lager yeasts vary in the number of chromosome bands present and in subtle electrophoretic mobilities of individual chromosomes. For example, chromosome fingerprints of a number of yeast cultures from the Stroh Brewery Culture Collection distinguished two unique fingerprints of type "Tuborg" and type "Carlsberg" (Casey 1996). These two strain types differ in the presence or absence of the small chromosomes I and X. By combining the techniques of pulsed-field gel electrophoresis and Southern blotting, the identity of individual chromosome bands was deduced. In a seminal paper by Casey (1986) the presence of three types of chromosome X in *S. carlsbergensis* was revealed using a DNA probe specific to chromosome X. Likewise, by varying the stringency of the hybridisation and washing conditions, the *S. cerevisiae* like, the non-*S. cerevisiae* homeologues and mosaic chromosomes, present in the lager strains, can be distinguished.

Electrophoretic karyotyping of wine yeasts from different sources reveals that strains differ in the number, electrophoretic mobilities and band intensities of chromosomes. Up to 21 chromosome bands have been identified in certain wine yeast strains (Castrejon et al. 2004). Hybridisation of chromosomes separated by pulsed-field gel electrophoresis, using chromosome-specific DNA probes, has allowed the

identification of individual chromosomes of wine yeast (Puig et al. 2000). Furthermore, karyotyping has revealed that the genomes of wine yeasts are subject to genetic rearrangements by undergoing recombination between homologous chromosomes and between paralogous or repeated DNA sequences (Codon et al. 1998; Infante et al. 2003). The recombination events, resulting in the generation of mosaic chromosomes in lager and wine yeasts, appear to occur during mitosis rather than being meiotic in nature (Puig et al. 2000). The resulting complex genomes of lager and wine yeasts lead to poor mating ability, poor sporulation and spore viability and thus contributes to the genetic isolation of these strains.

7.4.2 Comparative Genomic Microarray Analysis

7.4.2.1 Comparative Competitive Genomic Hybridisation of Lager Strains of Yeast

While pulsed-field gel electrophoresis allows the identification of the number of individual chromosomes and chromosome heterogeneity between yeast strains, it cannot reveal any information on the actual gene makeup of these industrial yeast strains. The development of whole genome microarray technology has allowed researchers to address this issue and to ask pertinent questions regarding the biological consequences of the hybrid nature of many industrial strains of yeasts. Such questions include what is the nature of the mosaic chromosomes in industrial strains of yeasts, how many copies of *S. cerevisiae* and non-*S. cerevisiae* genes are present in the genome and what effect does the presence of multiple non-identical genes have on the final transcriptome and proteome content of the yeast?

The technique of comparative competitive genomic hybridisation (CCGH) (Watanabe et al. 2004) to microarrayed *S. cerevisiae* genes on DNA chips can be used to determine the relative copy number of *S. cerevisiae* like genes, at single-gene resolution, in industrial strains of yeasts. In this case, the genomic DNA populations from two different yeast strains are differentially labelled by random priming with Cy3- and Cy5-tagged nucleotides by PCR amplification using enzymes such as Taq polymerase and hybridisations are carried out as described in Sect. 7.3.1. In this experiment, the differentially labelled DNA samples compete for hybridisation to their complementary sequence on the DNA chips. As with microarrays using two differentially labelled cDNA pools (Sect. 7.3.1), a ratio of hybridisation, reflected in red-to-green fluorescence ratios for each gene, is obtained. The ratio of hybridisation for the two competing DNA samples will depend on (1) the degree of sequence homology between the labelled DNA probes and the *S. cerevisiae* DNA arrayed on the chip and (2) the relative abundance (copy number) of the DNA sequence in the labelled DNA sample. In the simplest example, in which differentially labelled DNAs from two haploid strains of *S. cerevisiae* are competitively hybridised to *S. cerevisiae* DNA chips, one expects the ratio of red-to-green fluorescence to be 1.0 for all genes except for individual genes which may differ in the two strains. For polyploid and, in particular, aneuploid strains of yeasts, depending on the gene copy number and the homology between the reactants, the ratio of hybridisation (red-to-green fluorescence ratio) will be greater or less

than 1. This deviation from the control mean ratio of hybridisation is indicative of a higher or a lower copy number, respectively. The caveat in using CCGH analysis for industrial strains of yeasts is that only genes with extensive homology to *S. cerevisiae* will be detected.

Using this approach, Bond et al. (2004), Kodama et al. (2005) and Infante et al. (2003) have examined the copy number of *S. cerevisiae* like genes in lager and wine yeasts. A number of interesting findings have emerged from these studies. In one of the lager yeast studies (Bond et al. 2004), competitive hybridisations were carried out for two lager strains of yeasts and the yeasts were compared with a haploid *S. cerevisiae* strain. When the ratio of hybridisation for each individual gene was arranged according to the *S. cerevisiae* gene order, it was observed that the ratios of hybridisation for genes on a given chromosome were remarkably similar. However, in eight of the 16 chromosomes, distinct "jumps" in the ratios of hybridisation occurred at discrete loci along the chromosome. Examples of two of these jumps in the ratios of hybridisations are shown in Fig. 7.3. For chromosome III (Fig. 7.3a), a distinctive jump occurs at the MAT locus, between YCR039C and YCR040C. In lager strain 6701, genes to the left of the MAT locus show a ratio of hybridisation of 0.6, while for strain CMBS, the average ratio of hybridisation is 1.0. The genes to the right of the MAT locus in both strains show an average ratio of hybridisation of 1.7. It is interesting to note that the majority of the jump locations are conserved between the two lager strains of yeasts (they are definitely different isolates) and also between a third lager strain (Bond and James, unpublished data). However, each strain also possessed its own unique jumps; for strain CMBS a unique jump is observed on chromosome VIII after YHR165C and on chromosome XIII after YMR302C, while strain 6701 has a unique jump on chromosome X after YJR009C. The simplest explanation of the ratios of hybridisation data is that the jump locations represent regions where the homeologous chromosomes have undergone inter-and/or intra-chromosomal translocations. In fact, previous studies have used CCGH to identify genomic rearrangements such as translocations, deletions and amplifications in *S. cerevisiae* strains during experimental evolution conditions (Dunham et al. 2002). While CCGH analysis can reveal relative differences in copy number between two yeast species and/or strains, it does not allow an absolute quantitative determination of gene copy number. However, the relationship between the ratios of hybridisation and the gene copy number can be determined by combining CCGH analysis with quantitative real-time PCR. The technique of real-time-PCR allows the determination of the degree of amplification of known DNA quantities. By comparing the rate of PCR amplification of ORFs displaying unique ratios of hybridisation to the rate of PCR amplification of a known single copy gene, Bond et al. (2004) have shown that there is a direct correlation between the ratio of hybridisation and gene copy number for *S. cerevisiae* like genes in lager strains of yeasts.

An analysis of chromosome XVI in two lager strains of yeasts reveals the presence of four distinct regions with unique ratios of hybridisation (Fig. 7.3b). The region immediately following the left telomere up to the ORF YPL242C shows a ratio of 1.8. This is followed by a long stretch up to YPR159W, where a ratio of 1.0–1.2 is observed. Surprisingly, the region from YPR160C to YPR190C, encompassing 30 genes, many of which are essential in the haploid *S. cerevisiae* species,

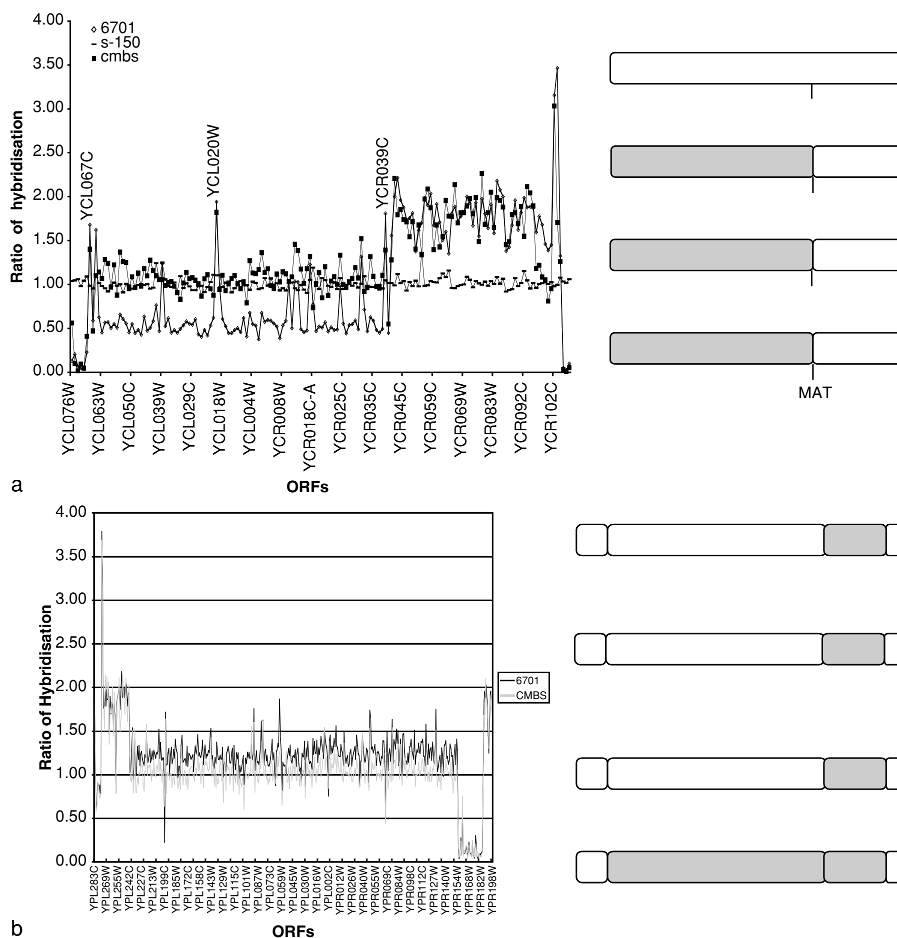

Fig. 7.3. Comparative competitive genomic hybridisation for the determination of the copy number of *S. cerevisiae* like genes in industrial yeast strains. (**a**) Ratio of hybridisation along chromosome III as determined by comparative competitive genomic hybridisation (*CCGH*) for the lager strains 6701 (*diamonds*) and CMBS (*squares*) relative to the haploid strain S-150B (*bars*). Schematic representation of chromosome III in strain 6701 as determined by quantitative-real-time PCR and CCGH. On the basis of these analyses the minimum copy number is one *S. cerevisiae* like chromosome III (*open box*) and three mosaic chromosome IIIs, where the genes to the left of the MAT locus are non *S. cerevisiae* like (*grey*) and those to the right of the MAT locus are *S. cerevisiae* like (*open boxes*). (**b**) Ratio of hybridisations along chromosome XVI for lager strains 6701 (*black line*) and CMBS (*grey line*) relative to the haploid strain S-150. Schematic representation of the minimum copy number and types of chromosome XVI in these strains as determined by CCGH and quantitative real-time PCR. *Open boxes S. cerevsiae* like, *grey boxes* non-*S. cerevisiae* like

shows a ratio of 0.2. From YPR190C to the telomere, the ratio once again rises to 1.8. The low hybridisation signal in the region YPR160C–YPR190C suggests that the *S. cerevisiae* genes in this region are absent in the lager strains or have significantly diverged from the *S. cerevisiae* sequence and most likely the region only contains non-*S. cerevisiae* gene equivalents. Southern blotting analysis of chromosome XVI, separated on CHEF gels, indicates that the latter is the case as a very weak hybridisation signal can be detected with DNA probes prepared from *S. cerevisiae* ORFs in this region (Usher and Bond, unpublished data).

Using the relationship between the ratio of hybridisation and the gene copy number one can deduce that the minimum chromosome XVI complement in these lager strains consists of three *S. cerevisiae* like chromosomes containing the non-*S. cerevisiae* region YPR160C–YPR 190C and one non-*S. cerevisiae* chromosome containing the telomeres and subtelomeric regions of an *S. cerevisiae* like chromosome (Fig. 7.3b). In a CCGH study with a number of lager strains of yeasts, Kodama et al. (2005) showed that the mosaic makeup of chromosome XVI is conserved amongst the lager strains although some variations do exist. These chromosomes most likely arose from recombination events between homeologous chromosomes. It is interesting to note that in the majority of cases, these recombination sites (as defined by the jumps in the ratios of hybridisation) are located at known sites of high genetic recombination (as in the case of the MAT locus on chromosome III) or at sites containing clusters of Ty or tRNA genes. However, the lager-specific jump sites, such as that observed at YHR165W in strain CMBS, do not contain any of these elements. These unique recombination sites may truly represent examples of adaptive evolution conferring specific selective advantages to that particular strain. Using CCGH analysis, Kodama et al. (2005) further showed that *S. pastorianus* (CBS 1538), *S. carlsbergensis* (IFO11023) and *S. monacensis* (CBS1503) lack certain *S. cerevisiae* like chromosomes. Remarkably, the *S. pastorianus* strain CBS1538 appears to lack *S. cerevisiae* like chromosomes II, III, IV, VI, VIII, XII, XV and XVI.

7.4.2.2 CCGH Analysis of Wine Yeast Genomes

Like lager yeasts, wine yeast strains show properties of aneuploidy, polyploidy and hybrid chromosomes. For example, a subgroup of wine yeasts, isolated from the flor vellum of aging sherry wines, have been shown to contain genetic heterogeneity as identified by electrophoretic karyotyping (Infante et al. 2003). Flor vellum is a biofilm that develops on the surface of sherry wines after the fermentative processes is complete. CCGH analysis of two flor yeast strains, *S. cerevisiae* var. *beticus* 11.3 and *S. cerevisiae* var. *montuliensis* 1.28, confirmed the aneuploid and hybrid nature of their chromosomes. By comparing the ratios of hybridisation for the two flor yeast strains, Infante et al. (2003) revealed that certain chromosomes, such as chromosome IV, show identical ratios of hybridisation (and therefore gene copy numbers), while chromosomes I, III, VI, X and XI show ratios of hybridisations 6 times that observed for chromosome IV. The aneuploid nature of the chromosomes is revealed by plotting the log ratios of the hybridisations between the two strains for each ORF as a function of their position on the *S. cerevisiae* chromosomes. As with the lager strains, the points at which the ratios of the hybridisations

change coincide with positions of Ty elements, LTRs and tRNA gene clusters. A number of other breakpoints correspond to positions of known meiosis-induced double-strand breaks (Gerton et al. 2000). Copy number difference between the two flor yeasts involved 38% of *S. cerevisiae* ORFs present in 116 regions of the genome.

Thus, the picture emerging for industrial yeast strains is one of aneuploidy arising from recombination events, mostly between homeologous chromosomes but also including non-homologous interchromosomal recombination events, resulting from the selective pressures experienced by these strains. This hypothesis, put forward by Infante et al. (2003), is supported by results from a number of model systems set up to examine genome evolution. In a study by Delneri et al. (2003), interspecies crosses between two different *S. mikatae* strains and an *S. cerevisiae* strain engineered to contain chromosomes that are collinear with the *S. mikatae* strain resulted in progeny whose genomes displayed widespread aneuploidy. The authors suggest that aneuploidy imposes genetic isolation on the strains, leading to stable genetic properties. In another experiment to examine the effects of selective pressures on the evolutions of genomes, Dunham et al. (2002) grew strains of *S. cerevisiae* for 100–500 generations in glucose-limited chemostats. Again using CCGH analysis, these authors found that these selective pressures resulted in the emergence of aneuploid strains showing gross chromosomal rearrangements such as amplifications, deletions and translocations. Experiments have also shown that spores of *S. cerevisiae*, *S. bayanus* (*S. uvarum*) and *S. pastorianus* are capable of mating to produce diploids (Delneri et al. 2003). However, upon sporulation, the spores produced are sterile and rarely produce meiotic offspring, leading to the genetic isolation of these strains and the maintenance of stable polyploid genomes.

That such chromosomal rearrangements result from selective pressures in nature was elegantly shown by Perez-Ortin et al. (2002) in the analysis of a wine yeast strain T73. In this strain, a reciprocal translocation between chromosomes VIII and XVI was observed. This genome rearrangement confers sulfite resistance to the strain by generating a fusion between the 5′ upstream region of the *SSU1* gene, encoded on chromosome XVI, and the promoter region of the *ECM34* gene from chromosome VIII. The *SSU1* gene mediates sulfite efflux in *S. cerevisiae* (Park and Bakalinsky 2000). Currently no known function has been ascribed to the *ECM34* gene. The resultant chimera (SSUI-R) results in higher expression levels of the *SSU1* gene, thus conferring sulfite resistance on this strain. The translocation event seems to be mediated by a short stretch of sequence homology between the two regions: The SSU1-R allele contains four repeats of a 76-bp sequence which is identical to a single copy of a 77-bp sequence in the *ECM34* gene. It is interesting to note that the site of translocation on chromosome VIII at YHL043W co-localises with a recombination site in the lager yeasts identified by CCGH (Bond et al. 2004), suggesting that this region may represent a particular “hotspot” of recombination common to wine and lager yeasts. However, an analysis of recombination sites identified in flor yeasts and lager yeasts shows few or no sites in common other than sites in subtelomeric regions. This lack of similarity may reflect the different adaptive pressures on these yeast strains owing to the different fermentation conditions encountered in beer making and wine making. It is interesting to note that none of the translocation sites

identified by sequencing in *S. bayanus*, *S. mikatae* and *S. paradoxus* strains (Kellis et al. 2003) have been identified by CCGH in the lager strains, suggesting that none of these species are the true parent of the hybrid lager strains. Alternatively, the recombination sites identified by CCGH may be *S. cerevisiae* specific. It is also possible that the strains have undergone further genomic rearrangements so that the original translocations have been lost or altered.

7.4.3 Comparative Proteomics to Reveal the Hybrid Constitution of Industrial Strains

The molecular details of the mixed constitution of industrial yeast strains can also be analysed by the application of proteomics.

7.4.3.1 Comparative Proteomics of Lager Strains

Boucherie and colleagues (Joubert et al. 2000) used 2D-PAGE in a comparative analysis of proteomes of different lager yeast isolates from various breweries. In agreement with the hypothesis that the genome of lager yeast is a hybrid from at least two different genomes, it was observed that many of the more abundant lager yeast 2D spots seemed to be duplicated (compared with what was found for the laboratory *S. cerevisiae* strain). The authors mentioned in their report that many of these duplicated spots exhibited the same relative abundance while displaying slight differences in their pI and/or M_r. For three of the duplicated non-*S. cerevisiae* spots, their identity as sequence variants of Pdc1p, Eno1/2p and Fba1p was confirmed by the use of microsequencing. Differential labelling of proteins from different strains and subsequent sample mixing followed by 2D-PAGE analysis allowed reliable scoring of the number of co-migrating proteins on a global scale. Independent analysis by two different labelling methods yielded essentially the same result: roughly 85% of the proteins in the lager strain K11 (patent no. FR 2 750 703-A1) co-migrated with proteins from *S. cerevisiae* (based on the analysis of about 300–500 proteins, respectively, in the two independent studies). Comparative proteome analysis via co-migration on 2D gels was also applied earlier to the analysis of strain variants of *S cerevisiae*, where one isolate from fish intestine was related to a laboratory strain (Andlid et al. 1999); for all 984 matched proteins in that study it was apparent that in total 98% of the proteins co-migrated, leaving only 16 strain-specific proteins in the fish isolate. Thus, in the comparison between *S. cerevisiae* and the lager beer strains a much lower similarity was seen compared with what was found between *S. cerevisiae* strains. The overlap to the 2D pattern of *S. cerevisiae* (Joubert et al. 2000) for the lager strain made possible the release of the first 2D map of an industrial yeast encompassing 185 identified proteins. The lager reference 2D pattern was later extended by MALDI-MS analysis to include an additional 30 lager-specific protein spots (Joubert et al. 2001).

The 2D pattern of the lager strain K11 was also compared with that of other type strains/species in an attempt to identify the non-*S. cerevisiae* genetic component of lager strains. It was reported that the proteome of lager brewing yeasts and of the type strains of *S. carlsbergensis*, *S. monacensis* and *S. pastorianus* could be

interpreted as the superimposition of two elementary patterns. One of them originates from proteins encoded by an *S. cerevisiae* like genome and the other apparently from the *S. pastorianus* NRRL Y-1551 strain. Surprisingly it was found that the two different type strains of *S. pastorianus* displayed rather different 2D patterns, despite supposedly being identical isolates. The reason for this discrepancy was not known, and complicates the final interpretation of their result. However, this work constitutes an important example for the use of 2D analysis for the comparative proteomics of yeast strains using spot position (x and y dimensions) as an indicator of protein identity.

More recent analysis of different lager strains adopted the procedure of liquid chromatography (LC) MS/MS analysis of resolved protein spots. In this work (R. Caesar, E. Pettersson, J. Gustafsson, A. Hashemi, M. Rudemo, A. Blomberg, unpublished results) MS/MS analysis of non-*S. cerevisiae* spots in the 2D gel and subsequent comparison to the complete proteomes of the yeasts within the *Saccharomyces sensu stricto* group revealed a phylogenetic tree with great similarity to the one obtained using gene sequence data. In this comparison the non-*S. cerevisiae* proteins in the total protein 2D pattern of the lager strains are clearly most similar to those of *S. bayanus* (Fig. 7.4).

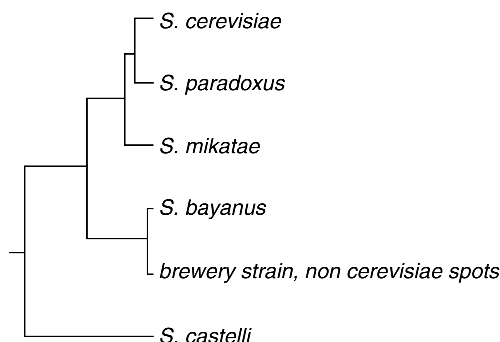

Fig. 7.4. Phylogenetic tree of the non-*Saccharomyces cerevisiae* proteins in the lager strain CMBS33. Seven dominant spots on a CMBS33 2D gel lacking corresponding spots on the gels from the laboratory strain BY4742 were identified as Eft1p, Shm2p, Ilv5p, Pdc1p, Met6p, Pgk1p and Pyk1p using liquid chromatography tandem mass spectrometry (MS/MS). The SEQUEST algorithm was subsequently used to correlate MS/MS spectra from these spots to theoretical mass data derived from a FASTA database containing the amino acid sequences of the identified proteins and their homologues in *S. paradoxus*, *S. mikatae*, *S. bayanus* and *S. castelli*. For each protein the peptide sequences identified from any of the species were compiled to a sequence for the CMBS33 form of the protein. In total, the peptides identified contained 1,237 amino acids covering 32% of the seven proteins. Multiple alignment of the regions covered by the MS/MS analysis were performed between the five *Saccharomyces* species and the brewery strain using ClustalW and a hierarchical tree based on the result was constructed. The brewery strain non *S. cerevisiae* spots differed with one amino acid from *S. bayanus*, with 36 amino acids from *S. cerevisiae*, with 34 amino acids from *S. paradoxus*, with 32 amino acids from *S. mikatae* and with 134 amino acids from *S. castelli*

7.4.3.2 Comparative Proteomics of Ale Strains

The first protein 2D map of an ale-fermenting yeast was recently presented (Kobi et al. 2004). In this analysis 205 spots corresponding to 133 different proteins were identified by MALDI-TOF analysis in the A38 ale strain. Comparison of the proteome of this ale strain with a lager brewing yeast and the *S. cerevisiae* strain S288c confirmed that ale strains are much closer to S288c than lager strains. A zoom-in on a central part of the 2D patterns of three different lager strains, *S. cerevisiae* S288c, and two different ale strains clearly displays these differences and similarities (Fig. 7.5). The A38 ale strain exhibited a very similar pattern to the *S. cerevisiae*

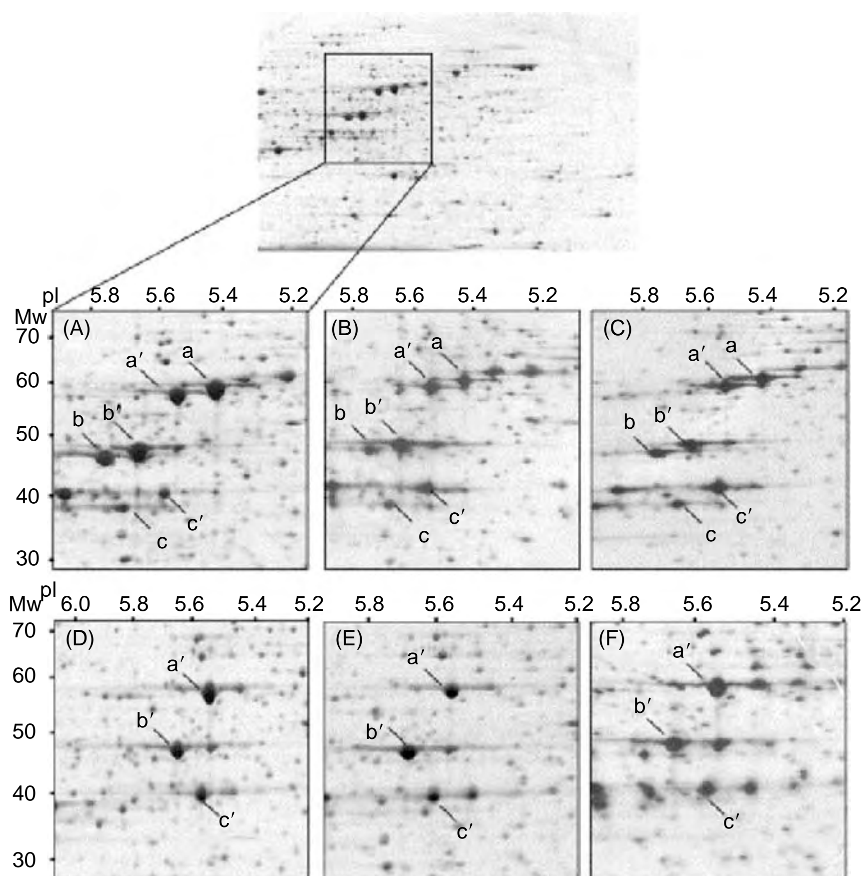

Fig. 7.5. The 2D patterns of ale strains are similar to the one found in laboratory strains. 2D polyacrylamide gel electrophoresis analysis of ale (**e**, **f**), lager (**a–c**) and laboratory (**d**) strains. The protein spots indicated are Pdc1p (*a* and *a*'), Eno2p (*b* and *b*') and Fba1p (*c* and *c*'). 2D images are displayed with the acidic side to the *right*. (Adopted from Kobi et al. 2004 with permission from Elsevier)

strain, and this observation could also be extended to other ale strains. However, this does not mean that the 2D patterns of these ale strains were identical to the laboratory strain S288C. On the contrary, looking at the entire gel for these strains, some discrepancies appear, e.g. the alcohol dehydrogenase isoform Adh4p is present in the ale-brewing strains but not in the laboratory strain under these growth conditions. This is interesting since this zinc-dependent Adh4 protein is reported to be regulated by zinc deficiency (Yuan 2000), which could indicate that the industrial ale strain might exhibit alterations in zinc metabolism. The Adh4p isoform was also found expressed under normal laboratory growth conditions in an industrial baker's yeast strain (Nilsson et al., unpublished results).

7.4.3.3 Comparative Proteomics of Baker's Yeast Strains

Four industrial baker's yeast strains with various fermentative capacities were compared with a laboratory *S. cerevisiae* strain during fed-batch cultivation. After 2D-PAGE analysis the strains were grouped according to differences in protein expression and 2D spot positional differences (Nilsson et al., unpublished results). It was found that the baker's yeast strains were more similar to each other than to the laboratory strain. However, overall the 2D patterns were rather similar among all strains and the most outstanding difference was found for the protein spots corresponding to alcohol dehydrogenase I and II, where all strains examined exhibited large variations. In particular it was noticed that the baker's yeast strains contained several alcohol dehydrogenase spots not present in the laboratory strain. Subsequent MALDI-MS analysis of these alcohol dehydrogenase spots revealed a rather large apparent variability in the alcohol dehydrogenase protein sequence, and some of the sequences seemed to be sequence hybrids between Adh1p and Adh2p.

7.4.4 Genomic Sequencing of Industrial Strains of Yeast

The ultimate characterisation of industrial yeast strains will require the complete genome sequence at a nucleotide level; however, this has been hampered by the polyploid nature of their genomes which requires sequencing and analysis of both the *S. cerevisiae* and the non-*S. cerevisiae* components of the genome. The first description of the sequencing of an industrial lager strains was reported by Suntory, Japan (Nakao et al. 2003). This group sequenced the lager strain Weihenstephan 34/70 (*S. pastorianus*) using a random shotgun approach. Two types of contigs covering 23.2 Mbp (95% coverage) were identified, displaying either 98% identity or 85% identity to the *S. cerevisiae* DNA sequence (Kodama et al. 2005; Nakao et al. 2003). The sequence data confirmed the findings of classical genetics of the presence of three types of chromosomes in the lager strains, *S. cerevisiae* like, non-*S. cerevisiae* like and mosaic chromosomes, arising from recombination events between homeologous chromosomes and identified 37 chromosomal varieties. In general, with the exception of a number of translocations and inversions, the gene synteny is identical to that of *S. cerevisiae*. The non-*S. cerevisiae* like DNA sequences most closely resemble those of *S. bayanus* (*uvarum*); however, the sequences are not identical. This may be due to the difference in strains of *S. bayanus* as only a single *S. bayanus* sequence

is currently available. Interestingly, this study identified 20 ORFs with no significant identities to *S. cerevisiae* ORFs. Furthermore, the recombination sites on the mosaic chromosomes, identified by CCGH analysis (Bond et al. 2004), are confirmed by the sequence analysis, although the Weihenstephan strain contains a number of unique mosaic chromosomes that are not present in the 6701 or CMBS strains characterised by CCGH analysis and vice versa (Fig. 7.6).

7.5 Analyses of the Industrial Process

The novel tools for genome-wide analysis of the yeast transcriptome and proteome are not only instrumental for a better description of industrial strains but also allow a more comprehensive analysis of the industrial-scale fermentation process. The environmental conditions experienced by yeasts in industrial settings are quite unique in their complexity and this complexity is rarely experienced by strains propagated under laboratory growth conditions. For example, lager and ale yeasts during industrial fermentation simultaneously experience a wide array of conditions such as anaerobiosis, high alcohol concentrations, high hydrostatic pressure and high cell density. Likewise, wine yeasts are exposed to a unique set of conditions such as high sugar concentrations, anaerobiosis, low pH and high ethanol concentrations during oenological fermentations. One might consider all of these conditions as imposing severe stress on the yeasts, and therefore it is of particular interest to examine the gene expression profiles in order to understand the stress responses of these yeasts

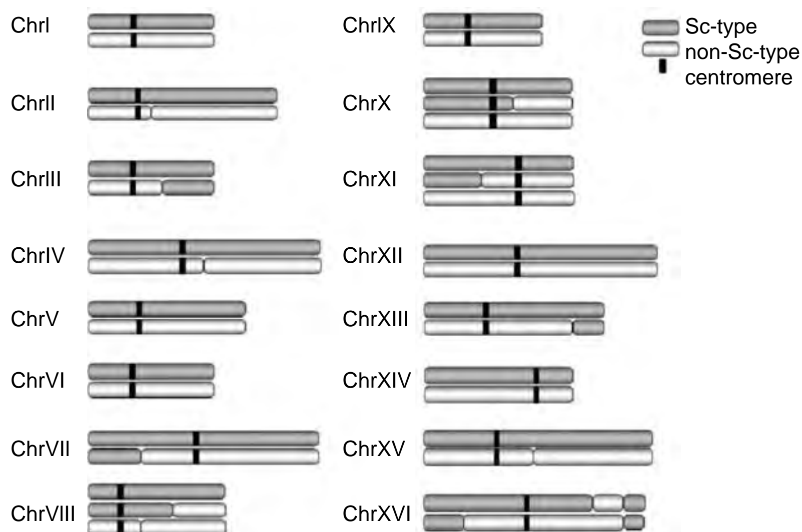

Fig. 7.6. Genome composition of the lager strain Weihenstephan 34/70 (*S. pastorianus*) as deduced by DNA sequencing. (Adopted from Kodama et al. 2005 with permission from Springer-Verlag)

to their environment and to examine the expression patterns of genes involved in metabolic processes, in particular those associated with carbohydrate metabolism.

A number of transcriptome datasets are currently available profiling the gene expression patterns in lager yeasts (Dawes et al. 2002; Higgins et al. 2003a, b; James et al. 2002, 2003; Olesen et al. 2002; Panoutsopoulou et al. 2001; Pugh et al. 2002) and wine yeasts (Backhus et al. 2001; Cavalieri et al. 2000; Erasmus et al. 2003; Rossignol et al. 2003) under fermentation conditions. It should be pointed out that in all transcriptome studies outlined in this section, it is only possible to examine gene expression of *S. cerevisiae* like genes in either lager yeasts or wine yeasts. The contribution of the non-*S. cerevisiae* genes to the overall gene expression patterns remains unexplored at this stage. Likewise the effects of multiple copies of similar but non-identical genes on the overall gene expression pattern are currently unknown, as is whether control of gene expression is conserved between the *S. cerevisiae* and non-*S. cerevisiae* genes. Currently these datasets have not been compiled into a format (website) that allows direct comparison of the different datasets; however, some comparisons have been carried out (James and Bond, unpublished data). While direct comparisons of the data are hampered by the varying experimental conditions used (parameters such as time points sampled during the fermentation, starting cell densities, industrial growth media and temperature used all vary between the datasets), an overall picture of the physiological state of the yeast under fermentation conditions can be deduced.

7.5.1 Beer Production

7.5.1.1 Gene Expression Patterns in Lager Yeasts Under Fermentation Conditions

Among the common gene expression themes that emerge from the analysis of lager yeast fermentations is the co-ordinate upregulation of genes affected by anaerobiosis and those required for ergosterol and fatty acid metabolism. The latter set of genes are required for continued cell membrane biosynthesis, a process requiring the presence of molecular oxygen. Thus, brewers generally actively aerate the wort at the start of the fermentation. This small quantity of initially added O_2 is sufficient to allow continued ergosterol biosynthesis during the fermentation. The co-ordinate upregulation of genes required for fatty acid and sterol metabolism is further verified by the upregulation of a number of genes involved in peroxisomal metabolism such as the PEX genes *PEX*, *5*, *10*, *14*, *21*, *11* and *18*. Likewise, the PAU gene family, initially identified as genes responding to anaerobic growth conditions, but more recently suggested to be sterol carriers (Wilcox et al. 2002), are co-ordinately upregulated as fermentation proceeds (James et al. 2003). High levels of ergosterol may be essential to protect yeast from ethanol stress (Alexandre et al. 1994). Another interesting finding from the transcriptome analysis was the co-ordinate upregulation during large-scale fermentation of genes involved in aldehyde metabolism (Fig. 7.1).

Of the genes that show decreased transcript levels on days 3 and 8, with respect to day 1, of fermentation, the most abundant classes are those required for protein and amino acid biosynthesis. These account for more than 30% of the downregulated genes. The co-ordinate downregulation of these genes most likely reflects the

low level of general cell metabolism occurring during industrial fermentation, where cells may only undergo one to two cell divisions. The co-ordinate downregulation of protein synthesis genes has also been observed in stationary-phase yeast cultures (Gray et al. 2004). The majority of genes involved in glycolysis are similarly downregulated as fermentation proceeds (Fig. 7.1). These genes are generally regulated by catabolite repression (glucose repression) and their downregulation may reflect continued levels of useable carbohydrates even at day 8 of fermentation.

7.5.1.2 Application of Proteomics to Analyse Ale Fermentation

Kobi et al. (2004) followed proteome changes during the fermentation process of the ale strain A38 in a 10-hl pilot device, for the first, second and third generation. The A38 strain was initially grown in aerobic conditions with saccharose as the sole carbon source before pitching in wort under anaerobic conditions for the first fermentation. To mimic the industrial process, the yeasts were harvested at the end of the fermentation and re-inoculated in the fresh wort for a second and then third generation. In particular, proteome analysis and comparison was performed during each generation (at the start and after roughly 200 h). A comparison between the beginning and the end of the first generation showed that 50 of the 85 differentially expressed proteins were repressed, mostly glycolytic enzymes, proteins involved in acetylcoenzyme A formation, proteins of the tricarboxylic acid cycle, and proteins involved in respiration. It is clear that most of the changes reveal an adaptation to anaerobic conditions. In addition, most of the proteins induced at later times in the fermentation process were protein fragments belonging to either proteins in carbon metabolism, or protein or amino acid biosynthesis pathways. It was suggested that intracellular proteolysis influenced the regulation of these proteins during the industrial fermentation process.

Many fewer changes in protein abundance were scored between the beginning and the end of the third generation, and the observed changes exhibited lower levels of response. Among the proteins that displayed at least a twofold change were proteins involved in methionine biosynthesis (e.g. Sah1p, Met6p and Met3p) as well as some involved in carbon metabolism (e.g. Fba1p, Adh1p and Ald6p); all the example proteins indicated displayed induction except Ald6p, which was repressed.

7.5.1.3 Stress Responses During Beer Fermentation

Considering the extreme environmental conditions experienced by yeasts during the fermentation process, one might expect the induction of stress genes in these cells. Stress-responsive genes are generally regulated by transcription factors such as the heat shock factor, Hsf1 and the Msn2/Msn4 proteins, which bind to heat shock elements (HSE) and stress responsive elements (STREs), respectively. Msn2/Msn4p are zinc-finger proteins that are activated by a number of stress conditions encountered by yeasts such as entry into the stationary phase, carbon source starvation and osmotic stress. Surprisingly, transcriptome analysis during fermentation indicates that genes under the control of Hsf1 and Msn2/Msn4 are downregulated as fermentation proceeds (James et al. 2003). These results are consistent with previous

data showing that Hsp104p levels were repressed during fermentation (Gray et al. 2004; Brosnan et al 2000). The lack of expression for stress-responsive genes appears to be unique to lager strains of yeasts. A comparative analysis of genes encoding heat shock proteins (HSP genes) in a haploid laboratory strain of *S. cerevisiae* and the lager strain 6701, grown under identical fermentation conditions, revealed that a subset of the HSP genes (*HSP104*, *HSP30*, *HSP26* and *HSP12*) are in fact highly induced late in fermentation (on day 8) exclusively in the haploid laboratory strain (James et al. 2002; James and Bond, unpublished results), a response not encountered for the lager strain. These results therefore suggest that the transcriptional regulation of HSE-/STRE-regulated HSP genes is significantly different in lager and laboratory strains of yeasts.

The regulation of genes responding to oxidative stress has also been extensively examined in lager strains of yeasts during fermentation (Higgins et al. 2003a; James et al. 2003). These genes are generally under the control of the transcription factor Yap1p. Expression levels of these genes are elevated on days 1 and 3 of fermentation most likely in response to the production of O_2 free radicals. The expression patterns of these genes differ in the various reported datasets (Higgins et al. 2003a; James et al. 2003; Olesen et al. 2002). This most likely reflects the different regimes for oxygenation of the media at the beginning of fermentation. A number of oxidative responsive genes are also induced late in fermentation; these include the genes *LYS7*, *SOD1*, *TSA1* and *MXR1* (James et al. 2002, 2003). The *MXR1* gene encodes the protein methionine sulfoxide reductase, which reverses the oxidation of methionine residues and which has been shown to be required for the reduction of dimethyl sulfoxide to dimethyl sulfide, which is a thioester of major importance for the aroma and flavour of beer (Hansen et al. 2002).

Kobi et al. (2004) reported that a number of stress proteins exhibited changes in ale yeasts during the first-generation fermentation, like Hsp26p and Ssa4p. However, the only stress proteins that also increased during the third generation of fermentation were Kar2p and Ssa1p (both these proteins encode chaperones that are required for protein folding). Yeast strains must cope with various stresses during the fermentation process; however, there is clearly no strong stress response in the ale strain.

7.5.2 Wine Fermentation

7.5.2.1 Gene Expression Patterns in Wine Strains Under Fermentation Conditions

The conditions for wine and lager fermentations differ in a number of important aspects, such as starting cell concentrations, growth media (wort, rich in maltose for beer, and grape juice, rich in fructose and glucose for wine), pH, temperature and length of incubation. However, yeasts in both fermentations share some common environmental conditions, such as anaerobiosis and high ethanol concentrations at the end of the fermentation. In general, carbohydrates do not become limiting during wine fermentations. Instead cells enter the stationary phase owing to limiting nitrogen concentrations and/or attainment of maximal cell density. Surprisingly, the expression patterns are remarkably similar in both lager and wine fermentations;

however, unique expression patterns are also observed under both conditions. As observed in lager strains of yeasts, there is a co-ordinate downregulation of genes involved in protein and amino acid biosynthesis and upregulation of PAU genes and genes encoding proteins involved in aldehyde metabolism. Significant differences between the expression patterns observed in the wine and lager fermentations include the downregulation of genes for ergosterol biosynthesis in wine fermentations and the upregulation of genes required for glycolysis. The former results from the inclusion of ergosterol in the culture medium, while the latter most likely reflects the concentrations of carbohydrates at different times during the wine and lager fermentations. One interesting similarity is the upregulation of genes encoding aryl alcohol dehydrogenases. The contribution of these genes to total alcohol synthesis is presently unknown. It has also been suggested that these genes are induced in response to oxidative stress (Rossignol et al. 2003).

Transciptome analysis during wine fermentation also revealed major changes in gene expression patterns as a result of nitrogen depletion as the fermentation proceeded and confirmed that growth arrest (entry into the stationary phase) is a direct consequence of nitrogen depletion (Rossignol et al. 2003). The major changes in the gene expression pattern associated with nitrogen depletion were the induction of genes required for the metabolism of poor alternative nitrogen sources such as genes required for proline, allantoin and urea utilisation, nitrogen permeases and genes encoding proteins required for the management of glutamate pools. All of these genes are under the control of the TOR pathway.

7.5.2.2 Stress Responses During Wine Yeast Fermentation

One of the major differences in gene expression patterns between wine and lager yeast fermentations is the upregulation of HSP genes in wine yeast (Rossignol et al. 2003). As mentioned before, this group of genes appear to be actively repressed during lager yeast fermentations. The expression patterns of HSP genes in wine yeasts most closely resembles that observed in haploid *S. cerevisiae* strains grown under fermentation conditions (James et al. 2002). The HSP genes, such as *HSP30*, *HSP26* and *HSP104*, which are under the control of STREs are particularly induced during wine fermentations. Such genes have previously been shown to be induced following diauxic shift and upon entry into the stationary phase. Both conditions see a shift from usage of fermentable carbohydrates to non-fermentable carbon sources such as acetate and ethanol. It is possible that conditions at diauxic shift are radically different in wine and lager production. Alternatively, the differential expression of HSP genes in wine and lager yeasts raises the possibilities that chromatin structure and/or the presence of homeologous non-*S. cerevisiae* genes contribute to these gene expression patterns.

In addition to HSP genes, 58% of genes previously defined as being regulated as part of the common environmental response (CER) and the environmental stress response (ESR) (Gasch et al. 2000) are upregulated during wine fermentations (Rossignol et al. 2003). Additionally, genes responsive to ethanol stress are also induced. Many of these genes are involved in cell wall biogenesis, suggesting that cell wall alterations may help yeasts cope with ethanol stress. It is interesting to note

that ten genes involved in cell wall biogenesis are also induced late in lager yeast fermentations (James et al. 2003).

A wild-type *S. cerevisiae* wine strain isolated from the natural must of spontaneous grape fermentation was analysed for proteome changes during semiaerobic growth conditions (Trabalzini et al. 2003). In particular the response of this wine strain to the exhaustion of glucose from the medium was of interest since this is believed to be the main reason for unwanted stuck fermentation during vinification. When glucose was depleted from the medium a large number of changes in protein abundance were apparent. Roughly 50 proteins displayed decreased amounts at the end of fermentation, usually in the range twofold to threefold repression and, interestingly, some of these proteins are currently functionally not characterised (e.g. Ybr025cp and Yir035cp). In addition, a large number of protein spots with low molecular weight increased during the later phases of fermentation. Identification of these spots indicated that they belonged to protein fragments of larger proteins, which indicated proteolytic breakdown as part of the cellular response. However, also well-known stress responsive proteins like Sod1p, Tsa1p and Ctt1p exhibited increased expression when glucose was depleted, indicating a clear stress response in this wine yeast.

7.5.2.3 Application of Proteomics to Understand Factors That Affect Wine Haze

The clarity of white wine is highly important for the winemaker. Bottles showing haziness, likely to be rejected by the consumer, result from the aggregation of grape proteins naturally present in wine. To prevent haze formation, winemakers usually lower the concentration of wine proteins through the use of bentonite. Unfortunately bentonite also removes wine aroma components, hence lowering wine quality. Thus, alternative methods of protein stabilisation are being investigated by the wine industry.

Mannoproteins from yeast cell walls are known to be released into the extracellular medium during yeast growth, in particular during the stationary phase (Dupin et al. 2000). One of the proteins released, invertase, was shown to lower haze formation, probably by competing with grape-derived proteins for some unknown factor(s) in wine that is required to form large highly light scattering protein aggregates that are responsible for the haze. To better characterise the protein components of wine a 2002 vintage Sauvignon Blanc wine was analysed by LC-MS/MS (Kwon 2004). Wine proteins were concentrated by the use of a 5-kDa cellulose membrane centrifugal filter tube, followed by salting-out precipitation in saturated ammonium sulfate aqueous solutions and subsequent centrifugation to isolate the protein pellets. Proteins were separated by SDS-PAGE and Coomassie visualised bands were cut out for trypsination and peptide elution. The LC-MS/MS analyses resulted in the identification of 12 different cell wall or plasma membrane associated proteins from *S. cerevisiae*: e.g. Gas1p, a GPI-anchored 1,3-glucosyltransferase, Pho3p, a periplasmic acid phosphatase, Suc4p, invertase, Bgl2p, *endo*-1,3 glucanase and Yju1p (Cwp1p), reported as a structural component of the cell wall. Many of these released yeast proteins could have an influence on the wine haze formation. It was proposed that MS identification of proteins could be used as a quality indicator of wine.

7.5.3 Industrial Production of High-Quality Baker's Yeast

Two aspects of industrial baker's yeast production have been investigated using proteomics. One important feature of baker's yeast performance is high fermentative activity even after long-term cold storage. To identify molecular effects during this storage regime on a baker's yeast strain, 2D analysis was performed before and after storage for 26 days at 4°C (Nilsson et al. 2001a). It was clear from this analysis that cold storage resulted in large changes in the protein content. However, even after this long period of storage the original state of the culture, e.g. exponential growth in the respiro-fermentative state or in the transition phase to respiration, could still be distinguished by analysis of the 2D pattern. The fermentative activity after storage was also shown to be dependent on the initial state, and thus it was concluded that the initial state at harvest was important for the long-term performance of this industrial yeast strain. The baker's yeast cells experience different types of starvation regimes during industrial production. To investigate the effects on these industrial strains from either nitrogen or carbon limited starvation, a differentially starved baker's yeast strain was analysed for protein changes (Nilsson et al. 2001b). It was found that for certain glycolytic enzymes a significant change in the amount of protein could be observed during these starvation regimes, e.g. Pdc1p decreased during nitrogen starvation, while Adh1p was downregulated during carbon starvation. However, the recorded changes in the protein levels did not correlate with the observed changes in fermentative capacity.

7.6 Future Perspectives

The complete genome sequence of the first industrial yeast strain, the lager yeast Weihenstephan 34/70 (*S. pastorianus*), will be instrumental in our analysis of these hybrid genomes. It will not only potentially indicate the full compliment of genes in these cells, but will also provide the basis for a number of novel technological possibilities in the analysis of industrial yeast strains. The genome sequence will soon be opened up for expression analysis of not only the *S. cerevisiae* part of the genome but will also put the non *S. cerevisae* part within experimental reach; not before long the first microarrays with a full complement of genes from industrial yeast will be available. In addition, it will make possible a more complete and precise proteome analysis by providing a better template for database searches using MS data. It will also make possible non-gel-based proteomics approaches that are based on whole cell trypsination prior to high-resolution LC-MS/MS analysis (Washburn et al. 2001). Hopefully these novel possibilities will lead to the publication of studies where combined transcriptome and proteome analyses are presented, which are missing at present. Of great general interest will be the analysis of differences in the regulatory components of these mixed genomes where industrial strains might have evolved/been selected to respond quite differently to the well-studied laboratory *S. cerevisiae* strains (see previous sections on the stress response). Hopefully the genome sequence and the novel experimental possibilities will also lead to the development of dedicated databases where transciptome and proteome data from industrial yeast strains can be presented, compared and analysed. The interplay between

the hybrid proteomes will be another important avenue for future studies, since the genome sequence puts within experimental resolution a view of the physical protein interaction network, by either two-hybrid analysis or co-immunoprecipitation (e.g. tandem affinity purification tagging), in particular regarding the mixed composition of protein complexes. These studies will also be of fundamental importance in our understanding of the functionality of hybrid protein complexes, where different components compete in the formation of complexes and play different functional roles in their final activity.

Many challenges remain and will require further advancements both in the tools of bioinformatics as well as in analytical techniques. One important aspect is the finite gene number for industrial strains, a number that even for the well-studied laboratory *S. cerevisiae* strain, as mentioned earlier, has changed over the years and most likely will continue to change. In this respect the mixed genomes of some industrial yeast strains will provide an even greater challenge in gene identification. However, maybe the greatest obstacle in our strive for a more detailed functional analysis of individual genes in the genetic background of industrial strains will be the difficulty in generating gene deletions in these mixed genomes of several similar and identical gene copies. This problem will certainly ensure that the evolutionary link to *S. cerevisae* will persist not only in the industrial production line but also in future functional analysis of non-*S. cerevisae* genes in the potent laboratory test-bed *S. cerevisiae*.

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Chapter 8

Carbohydrate Metabolism

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8.1 Introduction

Carbohydrate metabolism by yeasts has been the topic of scientific investigation for well over a century, owing to the central roles that yeasts play in food and beverage biotechnology and to the ease with which yeasts can be studied in the laboratory. The literature on the biochemistry, genetics, and molecular biology of yeast carbohydrate metabolism is too vast to cover thoroughly in this chapter. General reviews of the field (Zimmermann and Entian 1997; Kruckeberg and Dickinson 2004) as well as specialised reviews (vide infra) can be consulted for more detail. Most of the research on carbohydrate metabolism has been with the baker's or brewer's yeast, *Saccharomyces cerevisiae*. However, many other yeast species play important roles in the food sector, as agents of fermentation, as sources of biomass components, or as spoilage organisms. The roles of these other species are often due to unique features of their carbohydrate metabolism, and examples of these are given here.

8.2 Carbon Sources

Yeasts are chemoheterotrophic organisms, obtaining all of their energy and carbon from organic compounds. "Yeast" actually refers to a taxonomically and physiologically disparate group of organisms, including ascomycetes, basidiomycetes, and deuteromycetes (fungi imperfecti). Collectively, yeasts are able to grow on a wide range of carbon sources, including carbohydrates, alcohols, organic acids, amino acids, *n*-alkanes, and lipids. Carbohydrates are the commonest carbon source, and also the most important in terms of the biotechnological applications of yeasts. Yeasts are able to grow on monosaccharides, oligosaccharides, and polysaccharides (Barnett 1976). Among monosaccharides, metabolism of hexoses (glucose, fructose, mannose) is general, and is carried out largely by the glycolytic pathway (Käppeli 1986). Galactose utilisation additionally requires the action of the Leloir pathway (De Robichon-Szulmajster 1958; Leloir 1964; Douglas and Hawthorne

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1966). In contrast, pentose utilisation is quite restricted taxonomically (e.g. *Pichia stipitis* and a few other species; Maleszka and Schneider 1982; Verduyn et al. 1985), and requires xylose reductase, xylitol dehydrogenase, and xylulokinase activities and the pentose phosphate pathway.

Utilisation of oligosaccharides and polysaccharides is initiated by hydrolysis to the component monosaccharides, and expression of the appropriate hydrolase can also be taxonomically restricted. For example, not all yeasts are able to metabolise sucrose, presumably owing to the absence of invertase expression (Ahearn et al. 1977; Lachance et al. 2001). *Kluyveromyces* species are unusual amongst yeasts in expressing β-galactosidase activity, which is required for lactose utilisation (but see Sect. 8.10) (Algeri et al. 1978; Dickson and Markin 1980). *S. cerevisiae* var. *diastaticus* is an example of an amylolytic yeast; it expresses and secretes glucoamylase activity that is required for starch utilisation (Yamashita et al. 1985; Pretorius et al. 1986). Species of *Schwanniomyces* are amylolytic as well (Clementi et al. 1980). Pectinolytic (Wimborne and Rickard 1978; Gainvors et al. 1994) and xylanolytic (Kratky and Biely 1980; Lubomir and Peter 1998) yeasts have been isolated and characterised. In contrast, no yeasts with cellulolytic capability have been identified (but see Sect. 8.10). Yeast biomass and yeast extracts are, however, stimulatory to cellulolytic rumen bacteria, and *S. cerevisiae* is often used as a feed additive or probiotic for ruminants (Callaway and Martin 1997; Jouany et al. 1998).

8.3 Modes of Metabolism

A number of regulatory phenomena have been described for the modes of carbohydrate metabolism by yeasts. The *Crabtree effect* refers to the occurrence of alcoholic fermentation (rather than respiration) of glucose under aerobic conditions. This is a characteristic of *S. cerevisiae* and some other facultatively fermenting yeasts, and occurs during aerobic batch cultivation and during aerobic glucose-limited chemostat cultivation at high dilution rates (Fiechter et al. 1981; Postma et al. 1989). Numerous physiological differences distinguish Crabtree-positive species from Crabtree-negative species such as *K. lactis* and *Schw. castellii* (van Urk et al. 1989; Zimmer et al. 1997; Kiers et al. 1998; Zeeman et al. 2000), but the causal factor regulating the onset of Crabtree metabolism has not yet been identified and remains controversial (Diderich et al. 2001; Otterstedt et al. 2004; Thierie 2004).

The *Pasteur effect* refers to inhibition of fermentation by aerobiosis; for example, *P. anomala* displays respiratory metabolism under aerobic conditions even at high glucose concentrations, and switches to fermentative metabolism under hypoxia irrespective of the glucose concentration (Fredlund et al. 2004). In *S. cerevisiae* the Pasteur effect is largely confined to non-growing cells or to cells grown at very low dilution rates in aerobic, glucose-limited chemostats (conditions in which the glucose transport capacity is low; Lagunas 1986).

The *Custers effect* refers to the inhibition of fermentation by anaerobiosis; for example, the wine spoilage yeast *Brettanomyces* ferments glucose to ethanol and acetic acid only under aerobic conditions (Wijsman et al. 1984). The *Kluyver effect* refers to an inability to utilise some disaccharides anaerobically, although (some

of) their constituent monosaccharides can be metabolised under anaerobic conditions. For example, *Candida utilis* is able to ferment glucose but not maltose (4-α-D-glucopyranosyl-D-glucopyranose) anaerobically, and *S. cerevisiae* is able to ferment glucose but not trehalose (α-D-glucopyranosyl-(1,1)-α-D-glucopyranose) anaerobically (Sims and Barnett 1991; Malluta et al. 2000; Fukuhara 2003). In these situations the capacity to transport the disaccharide into the cell may be too low to sustain anaerobic growth.

8.4 Substrate Transport

Carbohydrate uptake is mediated by transport proteins which are integral in the plasma membrane. Transport of monosaccharides as well as disaccharides and oligosaccharides is mediated by proteins in the Sugar Porter family of the Major Facilitator Superfamily of solute transporters (Busch and Saier 2004). In yeasts, these proteins all display discernible sequence homology (Kruckeberg 1996).

Hexose transport is carried out by a facilitated diffusion (i.e. uniport) mechanism in *S. cerevisiae* (Kruckeberg 1996). Most other yeasts also display a hexose-H^+ symport mechanism, e.g. in *Schizosaccharomyces pombe* (Lichtenberg-Frate et al. 1997; Heiland et al. 2000) and *Kluyveromyces* species (Gasnier 1987; Postma and van den Broek 1990). A fructose-specific symporter, Fsy1, occurs in the *Saccharomyces sensu stricto* yeasts *S. pastorianus* and *S. bayanus* (Goncalves et al. 2000; Rodrigues de Sousa et al. 2004). Disaccharide transport is generally mediated by a symport mechanism as well. Examples include the maltose-H^+ and α-glucoside-H^+ symporters of *S. cerevisiae* (Serrano 1977; Cheng and Michels 1991; Stambuk et al. 1999) and the lactose-H^+ symporter of *K. lactis* (Dickson and Barr 1983; Chang and Dickson 1988). The uptake of sugars by proton symport may be adaptively advantageous as it allows for accumulation of the substrate against a concentration gradient; in other words, the nutrient can be taken up by the cell despite low extracellular concentrations. Furthermore, the substrate affinities of the cytosolic enzymes that act on transported sugars (e.g. hexokinase, β-galactosidase) are low enough that a concentrative mode of transport may be required to maintain a sufficient metabolic flux to sustain growth.

Some carbohydrates that are fermented by yeast are not transported intact, but are instead hydrolysed by extracellular hydrolases. Examples include sucrose and raffinose, which are typically cleaved to glucose and fructose (or, for raffinose, fructose and melibiose) in the periplasm by invertase; the monosaccharides are then taken up by the hexose transport system (Moreno et al. 1975; Lazo et al. 1977; Bisson et al. 1987). Strains of *S. cerevisiae* differ in their ability to utilise the melibiose; some express an extracellular melibiase that hydrolyses this disaccharide to glucose and galactose, both of which can then be taken up and metabolised (Buckholz and Adams 1981; Naumov et al. 1990).

S. cerevisiae has a family of 20 genes encoding hexose transporters and related proteins, the so-called *HXT* gene family (Boles and Hollenberg 1997; Kruckeberg 1996). Of these, only *HXT1–HXT7* encode transporters that are important for growth and metabolism of glucose (Reifenberger et al. 1995; Diderich et al. 1999a). The galactose transporter, encoded by *GAL2*, is also a member of the *HXT* gene family (Nehlin et al. 1989; Szkutnicka et al. 1989). Two members of the family,

encoded by *SNF3* and *RGT2*, have lost the ability to transport hexoses; instead, they function as sensors of the extracellular glucose concentration. This glucose signal is involved in regulating the expression of various *HXT* genes (Özcan and Johnston 1999). The remaining members of the family (*HXT8–HXT17*) are phenotypically silent, and may not be expressed under normal physiological conditions (Diderich et al. 1999a).

The affinity of Hxt hexose transporters for glucose differs; for example, Hxt1 and Hxt3 have a low affinity (approximately 100 mM), whereas Hxt2, Hxt6, and Hxt7 have a high affinity (approximately 1 mM; Reifenberger et al. 1997). The low-affinity transporters are expressed at high glucose concentrations (e.g. early in batch cultivation on glucose or in chemostat cultivation with high residual glucose concentrations), whereas the high-affinity transporters are expressed at low glucose concentrations (e.g. as batch cultures approach the diauxic shift or in chemostat cultures with low residual glucose concentrations; Diderich et al. 1999a). Thus, the substrate affinity of cellular glucose transport is appropriate for the glucose concentration in the environment. This regulation is achieved by the combined action of the Snf3/Rgt2 signal transduction system (Özcan and Johnston 1999) and by glucose repression of the high-affinity glucose transporter genes (Bisson 1988; Petit et al. 2000).

Sugar transporters are subject to inactivation when they are no longer required by the cell; this is true for the transporters of maltose (Lucero et al. 1993) and galactose (DeJuan and Lagunas 1986), and for the high-affinity glucose transporters (Busturia and Lagunas 1986). In all cases, the proteins are "tagged" by ubiquitination and removed from the plasma membrane by endocytosis; subsequently they are translocated to the vacuole and degraded by proteolysis (Riballo et al. 1995; Horak and Wolf 1997; Krampe and Boles 2002).

8.5 Glycolysis

Glycolysis oxidises glucose to pyruvate with the concomitant reduction of NAD^+ to NADH. The pathway consists of ten enzymes: hexokinase phosphorylates glucose and fructose at the expense of ATP, and phosphoglucose isomerase interconverts the two hexose-6-phosphates. Phosphofructokinase adds a second phosphate group to fructose-6-phosphate to form fructose-1,6-bisphosphate; this reaction consumes another ATP molecule. Aldolase then cleaves fructose-1,6-bisphosphate to form dihydroxyacetone phosphate and glyceraldehyde-3-phosphate. These can be interconverted by triose phosphate isomerase. The glyceraldehyde-3-phosphate formed by the last two reactions is oxidised to 1,3-bisphosphoglycerate by glyceraldehyde-3-phosphate dehydrogenase, with concomitant reduction of NAD^+; an inorganic phosphate is added in this reaction. The phosphoryl group is then transferred to ADP by phosphoglycerate kinase, to form ATP and 3-phosphoglycerate. The latter compound is rearranged to 2-phosphoglycerate by phosphoglycerate mutase, and further to phosphoenolpyruvate by enolase. A second phosphoryl transfer reaction occurs, catalysed by pyruvate kinase, to form pyruvate and a second ATP molecule. Note that two ATP molecules are consumed in upper glycolysis, and four are produced

in lower glycolysis (two each for the two three-carbon compounds produced by aldolase). Thus glycolysis yields a net of 2 moles of ATP per mole of glucose, via substrate-level phosphorylation (Stryer 1995; Zimmermann and Entian 1997).

Glycolysis is not only an important source of ATP, and of pyruvate for subsequent dissimilatory reactions, but also provides carbon skeletons for amino acid biosynthesis. For example, phosphoenolpyruvate is used for aromatic amino acid biosynthesis, 3-phosphoglycerate is used for serine biosynthesis, and pyruvate is used for biosynthesis of alanine, valine, and leucine.

The glycolytic pathway is ubiquitous amongst yeast species, despite the diversity in substrate range and metabolic modes displayed by yeasts. The biochemical basis for the differences in metabolism has not received much attention; from the limited number of studies done on yeasts other than *S. cerevisiae* it seems that the regulation of glycolytic enzyme gene expression is the primary source of diversity; in addition, differences in the kinetics and allosteric regulation of some enzymes has been noted among yeast species (Weusthuis et al. 1994; Passoth et al. 1996; Flores et al. 2000).

In *S. cerevisiae*, glycolytic enzymes are expressed to very high levels, especially during growth on glucose. Most of the enzymes occur as isoenzymes, and the genes encoding them are differentially expressed. For example, hexokinase activity is encoded by the *HXK1*, *HXK2*, and *GLK1* genes (the *HXK* gene products act on both glucose and fructose, whereas glucokinase, the enzyme encoded by *GLK1*, recognises only glucose). *HXK2* expression is stimulated by glucose, whereas transcription of *HXK1* and *GLK1* is repressed by glucose (Herrero et al. 1995).

8.5.1 Glycerol Biosynthesis

Glycerol biosynthesis is an important side-reaction of the glycolytic pathway. The dihydroxyactone phosphate formed by aldolase can either be metabolised further through glycolysis (after isomerisation by triose phosphate isomerase) or be reduced to glycerol-3-phosphate by glycerol-3-phosphate dehydrogenase, with concomitant oxidation of NADH to NAD^+. Subsequently, glycerol is formed by glycerol-3-phosphatase. Glycerol biosynthesis is important as a means of replenishing the pool of NAD^+ that is consumed by glycolysis.

Glycerol is also important to yeasts because it plays a role in protection of the cell from osmotic stress, since it is an effective compatible solute. In yeasts such as *S. cerevisiae* the enzymes required for glycerol formation are up-regulated in response to high osmolarity conditions in the environment. This response is mediated by the high osmolarity glycerol (HOG) signal transduction pathway (Hohmann 2002; Saito and Tatebayashi 2004). Some yeast species are osmotolerant, and are able to grow on substrates with low water activities such as jam and honey. These yeasts, such as *Debaryomyces hansenii* and *Zygosaccharomyces* species, can cause food spoilage. One physiological characteristic of these species is their high capacity for glycerol production, and their increased ability to retain glycerol within the cell for osmoprotection (Larsson and Gustafsson 1987; Larsson et al. 1990; Vindelov and Arneborg 2002).

8.5.2 Fates of Pyruvate: Ethanolic Fermentation

The pyruvate formed by glycolysis has two catabolic fates: it can be fermented to ethanol or it can be oxidised to CO_2 by the citric acid cycle. In either case, the NADH produced by glycolysis is re-oxidised to NAD^+. In fermentation, pyruvate is cleaved to acetaldehyde and CO_2 by pyruvate decarboxylase. The acetaldehyde so formed is then reduced to ethanol by alcohol dehydrogenase; NADH is oxidised by this reaction. *S. cerevisiae* has 20 genes for alcohol dehydrogenases. The two cytosolic isoenzymes most important for central carbon metabolism are encoded by the *ADH1* and *ADH2* genes (Lutstorf and Megnet 1968). Adh1 is the isoenzyme involved in ethanolic fermentation, and transcription of *ADH1* is up-regulated during growth on glucose (Denis et al. 1983). This regulation is due to the activity of the Rap1 and Gcr1 transcription factors (Santangelo and Tornow 1990). *ADH2* is glucose-repressed, and Adh2 functions primarily during gluconeogenic growth on ethanol; its transcription is activated via the action of the *ADR1* transcriptional activator (Denis and Young 1983; Young et al. 2002).

Acetaldehyde is also used in the production of cytosolic acetyl-coenzyme A (CoA) via the action of the acetaldehyde dehydrogenase and acetyl-CoA synthase enzymes. Cytosolic acetyl-CoA is used for the biosynthesis of lipids and amino acids, and can be translocated into mitochondria by the carnitine–acetyl transferase shuttle (for a review see Pronk et al. 1996).

8.5.3 Fates of Pyruvate: Respiration

The oxidative catabolism of pyruvate begins with its transport into the mitochondria, followed by conversion to acetyl-CoA and CO_2 with the reduction of NAD^+. The reaction is catalysed by pyruvate dehydrogenase, an enzyme complex which is composed of five different subunits which in *S. cerevisiae* are encoded by *PDA1* (E1α), *PDB1* (E1β), *LAT1* (E2), *LPD1* (E3), and *PDX1* (protein X), (reviewed in Steensma 1997). The E3 component (lipoamide dehydrogenase) also forms part of the related multienzyme complexes α-ketoglutarate dehydrogenase, branched-chain ketoacid dehydrogenase, and glycine decarboxylase (Dickinson et al. 1986; Ross et al. 1988; Dickinson and Dawes 1992; Sinclair et al. 1993; Sinclair and Dawes 1995).

It has long been held that in *S. cerevisiae* the flux of pyruvate to the fermentative and oxidative branches is a function mostly of the kinetic properties of pyruvate dehydrogenase and pyruvate decarboxylase. Pyruvate dehydrogenase has a tenfold higher affinity for pyruvate than pyruvate decarboxylase but its activity in the cell is quite low. Hence, low concentrations of pyruvate favour its oxidation via pyruvate dehydrogenase, whereas high concentrations of pyruvate (which occur when the flux through glycolysis is high, such as during growth on high glucose concentrations) result in "overflow" fermentative metabolism via pyruvate decarboxylase and alcohol dehydrogenase with the resultant formation of ethanol. Further control of the distribution of pyruvate between fermentation and respiration is effected at the level of gene expression, since glucose induces expression of pyruvate decarboxylase, and represses the expression of acetyl-CoA synthetase and the Lpd1 subunit of pyruvate dehydrogenase.

8.5.4 Acetate Metabolism

Acetate can be utilised as a carbon source by most yeasts. In *S. cerevisiae* it is converted to acetyl-CoA using ATP by acetyl-CoA synthetase. There are two isoenzymes: that encoded by *ACS1* is repressed by glucose, while that encoded by *ACS2* is constitutively transcribed (Steensma et al. 1993; van den Berg and Steensma 1995; van den Berg et al. 1996). The regulation of *ACS1* is complex. A carbon source responsive element (CSRE) and a binding site for Adr1 combine to mediate about 80% of derepression. The balance between repression by Ume6 and induction by Abf1 is also important (Kratzer and Schüller 1997). In anaerobic conditions Acs2 is used. An *acs2* mutant is unable to grow on glucose but grows on ethanol or acetate. Acs1 is the isoenzyme required for growth in aerobic conditions as an *acs1*Δ mutant can grow on all carbon sources. *acs1 acs2* double mutants are inviable (van den Berg and Steensma 1995). The cytosolic acetyl-CoA produced in this way can be used for lipid and amino acid biosynthesis, and can also be transported into the mitochondria by the carnitine–acetyl transferase shuttle. In *K. lactis* there are also two isoenzymes of acetyl-CoA synthetase encoded by *KlACS1* and *KlACS2* (Zeeman and Steensma 2003). The former is expressed at a low level on glucose or ethanol and induced on acetate or lactate; the latter is preferentially expressed on glucose and ethanol (Lodi et al. 2001; Zeeman and Steensma 2003).

8.5.5 Tricarboxylic Acid Cycle

Acetyl-CoA derived from ethanol or acetate via acetyl-CoA synthetase, from pyruvate via pyruvate dehydrogenase, or from fatty acid oxidation reacts with oxaloacetate to form citrate, the first intermediate in the tricarboxylic acid (TCA) cycle. The reaction is catalysed by citrate synthase. *S. cerevisiae* has three isoenzymes of citrate synthase with different subcellular locations: these are encoded by *CIT1* (mitochondrial matrix), *CIT2* (peroxisome), and *CIT3* (mitochondrial matrix) (McAlister-Henn and Small 1977). *CIT2* is taken as the classic example of a gene that is subject to the retrograde response – the situation in which the expression of nuclear genes is regulated by the mitochondria. (Key players in this phenomenon are the retrograde response genes *RTG1*, *RTG2*, and *RTG3*.) In wild-type cells the expression of *CIT2* is low, but in cells which are mitochondrially compromised the transcription of *CIT2* is greatly increased (Liao and Butow 1993; Chelstowska and Butow 1995). Metabolites produced in the peroxisomes can also be used in the TCA cycle (in the mitochondria). Thus, the retrograde regulation of *CIT2* can regulate the efficiency by which the cells use two-carbon compounds in anaplerotic pathways, especially the glyoxylate cycle. The retrograde response is also valuable in offering metabolic flexibility in a variety of different developmental scenarios (e.g. sporulation, filamentation).

Aconitase catalyses the stereospecific isomerisation of citrate into isocitrate. The enzyme is encoded by *ACO1* and is located in both the cytosol and the mitochondrial matrix. There is no genuine *ACO2* gene but Yjl200c has considerable sequence similarity to Aco1. The expression of aconitase is repressed by glucose and glutamate where its presence would be unnecessary. As one would predict, *aco1* mutants

are unable to grow on non-fermentable carbon sources and are glutamate auxotrophs on glucose because α-ketoglutarate (the precursor to glutamate) is not made (Crocker and Bhattacharjee 1973).

Next is the oxidative decarboxylation of isocitrate to α-ketoglutarate. There are three isoenzymes of isocitrate dehydrogenase. NAD-specific isocitrate dehydrogenase is an allosterically regulated octamer composed of four subunits of Idh1 and four of Idh2 (Lin and McAlister-Henn 2002). It is located in the mitochondrion. Both subunits can bind the substrate: Idh1 for allosteric activation by AMP and Idh2 for catalysis (Lin et al. 2001). There is a strong correlation between the level of isocitrate dehydrogenase activity and the ability to grow on acetate or glycerol (Lin et al. 2001). *S. cerevisiae* has two NADP-dependent isocitrate dehydrogenases: Idp1 (mitochondrial) and Idp2 (cytosolic). A mutation in either *IDH1* or *IDH2* renders it unable to grow on acetate or pyruvate but it can still grow on ethanol. In contrast, *idp1* or *idp2* mutants as well as *idp1 idp2* double mutants can grow on either acetate or ethanol. NAD-specific isocitrate dehydrogenase binds specifically and with high affinity to the 5′ untranslated leader sequences of all mitochondrial messenger RNAs (mRNAs). It is thought that this suppresses inappropriate translation because when *S. cerevisiae* has been disrupted for NAD-specific isocitrate dehydrogenase it displays increased mitochondrial translation (de Jong et al. 2000). However, despite the increased rate of synthesis, subunits 1, 2, and 3 of cytochrome c oxidase, and cytochrome b (all encoded by mitochondrial genes) are all reduced in the absence of NAD-specific isocitrate dehydrogenase owing more rapid degradation (de Jong et al. 2000). Evidently, several lines of evidence point to NAD-specific isocitrate dehydrogenase having a role in regulating the rate of mitochondrial assembly besides its role in the TCA cycle.

α-Ketoglutarate dehydrogenase catalyses the oxidative decarboxylation of α-ketoglutarate via succinyl-CoA to succinate. The multienzyme complex comprises a dehydrogenase (E1) encoded by *KGD1*, succinyl transferase (E2) encoded by *KGD2*, and lipoamide dehydrogenase (E3) encoded by *LPD1*. Mutants of *S. cerevisiae* in E1, E2, or E3 components are able to grow on glucose but not on acetate or glycerol (Dickinson et al. 1986; Repetto and Tzagoloff 1989, 1990). The complex assembles spontaneously in vivo. Succinyl-CoA ligase α and β subunits are encoded by *LSC1* and *LSC2*, respectively. All of the aforementioned are subject to catabolite repression.

Succinate dehydrogenase catalyses the conversion of succinate into fumarate. In *S. cerevisiae* the flavoprotein precursor of this enzyme is encoded by *SDH1*. The mature Sdh1 binds to Sdh2 (the iron–sulphur protein) to form an active dimer. Two hydrophobic proteins (Sdh3 and Sdh4) anchor the active dimer to the mitochondrial inner membrane. The expression of the *SDH* genes is repressed by glucose and derepressed on respiratory carbon sources (Lombardo et al. 1990; Scheffler 1998). The turnover of *SDH2* mRNA is crucial to the control of succinate dehydrogenase activity by the carbon source. Mutants defective in succinate dehydrogenase are unable to grow on all respiratory carbon sources. The situation in *K. lactis* is very different. Despite the fact that the orthologous gene *KlSDH1* is 84% identical to the *S. cerevisiae SDH1*, it is highly expressed under both fermentative and non-fermentative conditions. Furthermore, strains carrying mutations in the *KlSDH1* gene are still able to grow on lactate, but strangely cannot grow on acetate, ethanol,

or glycerol (Saliola et al. 2004). It would appear that in *Kluyveromyces* lactate is metabolised to pyruvate by lactate ferricytochrome c oxidoreductase (just as in *Saccharomyces*) and that the pyruvate is channelled into the TCA and glyoxylate cycles using enzymes which are differently regulated. In the *K. lactis Klsdh1* mutant both of the genes encoding acetyl-CoA synthetase are expressed more highly than in the wild type when grown on lactate as are its genes for the glyoxylate cycle enzymes malate synthase and isocitrate lyase when grown on both lactate and glucose. Since pyruvate decarboxylase is highly expressed (in both the mutant and the wild type) on lactate, the *Klsdh1* mutant can grow on lactate by having high levels of glyoxylate cycling and accumulating some succinate (Saliola et al. 2004). There must be an additional unknown special effect by lactate on gene expression or enzyme activity in *Kluyveromyces* because it is not clear why this mutant cannot grow on pyruvate.

Fumarase, which catalyses the conversion of fumarate to malate, is encoded by a single gene in all yeasts which have been examined. In *S. cerevisiae* it exists as separate cytosolic and mitochondrial forms. Various explanations have been proposed to explain this situation, including different transcription initiation sites to produce two different mRNAs (a longer one encoding the mitochondrial isoenzyme and targeting sequence and a shorter one for the cytosolic form) (Wu and Tzagoloff 1987), or dual translational initiation and selective splicing; the last two having been established for other proteins. However, the localisation and distribution of fumarase appears to be unique because there is only one translation product which is targeted to the mitochondria by an N-terminal presequence which is then removed by the mitochondrial processing peptidase. Some of the fully mature fumarase molecules are then released back into the cytosol. In vivo translocation into the mitochondria only occurs during translation and in vitro translation of the *FUM1* mRNA requires mitochondria (Sass et al. 2001).

Malate dehydrogenase catalyses the oxidation of malate to oxaloacetate. In *S. cerevisiae* the three isoenzymes, encoded by *MDH1* (mitochondrial), *MDH2* (cytoplasmic), and *MDH3* (peroxisomal), are all subject to catabolite repression. The majority (90%) of malate dehydrogenase activity is due to Mdh1, except when acetate or ethanol is the carbon source, in which case Mdh2 constitutes 65% of total malate dehydrogenase (Steffan and McAlister-Henn 1992). Oxaloacetate levels are crucial. If oxaloacetate levels were to become insufficient then further turns of the TCA cycle would not be possible. This could arise owing to the consumption of TCA cycle intermediates e.g. α-ketoglutarate and oxaloacetate in the formation of glutamate and aspartate (respectively), both of which contribute to the synthesis of other amino acids. The metabolic requirement is ensured in two ways: the anaplerotic (filling-up) glyoxylate bypass and Mdh2. Mdh2, like phosphoenolpyruvate carboxykinase and fructose-1,6-bisphosphatase, is critical in gluconeogenesis and is similarly rapidly inactivated and then proteolytically degraded if glucose is supplied to cells which had been growing gluconeogenically.

8.5.6 Mitochondria

It is impossible to describe the TCA cycle without mentioning its location – the mitochondria. Study of the function and biogenesis of these organelles has been

facilitated by the availability of respiratory-deficient *petite* mutants of *S. cerevisiae* which can live anaerobically by fermentation. Subsequently, for a variety of reasons, attempts were made to isolate similar mutants in other yeast species. This soon gave rise to the notion that there were "*petite*-positive" and "*petite*-negative" yeasts: being those from which it was possible or impossible (respectively) to isolate *petite* mutants (Bulder 1964a, b). Notable *petite*-negative species included *K. lactis*, *Sch. pombe*, and *Z. bailii*. For 3 decades researchers pondered why certain yeasts were apparently unable to survive without mitochondria. It was reasoned that these organisms must have certain functions needed for an anaerobic or a fermentative existence which were either contained within the mitochondria or encoded by mitochondrial genes. It now appears that the classification was fallacious and arose simply because in *S. cerevisiae petite* mutants arise at very high frequencies and in other supposed *petite*-negative yeasts the responsible *nuclear* genes had not been identified. However, the authors are still puzzled by the fact that, as far as we know, no-one has ever reported a *petite* mutant of *Z. bailii*. Maybe in this yeast certain vital functions required for anaerobic growth really do reside in its mitochondria.

8.5.7 The Glyoxylate Cycle

The glyoxylate cycle comprises two enzymes: isocitrate lyase and malate synthase. In *S. cerevisiae* isocitrate lyase is encoded by *ICL1* (Fernández et al. 1992). Isocitrate lyase catalyses the conversion of isocitrate into glyoxylate and succinate. The enzyme is a homotetramer. Its synthesis is induced by ethanol and repressed by glucose. Transcription of the gene is controlled by the global regulator Snf1. The enzyme is rapidly inactivated and then proteolytically degraded if glucose is added to gluconeogenically grown cells (Ordiz et al. 1996). As with the other enzymes, mentioned already, whose activity is similarly controlled, phosphorylation by cyclic-AMP-dependent protein kinase is the trigger. Malate synthase catalyses the formation of malate from glyoxylate and acetyl-CoA. There are two isoenzymes. *MLS1* encodes an enzyme with both peroxisomal and cytosolic locations. Mls1 is abundant in the peroxisomes of cells grown on oleic acid but in ethanol-grown cells it is mostly cytosolic (Kunze et al. 2002). *MLS1* is repressed by glucose. *MLS2* (better known as *DAL7*) encodes an enzyme required when allantoin is the sole source of nitrogen; it is subject to repression by NH_4^+ (Hartig et al. 1992). The different regulatory controls over the expression and activity of the two forms ensure that *S. cerevisiae* can metabolise C-2 compounds, allantoin, or both. It has been observed that *S. cerevisiae* which have been phagocytosed have up-regulated both *ICL1* and *MLS1* and that *C. albicans* lacking *CaICL1* have reduced virulence, leading to the suggestion that the glyoxylate cycle is an important component of fungal virulence (Lorenz and Fink 2001).

8.6 The Pentose Phosphate Pathway

The pentose phosphate pathway (hexose monophosphate pathway) is crucial to the operation of many other metabolic pathways. It can be considered to start with the dual glycolytic/gluconeogenic intermediate glucose-6-phosphate, which is converted to 6-phosphogluconolactone by glucose-6-phosphate dehydrogenase

(Zwf1) (Nogae and Johnston 1990; Thomas et al. 1991). It was once believed that this enzyme was the limiting step of the pathway in *S. cerevisiae*, but this is now known not to be the case (Dickinson et al. 1995) Glucose-6-phosphate dehydrogenase is an important source of NADPH, which is required for a variety of reductive biosyntheses. The subsequent conversion of 6-phosphogluconate to ribulose-5-phosphate catalysed by 6-phosphogluconate dehydrogenase (major isoenzyme Gnd1, minor isoenzyme Gnd2; Lobo and Maitra 1982) also yields NADPH. Ribulose-5-phosphate can be epimerised to xylulose-5-phosphate (catalysed by ribulose-5-phosphate 3-epimerase, Rpe1) or isomerised to ribose-5-phosphate by ribose-5-phosphate ketolisomerase, Rki1 (Miosga and Zimmermann 1996). Xylulose-5-phosphate and ribose-5-phosphate can then react together in a reaction catalysed by transketolase to produce glyceraldehyde-3-phosphate and sedoheptulose-7-phosphate. There are two isoenzymes of transketolase: Tkl1 and Tkl2. Tkl1 is the major isoenzyme (Fletcher et al. 1992; Schaaff-Gerstenschläger et al. 1993; Sundström et al. 1993). The glyceraldehyde-3-phosphate can re-enter glycolysis/gluconeogenesis or can undergo further reaction with sedoheptulose-7-phosphate to yield fructose-6-phosphate and erythrose-4-phosphate; the latter reaction being catalysed by transaldolase (Tal1). The fructose-6-phosphate formed in the transaldolase reaction can also re-enter glycolysis/gluconeogenesis or can undergo a transketolase-catalysed reaction with erythrose-4-phosphate to produce glyceraldehyde-3-phosphate and xylulose-5-phosphate.

The pentose phosphate pathway fulfils many metabolic requirements: the provision of NADPH, ribose skeletons (needed for the synthesis of histidine, tryptophan, and purine ribonucleotides and deoxyribonucleotides), and erythrose-4-phosphate for the synthesis of the aromatic amino acids phenylalanine, tyrosine, and tryptophan.

8.7 Gluconeogenesis

When yeasts are growing on non-fermentable carbon sources they use gluconeogenesis to synthesise glucose-6-phosphate, which is required in various biosynthetic reactions, including the formation of ribose-5-phosphate (via the pentose phosphate pathway, see Sect. 8.6) for the synthesis of histidine and of purine ribonucleotides and deoxyribonucleotides; and erythrose-4-phosphate (also via the hexose monophosphate pathway) for the synthesis of aromatic amino acids. The carbon skeletons to be built into glucose-6-phosphate originate from the TCA and glyoxylate cycles in the form of oxaloacetate which is converted into pyruvate by phoephoenolpyruvate carboxykinase (the first enzyme unique to gluconeogenesis). The pyruvate is then converted to 2-phosphoglycerate and subsequently by "reverse glycolysis" as far as fructose-1,6-bisphosphate. Here the second enzyme unique to gluconeogenesis (fructose-1,6-bisphosphatase) converts the fructose-1,6-bisphosphate to fructose-6-phosphate. The fructose-6-phosphate is subsequently converted to glucose-6-phosphate by phosphoglucose isomerase. Two other enzymes (isocitrate lyase and malate synthase) are required to compensate for the extraction of oxaloacetate from the TCA cycle which would otherwise cease owing to depletion of oxaloacetate. These two activities which comprise the glyoxylate bypass have already been described (Sect. 8.5.7).

It would clearly be metabolically futile for cells which are growing on a nonfermentable carbon source to simultaneously use glycolysis, or to operate gluconeogenesis when glucose is plentiful. *S. cerevisiae* avoids such wasteful futile cycling by controlling the activities of the key enzymes phosphoenolpyruvate carboxykinase and fructose-1,6-bisphosphatase. Phosphoenolpyruvate carboxykinase is encoded by *PCK1* (Valdes-Hevia et al. 1989). This gene's expression is strongly up-regulated in cells growing on gluconeogenic carbon sources (Mercado et al. 1994). *FBP1*, which encodes fructose-1-6-bisphosphatase (Sedivy and Fraenkel 1985; Entian et al. 1988), and *PCK1* are both repressed in cells growing on glucose (Sedivy and Fraenkel 1985; Mercado et al. 1991) This glucose repression is extremely sensitive as it is triggered at glucose concentrations of only 0.005% glucose (Yin et al. 1996) and gluconeogenically grown cells rapidly inhibit and proteolytically degrade phosphoenolpyruvate carboxykinase and fructose-1,6-bisphosphatase if glucose becomes available (Gancedo 1971; Haarasilta and Oura 1975; Müller and Holzer 1981). The artificial expression of *FBP1* and *PCK1* when glucose is present resulted in an increase in generation time of approximately 20% (Navas et al. 1993).

8.8 Trehalose, Glycogen, and Cell Wall Glucans

Trehalose is produced from glucose-6-phosphate in two steps. First, UDP-glucose (or ADP-glucose) and glucose-6-phosphate are converted into trehalose-6-phosphate and UDP (or ADP) by trehalose-6-phosphate synthase (Vuorio et al. 1993). Then, the phosphate group of trehalose-6-phosphate is removed by trehalose-6-phosphate phosphatase (De Viriglio et al. 1993). Both enzymes are present in a complex (Thevelein and Hohmann 1995; Ferreira et al. 1996). Trehalose is important to many organisms (not just yeasts) in resistance against many adverse conditions, including heat, cold, dehydration, osmotic stress, solvents, and free radicals; hence, its synthesis and degradation are highly regulated. In *S. cerevisiae* there are two trehalases which hydrolyse trehalose into two molecules of glucose. The neutral trehalase (Nth1) is cytosolic; the acid trehalase (Ath1) is vacuolar (Kopp et al. 1993; Destruelle et al. 1995).

Glycogen synthesis also uses UDP-glucose but results in a polymer, not simply a disaccharide as with trehalose. An α-1,4 glucosyl "primer" is required initially; this is formed from UDP-glucose (by Glg1 and Glg2 in *S. cerevisiae*; Cheng et al. 1995). Glycogen synthase performs the subsequent elongation. Gsy1 and Gsy2 represent the minor and major isoforms, respectively (Farkas et al. 1991). Gsy3 forms α-1,6 branch points. The breakdown of glycogen into glucose and glucose-1-phosphate is accomplished by glycogen phosphorylase (Gph1) (Hwang et al. 1989). Phosphorylation inactivates glycogen synthase and activates glycogen phosphorylase; dephosphorylation has the opposite effect on both activities (for a complete description see Stark 2004). Dual reciprocal control is obviously efficient as phosphorylation will increase the availability of glucose units by stopping glycogen synthesis and starting glycogen breakdown; whilst dephosphorylation stops glycogen breakdown and simultaneously starts glycogen synthesis.

The cell wall of yeasts is composed of glucans, mannans, chitin, and mannoproteins (as well as other minor components). The biosynthesis of these structural

carbohydrates is complex. As with glycogen and trehalose, the hexose molecules that make them up are initially charged by condensation with UTP (or ATP). In the case of glucose monomers, glucose-6-phosphate is converted to glucose-1-phosphate by phosphoglucomutase (encoded in *S. cerevisiae* by *PGM1* and *PGM2*). Glucose-1-phosphate then combines with UTP to form UDP-glucose in a reaction catalysed by UDP-glucose pyrophosphorylase (encoded by *UGP1* in *S. cerevisiae*; Daran et al. 1995). Synthesis of glucans and other cell wall polysaccharides from UDP-glucose and other constituents is a complex process involving multiple proteins, and its description is beyond the scope of this chapter (for reviews, see Cid et al. 1995; Shahinian and Bussey 2000; Smits et al. 2001). The glucan fraction of the yeast cell wall has received considerable attention recently as a candidate nutraceutical, as it displays immunostimulatory, anti-mutagenic, and anti-oxidant properties (Krizkova et al. 2003; Lee et al. 2001).

8.9 Regulation

8.9.1 Glucose Repression

Glucose is considered the preferred carbon source of *S. cerevisiae* because if this sugar is present at levels above about 0.1% (w/v) then the utilisation of other carbon sources is prevented. *S. cerevisiae* operates its metabolism in this way partly by repressing the transcription of genes required for the utilisation of the alternative carbon sources. Target genes of this glucose repression pathway include the *SUC* genes encoding invertase, *GAL* and *MAL* genes involved in utilisation of galactose and maltose (respectively), and the *FBP1* gene encoding fructose bisphosphatase. Genes required for utilisation of non-fermentable carbon sources (encoding proteins involved in mitochondrial biogenesis, respiration, the TCA cycle, etc.) are also repressed by glucose. Other fermentable carbon sources (e.g. fructose, galactose, and maltose) can exert repressive effects on members of this set of target genes as well, though they are generally less potent than glucose. The metabolism of *K. lactis* is not subject to glucose repression to anything like the extent seen in *S. cerevisiae* (Schaffrath and Breunig 2000), and the oxidative metabolism of *Schw. occidentalis* is also famously not repressed by high sugar concentrations (Poinsot et al. 1987; Zimmer et al. 1997).

Glucose repression in *S. cerevisiae* involves the binding of transcriptional repressors to the promoters of affected genes; these repressors, the Mig1 (Nehlin and Ronne 1990) and Mig2 (Lutfiyya and Johnston 1996) zinc-finger proteins, require the Ssn6 and Tup1 co-repressors for activity (Keleher et al. 1992; Vallier and Carlson 1994). Mig1 is localised in the nucleus in glucose-grown cells, and upon removal of glucose it is rapidly phosphorylated and translocated from the nucleus to the cytoplasm (De Vit et al. 1997). Derepression requires the Snf1 protein kinase complex (Carlson et al. 1981; Carlson 1999). This heterotrimeric complex includes the Snf1 (α) catalytic subunit, which has a catalytic domain and an autoregulatory domain. The other components of the complex are a regulatory (γ) subunit Snf4 involved in responding to glucose, and an oligomerisation factor (β subunit) (Sip1, Sip2, or Gal83) that mediates interactions with the downstream targets and sets the

subcellular localisation of the complex (Schmidt and McCartney 2000). Each β subunit has a unique pattern of localisation (Vincent et al. 2001). During growth on glucose all β subunits are located in the cytoplasm. On shifting the cells to a nonfermentable carbon source Sip1 localises around the vacuole, Sip2 remains cytoplasmic, and Gal83 becomes enriched in the nucleus. It has been shown recently that the cyclic-AMP-dependent protein kinase (protein kinase A) pathway maintains the cytoplasmic localisation of Sip1 in glucose-grown cells. Furthermore, the Snf1 catalytic subunit mislocalises to the vacuolar membrane in cells which lack protein kinase A (Hedbacker et al. 2004). This explains the long-known, but hitherto unexplained genetic interactions between *SNF1* and the cyclic AMP signalling pathway. The Snf1 kinase phosphorylates Mig1 (Treitel et al. 1998; Smith et al. 1999), resulting in its dissociation from promoter DNA and thus permitting transcription of target genes.

The activity of the Snf1 kinase is regulated by glucose; in the presence of the hexose the catalytic domain is inactive owing to intramolecular interaction with the autoregulatory domain. As glucose concentrations decline, the Snf4 protein liberates the Snf1 catalytic domain, which is then able to phosphorylate Mig1. The process is reversed by the Glc7-Reg1 protein phosphatase, which restores the inactive conformation of the Snf1 catalytic and autoregulatory domains at high glucose concentrations.

The signal initiated by glucose is not yet understood. Two models are currently favoured. The first is based on the homology between Snf1 and the AMP-activated protein kinase (AMPK), which regulates energy metabolism in mammalian cells. AMPK is activated by high AMP concentrations and low ATP concentrations, and it is proposed that changes in adenylate concentrations in response to glucose availability could affect Snf1 in a similar way (Hardie and Carling 1997). The second model is based on two types of mutations that result in expression in the presence of glucose of normally glucose repressible genes. Mutations in *HXK2* encoding the predominant hexokinase in glucose-grown cells lead to derepression, as do mutations that restrict cellular glucose transport activity. Hence, the intracellular concentrations of glucose, glucose-6-phosphate, or a related non-glycolytic metabolite are implicated in signalling the extracellular glucose concentration to the repression apparatus. The role of Hxk2 is worthy of special note because it has been suggested to have intrinsic regulatory functions as well as a catalytic function in glycolysis. Mutations in the *HXK2* gene are known which separate catalytic activity from the protein's role in glucose repression (Hohmann et al. 1999; Mayordomo and Sanz 2001a). Also, Hxk2 has been shown to translocate into the nucleus and act directly on the *SUC2* promoter (Herrero et al. 1998; Randez-Gil et al. 1998a, b). These observations are complicated by results which demonstrate that hexokinases from many other organisms are able to replace Hxk2 in exerting glucose repression (Rose 1995; Petit and Gancedo 1999; Mayordomo and Sanz 2001b). This implies that the catalytic activity of the enzyme is sufficient for its regulatory role.

Another line of evidence supporting the second model of Snf1 regulation by glucose is the identification of a trio of protein kinases that act upstream of Snf1, namely Pak1, Tok1, and Elm1 (Nath et al. 2004; Sutherland et al. 2003). The signalling pathway between glucose and these protein kinases remains to be elucidated.

8.9.2 Activation

A number of mechanisms are known in *S. cerevisiae* which bring about transcriptional activation of genes in conditions where glucose is low or absent. Two are described here. The first involves a complex of the Hap2, Hap3, Hap4, and Hap5 proteins, which binds to the promoters of target genes in the absence of glucose and activates their transcription. In *S. cerevisiae* the target genes are mostly involved in respiration and the utilisation of non-fermentable carbon sources. Regulation of the Hap complex takes place at the level of transcription of its components (Pinkham and Guarente 1985; Forsburg and Guarente 1989), although other levels of control probably exist as well. The mechanism results in expression of genes (e.g. *FBP1*, *PCK1*, *ICL1*, *MDH2*, and *JEN1*, which encodes the lactate transporter) required for the utilisation of non-fermentable carbon sources. These genes are under control of Cat8, a zinc-finger DNA-binding protein that activates transcription of target genes (Hedges et al. 1995; Randez-Gil et al. 1997; Bojunga and Entian 1999; Haurie et al. 2001; Roth and Schuller 2001). The *CAT8* gene itself is repressed by the glucose repression pathway (Hedges et al. 1995). Despite possessing *HAP* genes orthologous to those in *S. cerevisiae*, the Hap complex seems to be of lesser importance in *K. lactis* because *hap* mutations have little effect on respiratory metabolism in this organism (Nguyen et al. 1995).

It has long been believed and often written that, in *S. cerevisiae*, glucose repression is always stronger than oxygen induction. In other words, the conventional wisdom has been that when glucose levels are high *all* of the genes required for oxidative metabolism are repressed. However, very recent two-dimensional transcriptome analysis in chemostat cultures has revealed a subset of 35 genes for which induction by oxygen supersedes glucose repression (Tai et al. 2005). The mechanism(s) by which this is accomplished are not understood for every gene but it is noteworthy that some of the genes are known targets of the Hap2, Hap3, Hap4, Hap5 complex and that the expression of *HAP4* (which encodes the regulatory subunit of the Hap2, Hap3, Hap4, Hap5 complex) is identical to the aforementioned 35 genes.

8.9.3 Metabolic Integration

The regulatory pathways discussed earlier reflect some of the complexity of the regulatory networks that act on carbon metabolism in yeasts. Additional layers of regulation stem from the occurrence of forked regulatory pathways and cross talk between pathways. It should always be remembered that no living cell's metabolism distinguishes between, for example, "carbon metabolism" and "nitrogen metabolism": these are merely convenient conceptual divisions for the human mind. In reality, the pathways are integrated to allow optimal growth and reproduction in the individual organism's environmental niche. Consequently, there are "cross-pathway" regulatory phenomena which operate on individual genes and proteins, on individual pathways, and on groups of pathways. For example, some genes encoding enzymes of carbon metabolism (e.g. *PYC1*, *LPD1*) are regulated by the nitrogen source. Conversely, glucose limitation induces *GCN4* (the "global" transcriptional activator controlling depression of amino acid biosynthetic pathways) (Yang et al. 2000).

The integration of carbon catabolism with amino acid biosynthesis (anabolism) is readily observed in the TCA cycle, which, besides being essential for oxidative metabolism, is also required for the provision of intermediates for amino acid biosynthesis. For example, α-ketoglutarate is essential in the biosynthesis of glutamate, glutamine, proline, lysine, and arginine. Additionally, glutamate, which is derived from α-ketoglutarate via NADP-dependent glutamate dehydrogenase, is itself used in the biosynthesis of aspartate, and hence threonine, methionine, and cysteine and also tyrosine, phenylalanine, serine, leucine, isoleucine, and histidine. Hence, as noted before, *aco1* (aconitase-less) mutants of *S. cerevisiae* are glutamate auxotrophs on glucose. Mutants of this yeast defective in the major (mitochondrial) NAD-specific isocitrate dehydrogenase also show glutamate auxotrophy which can be partially compensated by overexpression of the (mitochondrial) NADP-dependent enzyme Idp1. Recent studies of the kinetic properties of the isoenzymes of isocitrate dehydrogenase have led to the conclusion that Idp1 has an ancillary role in glutamate biosynthesis and that the role of Idp2 is equilibration of isocitrate and α-ketoglutarate levels (Contreras-Shannon et al. 2005). Thus, perhaps independent of genetic controls, a high degree of evolutionary fine-tuning has occurred to the kinetic parameters of the different isoenzymes of isocitrate dehydrogenase. Many other examples are evident at this important node of metabolism (Dickinson 2004).

8.9.4 Metabolic Control

Cellular metabolism is subject to *regulation* at the level of gene expression, enzyme half-life, etc. and by the suite of enzymatic activities encoded by the yeast genome. Metabolism is also subject to *control*; in other words, under given conditions of gene expression and substrate availability, the flux through a metabolic activity is set by the activities of the pathway enzymes. Metabolic Control Analysis has pointed out that the control of metabolic flux is in principle distributed amongst all of the steps in a pathway; some steps may have high control, while others have negligible control (Kacser and Burns 1981; Fell 1997). This is in disagreement with the textbook view that single enzymes can be the “rate-limiting step” of a pathway. The most well-known example is that of phosphofructokinase (or in some textbooks hexokinase or pyruvate kinase), which owing to complex allosteric regulation is considered to be the rate-limiting step of glycolysis. Both experimental (Heinisch 1986) and theoretical (Cornish-Bowden 2004) studies have discredited this point of view. Indeed, the preponderance of control over yeast glycolysis lies in the first step, viz. transport of glucose over the plasma membrane, and not in any of the enzymatic steps of the pathway (Ye et al. 1999; Diderich et al. 1999b).

8.10 Metabolic Modelling and Functional Genomics

Carbohydrate metabolism in yeasts has been subject to numerous modelling efforts over the last few decades. The goals and theoretical bases of these efforts have varied widely, and a comprehensive review is not feasible here. Metabolic models can aid in the description and interpretation of experimental data, and they can have a heuristic value in the design of experiments. The input data for metabolic models

can include metabolic fluxes and intracellular metabolite concentrations (e.g. flux balance analysis and metabolic control analysis) or information about enzyme kinetics (substrate and inhibitor affinity, catalytic centre activity, allosteric effector effects, etc., e.g. biochemical systems theory and kinetic parameter modelling). The advent of functional genomics, such as the ability to screen thousands of single-gene knockouts in parallel for their metabolite profile (the "metabolome"), makes metabolic modelling, combined with other types of bioinformatics, crucial for data reduction and identification of interesting trends and phenotypes (Oliver et al. 2002). Simultaneous determination of dozens or hundreds of metabolites in a cell extract is generally performed with NMR or various mass spectroscopic techniques. The large and convoluted data sets generated by these techniques require computational analysis to discriminate the identities and concentrations of individual metabolites. Alternatively, the overall pattern and abundance of metabolites (metabolite fingerprint) can be compared between two or more strains or cultivation conditions in order to identify trends. An example of the latter approach was the comparison of a set of *S. cerevisiae* strains grown under identical conditions: a wild-type strain, and mutants either in fructose-6-phosphate 2-kinase genes or in respiratory functions. The metabolite composition of the cellular extracts of the strains was determined from ^{1}H-NMR spectra. Statistical analysis of the spectra to generate the metabolite fingerprints was performed using principle components analysis (to reduce the large number of variables in the raw spectra to a smaller number of correlated variables) followed by discriminant function analysis (to determine which of the correlated variables discriminate among the strains); however, the identity of individual metabolites was not determined. From the statistical analysis, the strains were easily resolved into the three classes, namely wild type, respiratory mutants, and fructose-6-phosphate 2-kinase mutants (Raamsdonk et al. 2001). The authors propose that this approach could be generally useful in determining the cellular roles of those genes of unknown function identified by genome sequencing projects. It could also be applied to assess the effects of cultivation conditions, stress, or other environmental parameters on yeast physiology.

Applications of metabolic modelling in biotechnology include providing a rational basis for metabolic engineering. For example, the conclusion from metabolic control analysis that in *S. cerevisiae* the glucose transport step exerts high control on glycolytic flux (Sect. 8.9.4) made the transport step a rational target for reducing the flux to pyruvate. It was hypothesised that decreasing the size of the cytosolic pyruvate pool would prevent overflow into the fermentative pathway (Sect. 8.5.4), and that consequently a strain with a sufficiently diminished transport capacity would respire glucose under aerobic conditions. A series of strains of *S. cerevisiae* were constructed with diminished glucose transport capacities, and were screened for respiratory glucose metabolism. One was found that actually displayed Crabtree-negative glucose metabolism, i.e. it respired glucose even at high glucose concentrations. As a result, the strain had a high biomass yield and a negligible ethanol yield during aerobic batch cultivation (Otterstedt et al. 2004).

Metabolic engineering can be defined in general as the use of genetic engineering to modify cellular metabolism. The goals of metabolic engineering can include changing the mode of cellular metabolism (see earlier) or changing the substrate

range or product output of the cell. For example, as noted before, *S. cerevisiae* is not able to metabolise xylose, lactose, or cellulose (Sect. 8.2); however, strains of this species have been engineered to gain these functions. Lactose metabolism was conferred by transformation of the *LAC4* (encoding β-galactosidase) and *LAC12* (encoding lactose-H^+ permease) genes from *K. lactis* into *S. cerevisiae* (Sreekrishna and Dickson 1985; Rubio-Texeira et al. 1998). Cellulose utilisation by *S. cerevisiae* has been engineered by transformation with three fungal genes that encode secreted enzymes capable of degrading cellulose polymers to glucose (endoglucanase and cellobiohydrolase from *Trichoderma reesei* and β-glucosidase from *Aspergillus aculeatus*). The resulting strain ferments cellulose to ethanol with a high yield (Fujita et al. 2004).

Metabolic engineering for xylose utilisation (for production of biofuel ethanol from plant waste) has received considerable attention, with only moderate success. Genes encoding xylose reductase (NADP-dependent) and xylitol dehydrogenase (NAD-dependent) from *P. stipitis* have been transformed into *S. cerevisiae*, and numerous other alterations have been engineered or selected in the resulting strains as well. However, the rates and yields of ethanolic fermentation from xylose are low compared with those from glucose, and a significant portion of the pentose is converted to xylitol. The low efficiency has been attributed to a redox imbalance created by the different cofactor specificities of the heterologous enzymes, to low xylose transport capacity, or to insufficient flux through the pentose phosphate pathway. The challenges of this important undertaking, and the prospects for the future, have been reviewed (Hahn-Hägerdal et al. 2001; Jeffries and Jin 2004).

8.11 Concluding Remarks

Yeasts have been domesticated for food biotechnology for millennia, and have contributed to food spoilage for even longer. The elucidation of the roles of yeasts in the food sector, starting with the discoveries of Pasteur, has highlighted the importance of carbohydrate metabolism – in fermentation, in biomass formation, and in tolerance of extreme environments. In the twenty-first century the economic importance of these organisms in food is likely to grow, and this will be stimulated by continued research into basic and practical aspects of the carbohydrate metabolism of the yeasts.

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Chapter 9

Yeasts as Biocatalysts

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9.1 Introduction

When looking at a biological reaction (a reaction due to a microorganism) it appears clearly that it can take place in two different ways:

– A system where the microorganisms are suspended in the medium (here considered as homogeneous): that is the case of the fermentations in winemaking or in brewing, for example.
– A system where the microorganisms are not free in the medium but where they are attached to a support, giving a two-phase system. Such a system is illustrated in nature by biofilms, for example.

In the controlled use of microorganisms in an industrial frame, like alcoholic fermentation, free cells of microorganisms were used first, surely because of tradition, which existed prior to knowledge of the biological nature of the reaction. In oenology, and also in the major part of industrial fermentation processes, this concept of free cells is largely pre-eminent. In some others fields of applications, in contrast, as early as 1960s the purpose was to attach the microorganisms and some industrial applications quickly followed the conceptual developments: electrodes with attached enzymes, bacterial beds in water treatment, adsorbed bacteria for vinegar production, etc.

Thus it appears quite logical to ask:

– Why should the microorganisms be attached?
– How should it be done?
– What interest and what applications are there for the immobilized yeasts in the food industry?

9.1.1 Why Attach the Microorganisms?

In the fermentations in the food industry the main expected interests in the immobilization of microorganisms are first the increase of the reaction rates due to the high cell concentration, second an easier operation in continuous mode and third

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the easy separation of the microbial cells at the end of the fermentation step. Moreover it allows the reuse of the catalyst for some cycles of production. As it becomes possible to increase the reaction rates it is also possible to have better control of the reaction. Indeed the reaction rate is the product of the specific rate of a cell and the number of cells. To increase the reaction rate it is possible to increase the specific rate, the number of cells or both together. Increasing the specific rate requires giving the microorganism the best environmental conditions, but these conditions are rarely those encountered in the practice of industrial fermentations (low pH, high sugar concentration, alcohol) where the inhibition or limitation mechanisms generally dominate. In contrast, the sole apparent limitation to the increase of the number of microbial cells is the saturation of the culture medium. However, it is worth noting that the microorganisms may slightly modify their metabolism when submitted to excessive conditions of cellular concentrations.

Also some works deal with the immobilization process in order to allow good control of different microorganisms working together (co-immobilization). This co-immobilization may also concern microorganisms of the same kind (for example, different yeast genus or species), very different microorganisms (yeasts and bacteria, for example) or a microorganism and an enzyme. This co-immobilization may be realized using different processes: adsorption, entrapment or membrane retention.

9.1.2 How to Immobilize the Microbial Cells?

The immobilization of microbial cells may be carried out by different methods which (for most of them) are based on the methods initially proposed for the immobilization of enzymes by Chibata (1979). These methods may be classified in four categories: adsorption, covalent union, inclusion and retention of microbial cells without a support.

Each of these methods will be briefly presented and their applications to the use of yeasts in the food industry will be developed.

9.1.2.1 Adsorption

The method based on the adsorption phenomena of microbial cells on a support is certainly the oldest method used in the food industry (Linko and Linko 1984). The adsorption is the result of electrostatic attractions between the support and the microbial wall, which is charged negatively (Kolot 1980). The supports used can be of very different natures: wood, bricks, PVC, silica, bentonite, fragments of vegetables. The affinity of a microbial cell to a support varies depending on each microorganism/support pair and is very difficult to forecast. Generally yeast cells have better adsorption characteristics than bacterial cells (Navarro 1980).

Adsorption is a reversible phenomenon that depends on the age of the cells, cellular wall composition, pH and ionic composition of the medium. A desorption phenomenon can take place, leading to a hybrid system where the free cells are associated to immobilized cells. However, this desorption can be compensated with the growth of the microorganism cells on the support, which induces a permanent regeneration of the "biocatalytic system".

In the recent works dealing with the immobilization of yeast cells the supports most used for the different applications in winemaking, brewing, ethanol production, etc. are cellulose and cellulose derivates (Koutinas et al. 1995; Bardi et al. 1996; Viljava and Lommi 2000), (diethylamino)ethyl (DEAE) cellulose (Lommi and Ahvenainen 1990; Linko and Kronlof 1991; Kronlof and Linko 1996; Andersen et al. 2000), fragments of vegetables (apple cuts, Kourkoutas et al. 2002b; dried raisin berries, Tsakiris et al. 2004; cane stalks, Chen 2001; pieces of figs, Bekatorou et al. 2002), gluten pellets (Smogrovicova et al. 1999; Bardi et al. 1997a), wood chips (Linko et al. 1998; Pajunen et al. 2000; Viljava and Lommi 2000), quince (Kourkoutas et al. 2003), minerals such as aluminium (Loukatos et al. 2000), ultraporous fired bricks (Opara and Mann 1988), kissiris (a glassy volcanic rock; Bakoyianis et al. 1993), ceramics (Zhang et al. 1992; Horitsu 1993; Cheng et al. 2000), porous glass (Kronlof and Linko 1992; Breitenbuecher and Mistler 1994; Yamauchi et al. 1994; Kronlof and Linko 1996) and silicon carbide (Masschelein and Andries 1996; Tata et al. 1999) and a flocculent strain has been absorbed into a sponge (Scott and O'Reilly 1995).

The adsorption is obtained by keeping the microbial suspension in contact with the support. After incubation the free cells are eliminated by several washings of the support as explained by Kourkoutas et al. (2002b) for yeast cells onto apple cuts.

As far as the industrial applications are concerned, the main fields are winemaking, brewing and alcohol production. Nevertheless, even though a lot of work has been done, few of the industrial applications are being used at this time.

9.1.2.2 Immobilization by a Covalent Link

In order to avoid the desorption phenomenon it is possible to establish true covalent binding between the microbial cell and the support. This is done using a union agent and the support is then called an "activated" support. Glutaraldehyde is the most commonly used agent, especially when the supports are made of proteins (Phillips and Poon 1988). In this way, the attachment becomes irreversible and the biocatalyst offers great stability. But the union agents are generally highly toxic against the microbial cells and induce a decrease of their activity. That is why this method of immobilization is no longer used for the immobilization of microbial cells, but it remains interesting for the immobilization of enzymes.

9.1.2.3 Inclusion

In this system the microbial cells are incorporated in the matrix of a more or less rigid polymer. These polymers are synthetic, such as polyacrylamide or cellophane, but they also can be made of proteins (gelatine, collagene) or polysaccharides (cellulose, alginate, agar, carragheenans, etc.). Inclusion is a technique that is easy to manage and it leads to products having good stability but weak mechanical resistance. In some cases this weakness may be a problem (mechanical stirring of the reactors, growth of microbial cells into the matrix leading to the breaking of the matrix) and could be a disadvantage for long-term continuous operation as discussed for brewing by Virkajarvi (2001). Also the polymer may be a limiting factor for the free

diffusion of the solutes or gases required (or produced) by the microorganism (Hannoun and Stephanopoulos 1986). It is considered that diffusion is not the limiting step for compounds having a molecular weight of less than 5,000. It is obvious that for use in the food industry the support has to be safe for the consumer (stable and non-toxic support) and is allowed by the regulation in force for the specific use considered. At this time immobilization by inclusion is the most widespread process and thus it is interesting to present the main supports used.

The main materials for inclusion of microbial cells are:

– κ-Carragheenan: This polysaccharide extracted from marine microalgae is commonly used as a food additive. It jellifies when the temperature reduces (room temperature) after the dissolution obtained by heat treatment (60–80°C). Different works dealt with the characterization of the optimal conditions to immobilize the cells into the gel and it was observed that the mechanical strength increased with increasing carragheenan concentration, corresponding to a decreased cell release. To avoid the cell release, which may be a major disadvantage in some cases, Nunez et al. (1990) proposed to treat the beads with $Al(NO_3)_3$; this treatment was shown to be efficient to induce gel hardening but it was observed that cell viability and diffusion were reduced. Recent examples of the use of carragheenan beads with 4.5×10^9 cells mL^{-1} in brewing were given by Mensour et al. (1996) and Pilkington et al. (1999).
– Agar and agarose: Agar is a polymer issued from some marine macroalgae and agarose is obtained from agar by separation and purification. The gelation is induced in the same way as for carragheenan. Even though the procedure to immobilize cells in this polymer is simple, it is not widely used owing to the low mechanical strength that makes this gel unstable compared with alginate or carragheenan.
– Chitosan gel: This polysaccharide is obtained from chitin extracted from crustacean cells. Its gelation occurs by an ionotropic reaction like alginate. It was used quite early but it appeared that acid-soluble chitosan affected cell viability and so that it is not used for cell entrapment, except in some cases to coat alginate beads to avoid cell release.
– Poly(vinyl alcohol) (PVA): PVA can form a gel when treated with UV radiation but this causes cell death. Also it is possible to make a gel by treatment with boric acid, but this acid may be toxic for some microorganisms. It is also possible to induce gelation by some freeze–thaw cycles, and recently Martynenko et al. (2004) proposed a process for champagnizing involving the use of champagne yeasts immobilized into PVA cryogels (PVACs). At this time this polymer does not appear to be used much for cell entrapment.
– Calcium alginate gel: Alginic acid is a complex heteropolysaccharide extracted from some species of algae. Its gelation is obtained by contact with a calcium solution. Its composition varies a lot depending on the source, and the nature of the alginate must be well defined when making the beads. Indeed the rheological properties of the gel depend on the composition of the alginate (sequences and ratios of L-glucuronic acid and D-mannuronic acid; arrangement of the monomers).

At this time, to our knowledge, this polymer is the one most commonly used for cell entrapment in the food industry: for example, calcium alginate beads

have been retained with a capacity of 1.2×10^9 cells mL^{-1} of gel beads (Linko and Linko 1981; Hsu and Bernstein 1985; Patkova et al. 2002). Indeed, this polymer is already allowed by the regulations for some applications in the food industry, such as a binding or thickening material, and also has good mechanical properties. The method is as follows. A solution containing yeast cells and alginate is driven to a solution of calcium chloride. A mechanical device allows the continuous film to be separated into droplets. As soon as the alginate meets the calcium chloride solution it gels quickly giving small spheres (1.5–2-mm diameter) called beads. In these beads the yeast cells are held prisoner in the frame of the alginate gel. The first works dealing with the use of these kinds of beads rapidly showed that the cells entrapped at the surface of the beads were able to multiply and thus to release free cells into the medium. In order to avoid this phenomenon, it was proposed (1980s) to make an external layer of sterile alginate using two concentric pipes. The internal pipe brings the alginate–yeast cell solution, while the external pipe brings the solution of sterile alginate. The critical points are the regularity of the spheres, the continuity of the external layer and the firmness of the gel (depending on the nature and the concentration of the alginate and the residence time in the calcium chloride solution). It is undoubtedly the method of inclusion which presents the most advanced industrial applications, particularly for some steps in winemaking. Recently a method to obtain dry double-layered beads was developed by Proenol (Portugal) and so it is possible to find on the market an industrial product which is easy to carry, with a long storage life (2 years) and easy to use (no problem of adhesion of beads to the walls of pipes or bottles as was the case with the wet beads).

9.1.2.4 Cell Retention Without External Support

It is also possible to increase the microbial cell concentration by using a natural process such as flocculation or by confining the cells to a part of the reactor by way of a membrane. Flocculation is a natural phenomenon resulting in cell aggregation. It involves the setting up of ionic bonds between sites of cell wall and cations of the medium. In some cases, these ionic bonds are strengthened by the production of filaments at the surface of the yeast cells (Teixeira 1988). But not all the cells are able to flocculate and also natural aggregates are often unstable and sensitive to the shear. Nevertheless, this spontaneous mechanism of flocculation is used in the waste-treatment process (activated sludge).

In the field of fermentations for the food industry the main applications at this time concern alcohol production (Zani-floc process in Brazil), some kinds of beers, and sparkling wine making (second fermentation). In the case of confinement, the free cells of the microorganism are kept in a part of the reactor thanks to a membrane or are retained inside a hollow fibre. This device makes it possible to reach a very high cell concentration (more than 100 g dry weight L^{-1}). For sure the membrane is needed to be freely permeable to solutes and gases. The great interest in this process is that it leads to a sterile medium at the end of the fermentation step. Different bench tests were made for alcoholic beverage production using a device

coupling fermentation and ultrafiltration or using a specific hollow fibre device for sparkling wine making (Jallerat et al. 1993) but to our knowledge none of them are being used at the industrial level.

9.1.3 Impact of Immobilization on Cell Physiology and Fermentation Activity

The possible effects of immobilization on cell activity were studied early on and the different authors reported very different conclusions. In fact, physiological reactions of cells vary depending on the method of immobilization used. For example, it is clear that the entrapment or adsorption of cells results in changes in their micro-environment and thus affects their metabolism. Anyway, in all the cases of cell immobilization, the main factor which likely influences the cell behaviour is the mass transfer limitation (Onaka et al. 1985) as it results in gradients of oxygen, substrates and products. Thus, immobilization may influence the cell physiology and activity via a lot of mechanisms which are still poorly characterized and which may act in opposite ways. For sure the effects depend on the immobilized complex size and the type and the concentration of the polymer or the matrix used for the entrapment or the adsorption.

9.1.3.1 Mass Transfer Limitations and Cell Physiology

Different studies led to different conclusions so it is quite difficult to have a clear view. As far as entrapped yeast cells are concerned, it appeared from different studies quoted by Groboillot et al. (1994) that the size of the beads may act as well as the alginate molecular weight, the ratio between guluronic and mannuronic acids (G/M) and the alginate concentration, and, for example, it was observed that the ethanol productivity of immobilized yeast cells increased when the alginate concentration or G/M decreased. This was explained by the fact that a weak gel probably facilitates the transport of substrate and product, thus enhancing the cell activity. It was also observed that mass transfer limitations may be due to cell concentration (or growth). Nevertheless, it seems well accepted by many authors quoted by Martynenko and Gracheva (2003) that respiratory and fermenting activities are higher in immobilized cells than in free cells.

But the use of an immobilized cell system may also be an easy way to reduce glucose inhibition. In most fermentation processes the diffusional limitations are generally recognized as a major disadvantage since they reduce the glucose uptake, but they may also be beneficial for the uptake of other sugars which are repressed by the glucose level. This was proved by Willaert (1999): studying the brewing by immobilized cells in calcium alginate, he established that the glucose concentration was high in the outer layer of the gel matrix but went to zero in the core of the gel. The cells which were located on the surface of the gel consumed most of the available glucose and the maltose (or maltotriose) uptake was repressed because of the glucose content. In contrast, cells located in the core were not repressed by glucose and thus maltose uptake was not repressed. The author suggested that the same mechanism may act for amino acid uptake.

9.1.3.2 Effect of Immobilization on Cell Morphology

Many changes in the morphology of yeast cells entrapped in calcium alginate were described by different authors. The well-documented review of Martynenko and Gracheva (2003) quotes studies dealing with the physiological activity and morphological changes of immobilized cells in the special case of the Champagne process. It was said that the adaptation of yeast cells to immobilization was accompanied by vacuolization. Also the thickness of the cell wall increased and ribosomes became scarcely visible. It was also shown that the specific rate of cell division was very low: the yeasts proliferated at the periphery of the bead, while the number of cells in the core remained constant.

9.1.3.3 Effect of Immobilization on Cell Physiology

Fumi et al. (1994) established that immobilized cells of *Saccharomyces cerevisiae* in alginate beads showed some alterations during alcoholic fermentation: they observed that the percentage of phosphomonoesters with respect to total phosphorous increased from 1.8% for free cells to 30.8% for immobilized ones and that the polyphosphates contents were, respectively, 56.7 and 22.6%. Grego et al. (1994) noticed that the immobilized cells of *S. cerevisiae* exhibited a slightly increased ethanol stress resistance and explained it by the impact of the ethanol stress on the fatty acid composition being smaller in the immobilized cells than in the free cells. For the enzyme activity Sarishvili and Kardash (1980) noticed that yeast cells immobilized on solid supports exhibited a greater activity of some enzymes (NAD or NADP-dependent glutamate dehydrogenase, alcohol dehydrogenase and malate dehydrogenase) than suspended cells and thus they suggested that a process using immobilized cells could be carried out at lower temperatures and shorter times.

9.1.3.4 Immobilization and Effects on the Product

Concerning the composition of the product after the use of immobilized cells it is generally assumed that there are not great differences with the product obtained with suspended cells (Busova et al. 1994). Jallerat et al. (1993) compared the second in-bottle fermentation using free cells or cells retained in a hollow fibre cartridge: they did not note any difference in the time needed for the fermentation and in the organoleptic qualities of the wines. Recently Tsakiris et al. (2004) assumed that the wines obtained using yeast cells immobilized onto raisin berries had the same aromatic profiles as the wines obtained using free cells even though, according to Balli et al. (2003), the glycerol content was slightly higher in wines obtained with immobilized cells on delignified cellulosic material and gluten pellets than in wines obtained with free cells. Studying the fermentation of white wines using different sorts of immobilized yeast cells, Yajima and Yokotsuka (2001) established that the concentrations of undesirable products (methyl alcohol, ethyl acetate, etc.) were lower in wines made using immobilized cells (in alginate beads) than in wines produced with free cells. But Bardi et al. (1997b) observed that the immobilization of yeast cells on delignified cellulosic material or gluten pellets led to a higher production

of ethyl acetate (compared with free cells). The same behaviour (greater ethyl acetate production) was observed with yeast cells immobilized on a kissiris support (Bakoyianis et al. 1993). So it seems obvious that it is difficult to draw a strong conclusion and that the effects of the immobilization on sensory evaluation of the product depend on the field of application and on the sort immobilization process. As an example of this we can quote the work of Ageeva et al. (1985): they noticed that yeast cells immobilized on different clay materials did not behave the same in regard to the volatile product synthesis.

9.2 Immobilized Yeast Cells and Winemaking

In winemaking different possibilities for the use of immobilized yeast cells have been described. Surely this area of application is the one where most work has been done. To analyse these data we can classify them according to the step in winemaking where the immobilized cells act: demalication of must (or wine), alcoholic fermentation and treatment of sluggish or stuck fermentations, in-bottle fermentation in sparkling wine making.

9.2.1 Demalication of Musts or Wines

L-Malic acid is one of the two main acids in musts and its concentration depends on grape variety and climatic conditions. Winemakers often rely on malolactic fermentation (MLF) to deacidify the must and thus to achieve the biological stability of the wine as well as to ensure good organoleptic qualities. MLF is performed by lactic acid bacteria (*Oenococcus oeni*) and many factors such as low pH and sulphur dioxide level could affect these bacteria and, in some cases, this MLF becomes impossible. The yeast *Schizosaccharomyces pombe* has been proposed as an alternative to MLF but it was quickly proved that a too important development of these yeasts leads to some off-flavours. So, some wineries have set up a two-step process: first the must is inoculated with *Schiz. pombe* for the consumption of L-malic acid and in a second step the must is inoculated with a selected strain of *Saccharomyces* in order to achieve the alcoholic fermentation. However, it appeared that the complete elimination of free cells of *Schiz. pombe* was not possible and that the risk of obtaining some off-flavours was always present. So, a process based on the use of immobilized cells of *Schiz. pombe* has been proposed. The first works of Magyar et al. (1987) established clearly the feasibility of the process using cells of *Schiz. pombe* entrapped in alginate beads. Later, Taillandier et al. (1991) and Ciani (1995) analysed a continuous process using immobilized cells of *Schiz. pombe*. But all these experiments were made at a laboratory or pilot scale owing to the impossibility to obtain and to store great quantities of entrapped cells of *Schiz. pombe*. More recently Silva et al. (2002b, 2003) described experiments using cells entrapped in dried double-layered alginate beads (see Sect. 9.1) on a laboratory scale as well on a winery scale. The beads were placed into nylon bags and these bags were poured in the fermentation tank and shaken daily to agitate the cells and improve the diffusion of solutes and the release of carbon dioxide. To stop the deacidification reaction at the desired level it was enough to remove the bags from the tank and to add to the tank the suitable

strain of *S. cerevisiae* to achieve the alcoholic fermentation. It was shown that the process was efficient and led to wines of good quality. Also the reuse of these beads was studied and it was shown that they maintained good activity for at least five cycles. The process developed by the Portuguese company Proenol to produce these beads of entrapped cells of yeasts makes it possible to store them for more than 2 years without any loss of activity. Thus, it can be concluded that this process is now well established and ready to be used in wineries. Some experiments were done using these entrapped cells of *Schiz. pombe* on wines (red or white) after alcoholic fermentation (unpublished data) and the first results were promising.

9.2.2 Alcoholic Fermentation

Many papers deal with the use of immobilized cells of yeasts (generally *S. cerevisiae*) to achieve the alcoholic fermentation of musts (red or white). The main purpose is always to ensure better control of this important step in winemaking: low-temperature fermentations, improvement of organoleptic characteristics, increase of reaction rates, good achievement of sugar consumption, etc.

Gorff (1988) patented a process using yeast cells immobilized on derivatized cellulose and later Divies et al. (1990) patented a process to entrap the yeast cells in calcium alginate beads. The same year Sarishvili et al. (1990) described a "technology for manufacture of dry red wines with immobilized yeast": the cells were immobilized on beech, oak or polyethylene and the authors observed that the quality of wines was improved. Malik et al. (1991) then tried ten different strains immobilized in alginate and noticed a reduction in their acidification potential compared with that of unbound cells. But most of the studies on this subject are due to the Department of the Chemical University of Patras (Greece). As early as 1992 Bakoyianis et al. (1992) published a paper dealing with the use of a psychrophilic and alcohol-resistant yeast strain immobilized on kissiris in a continuous process for making wine at low temperature. Later they showed (Argiriou et al. 1996) that this yeast strain was more efficient if some preservation treatments at 0°C were made. Bardi and Koutinas (1994) described experiments where different supports were tested as well as different conditions of fermentation: immobilization of cells on delignified cellulose and use of them in 55 repeated batch cultures at low (10°C) or room (30°C) temperature: the main result was that the fermentation rates are increased (threefold) compared with those for free cells. Also the stability of the biocatalyst was proven. Bakoyianis et al. (1998) using cells of *S. cerevisiae* immobilized on different supports (alumina, kissiris and alginate) compared the volatile by-products obtained at different temperatures in a continuous process. It was observed that the levels of 1-propanol, isobutyl alcohol and amyl alcohols were less than those synthesized by free cells for all supports and temperatures studied. But the most original studies from this group dealt with the immobilization of yeast cells onto supports such as apple pieces or raisin skins. Kourkoutas et al. (2001, 2002b) proposed using a psychrophilic and alcohol-resistant yeast strain immobilized on apple cuts for speeding up the fermentation. They noticed excellent taste and aroma of the wines produced and concluded that this process could be accepted by the industry for scaling up the winemaking process. Tsakiris et al. (2004) immobilized yeast cells

on dried raisin berries and obtained good stability of the device. The wines were not different from those obtained using free cells.

All these data show clearly that the alcoholic fermentation in winemaking may be realized without any damage by using immobilized cells of the suitable yeast strain, and a batch process as in the continuous process. But the main problem in developing these processes to an industrial scale is linked to the legislation for winemaking. At this time the sole support clearly allowed (for some applications) is calcium alginate gel. Also the continuous process is not allowed for all types of wine, but that is another problem.

9.2.3 Treatment of Stuck and Sluggish Fermentations

Sluggish and stuck fermentations are some of the most challenging problems that can occur during the winemaking process. The causes can be attributed to nutritional deficiencies of the must, the presence of high levels of inhibitory products, inadequate temperature, and residual toxic products. Although the causes are numerous, the main result is the decrease of cell growth, fermentative activity and viability of the yeast population. To try to reinitialize the fermentation an inoculation of activated yeast cells into the fermentation tank is usually carried out, but this procedure is not always efficient. Silva et al. (2002a) suggested the use of yeast cells immobilized into double-layered alginate beads. The results obtained on a laboratory scale as well as at the winery level showed very good efficiency of the device to treat the stuck fermentations. The great success of these immobilized cells can be explained by an adaptation of the cells to high concentrations of alcohol during the immobilization step.

9.2.4 Special Applications

9.2.4.1 Sweet Wine Making

Sweet wines are wines where the fermentation is stopped before the complete utilization of sugars. At this time to stop the fermentation activity of yeast cells in this kind of winemaking strong quantities of sulphur dioxide are employed, but this product has some disadvantages for consumer safety and the aim of the legislation is to reduce its content in wine. The use of immobilized cells was investigated by some researchers in order to find another solution for making sweet wines as it made it possible to stop the reaction by removing the particles (containing the cells) from the medium. Okuda et al. (2001) described an original process: the fermentation was carried out by immobilized yeast cells which were removed from the medium at the desired level of alcohol (or remaining sugar). In order to ensure the microbial stability of the wine they added an antimicrobial substance isolated from paprika seeds. In this way, they obtained a very stable sweet wine with no viable cells. Kourkoutas et al. (2004) suggested producing semi-sweet wines by using cells of *Kluyveromyces marxianus* immobilized on delignified cellulosic material, quince or apple pieces. The fermentation was run at high temperature and 3–4% of alcohol was synthesized. The final alcohol level was obtained by the addition of potable

alcohol to the fermented must. In these conditions it was said that the semi-sweet wine obtained showed good flavour and aroma and may be blended with other products to improve their quality. This idea to use immobilized yeast cells other than *Saccharomyces* was already developed by Crapisi et al. (1996). It was expected that the bad alcohol production activity of this kind of yeast and also its ability to produce aroma could lead to a partially fermented and aromatic beverage. It was established that it was effectively possible to carry out the fermentation by immobilized apiculate yeast species and that the wine bouquet was not affected by the use of these yeast species. Silva et al. (2002c) described a process to obtain sweet wines by using cells of *S. cerevisiae* entrapped in double-layered alginate beads and showed that it was a great way to make such wines. The process was tested in wineries. For sure the must had to be prepared in order to have a very small population of indigenous yeast cells. The beads containing the cells of the selected yeast strain were placed in nylon bags so that they were easy to remove from the tank after they had worked. When the desired level of remaining sugar was reached, the bags were taken out of the tank and the wine was stabilized to prevent the further development of free cells. As a conclusion, it appears clearly that the use of immobilized cells associated with a treatment of stabilization can be an efficient and easy way for making sweet wines with reduced sulphur dioxide contents.

9.2.4.2 Sparkling Wine Making

In sparkling wines according to the traditional method, which is the method used for the Champagne process, the problem is to eliminate yeast cells without taking the wine out of the bottle at the end of the in-bottle fermentation. Traditionally it is made by the so-called operation of *remuage*, which requires special know-how.

Thus, for more than 20 years, other solutions have been sought and tested and there is certainly the field of winemaking, where the possible use of immobilized cells has been investigated the most. For sure the simplest method would be to filter the wine from one bottle to another, but if this is done, the wine is not allowed to be called Champagne.

For the last 20 years, entrapped cells have been tested. All the first experiments were done using homogeneous alginate and cell beads. Obviously, the results were quite bad, for it was always observed that yeast cells were able to escape the bead and to grow in the medium, which resulted in a troubled wine. But as early as the 1990s double-layered alginate beads were tested (Zamorani et al. 1989; Crapisi et al. 1990; Godia et al. 1991). All the studies made clear that the use of entrapped cells in double-layered alginate beads led to a perfectly clear wine and that there was no difference with a wine obtained following the traditional method. Nevertheless it must be noted that these applications remained at a laboratory or a pilot scale, except for a quite large-scale application by Moët et Chandon (France). This was due to the difficulty to produce regular and easy-to-use alginate beads at an industrial level. As an example, using a laboratory apparatus we (in our group in 1992) were only able to make 500 g of wet beads per day. Moreover wet beads were difficult to place in the bottle as they stuck to the walls of pipes. Also a special machine was needed to put a constant and fixed quantity of the beads in the bottles. From this point of

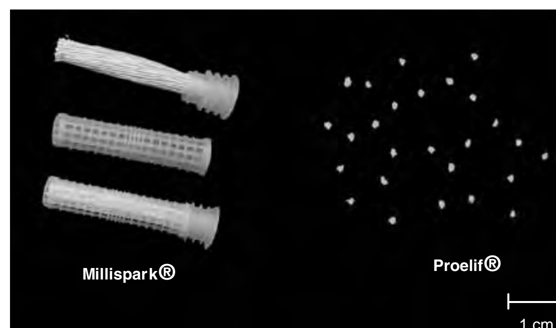

Fig. 9.1. The Millispark device (*left*) and dried double-layered beads (Proelif) (*right*)

view, great progress was made recently (1997) by Proenol (Portugal) and our laboratory working together. So, at this time we are able to produce more than 35 kg of dry beads per hour. These beads are dried so that they can be stored before use for more than 2 years without any loss of activity and also they are easy to put in the bottle thanks to a machine which was developed at the same time.

Another attempt was to place yeast cells in a sort of little cartridge with a membrane or some hollow fibres which separates the cells from the medium: the concept is like a tea bag. This device (called Millispark) developed by Millipore in 1993 (Jallerat et al. 1993) has proven to be very efficient but its development at the industrial level was not possible owing to a too high price.

Figure 9.1 illustrates the Millispark device and dried beads (Proelif) produced by Proenol.

9.3 Ethanol Production

More than 2×10^{10} L of pure alcohol is produced in the world each year and half of that is made in Brazil from sugar cane. It is without doubt the most important fermentation process, and as such the most studied. This alcohol, obtained from the distillation of different kinds of wines, is for the most part used for industrial purposes, such as additives for fuel, solvents for pharmaceutics or food ingredients. The wines to be distilled come from different substrates: sugar (sugar cane or beet root), hydrolysates of starch (maize, wheat or rice), sugar from industrial waste such as lactose from whey or sugars from biomass such as xylose, cellulose or hemicellulose. For sure the main challenge of the fermentation process is in this case to reach the best yield (alcohol produced/sugar used) as well as the highest reaction rates (in order to maximize the use of the fermentation tanks). In contrast to what we observed for the production of drinks, no attention is paid to the organoleptic quality of the wine. The most important thing is to avoid the synthesis of secondary products which can affect the yields and also the distillation process. For these different reasons it is obvious that a lot of studies have been made for 20 years in order to increase the yields, the reaction rates and to minimize the operational costs.

Among these studies a lot deal with the use of immobilized cells. Different processes using different kind of substrates, different kinds of yeasts and also different kinds of apparatus have been described and we will focus here on the most familiar ones or on those developed at the industrial level.

9.3.1 Alcohol from Sugar (Sucrose)

Saccharose (or sucrose) is the main component of sugar cane or sugar beet root. It is extracted from the plant by grinding and water diffusion and the medium obtained contains 120–140 g L^{-1} of sugar. It appears well established that the immobilized cells are more efficient than the free ones: Sree et al. (2000) using a repeated batch fermentation system (*S. cerevisiae* immobilized in alginate beads) noticed that more ethanol was produced by immobilized cells compared with free cells. The maximum amount of ethanol produced by immobilized VS3 cells using 150, 200 and 250 g L^{-1} glucose was 72.5, 93 and 87 g L^{-1} ethanol at 30°C. Using immobilized yeast cells some authors compared the method of immobilization as well as the efficiency of the reaction according to the process used. For example, we can quote the work of Goksungur and Zorlu (2001): they compared the continuous production of ethanol from beet molasses by calcium alginate immobilized *S. cerevisiae* in a packed-bed bioreactor to that obtained in a continuous stirred reactor. They showed that (with a temperature of 30°C and a dilution rate of 0.22 h^{-1}) maximum ethanol (4.62% v/v), yield (0.43 g g^{-1}) and volumetric productivity (10.16 g L^{-1} h^{-1}) were obtained from the beet molasses medium containing 10.90% (w/v) total sugar with 2.0–2.4-mm diameter beads prepared from 2% (w/v) sodium alginate solution. At higher substrate concentrations, substrate was recirculated through the packed-bed bioreactor to increase yields and to decrease residual sugar content. The bioreactor system was operated at a constant dilution rate of 0.22 h^{-1} for 25 days without loss of capacity. In the continuous stirred bioreactor (compared with the packed-bed bioreactor) lower ethanol concentration (3.94% v/v), yield (0.36 g g^{-1}) and productivity (8.67 g L^{-1} h^{-1}) were obtained. Dealing with the continuous fermentation of sugar cane syrup using immobilized yeast cells (*Saccharomyces* sp.) onto chrysotile (fibrous magnesium silicate) in a packed-bed reactor, Wendhausen et al. (2001) showed that the activity of the cells was higher when immobilized, mainly for fermentation of 30–50% w/v glucose solutions. In medium containing 30% w/v glucose, the initial fermentation rate increased 1.2–2.5 times. The yields were in the range 0.41–0.49 g g^{-1} for the immobilized cells and 0.37–0.43 g g^{-1} for the free cells. An average productivity of 20–25 g L^{-1} h^{-1} was obtained in the first 20 days and an average of 16 g L^{-1} h^{-1} was obtained after 50 days of operation. In order to increase the efficiency of immobilized cells, Nagashima et al. (1983) suggested adding some ergosterol and oleic acid to the alginate matrix. In this way they were able to increase the ethanol content of the medium to 57 g L^{-1} instead of 47 g L^{-1} in the same operating conditions but without sterol addition. An example of the use of immobilized cells for ethanol production from molasses on an industrial scale was given by Shi et al. (1995): yeast cells, suspended in the low concentration sodium alginate solution, were immobilized on the fluffy chemical fibre matrix to initiate the associated immobilization. Under factory conditions (four fermentors of 6.5 m^3), the ethanol

production was carried out continuously for 99 days by flowing diluted molasses (16.5–18% w/v sugar), resulting in an ethanol productivity of 6.21 g L^{-1} h^{-1} and an average ethanol concentration in the fermented mash of 9.44% (v/v). It was also proved by Murakami and Kakemoto (2000) that sodium alginate was a better support than κ-carragheenan gel because of its better mechanical strength.

But a natural phenomenon such as flocculation was also used at the industrial level in order to increase the efficiency of the process: Xie et al. (1999) described an industrial plant composed of four air-lift suspended-bed bioreactors in parallel with a total volume of 400 m^3 using cells able to self-flocculate. The process ran for more than 6 months in continuous operation: the effluent contained 70–80 g L^{-1} of ethanol and less than 5 g L^{-1} of residual sugar and an ethanol productivity of 7–8 g L^{-1} h^{-1} was achieved (to be compared with 2–4 g L^{-1} h^{-1} usually observed in classical process).

Studies were also made to appreciate the possible use of yeasts other than *Saccharomyces* and also different immobilization matrices: for example, Gough et al. (1998) analysed the production of ethanol from molasses (140 g L^{-1} sugar) at 45°C using a *Kluyveromyces marxianus* strain immobilized in calcium alginate gels and PVAC. The immobilized cells were used as a biocatalyst in fed-batch reactor systems for prolonged periods. When each system was operated on a fed-batch basis for a prolonged period of time, the average ethanol concentrations produced in the alginate- and the PVAC-immobilized systems were 21 and 45 g L^{-1}, respectively, while the yields remained high (0.41–0.45 g g^{-1}). The results suggested that the PVAC-based immobilization system might provide a more practical alternative to alginate for the production of ethanol by *K. marxianus* IMB3 in continuous or semi-continuous fermentation systems. Love et al. (1998) also tried a mixed matrix made of alginate and kissiris with the same yeast strain and reported good efficiency of the system.

If alginate seems nowadays the most used matrix for immobilization of yeast cells for alcoholic fermentation it must be noted that some different matrices were reported as more efficient. Zhang et al. (1996) suggested that the properties of the ceramic supports compared with those of a calcium alginate gel indicated that the ceramics were the better of the two types of material and had potential for industrial application. Harris and Ghandimathi (1998) immobilized yeast cells of *S. cerevisiae* in a natural rubber coagulum and used them for repeated batch fermentation of molasses. The authors pointed out the fair stability and efficiency of the process and that rubber was inert compared with alginate.

However, the use of a reactor with a very high level of immobilized cells may also have some negative effects and, for example, Yadav et al. (1996) observed that the productivity and the efficiency of a column reactor packed with gel beads might be affected by problems due to gas hold-up and mass transfer effects.

9.3.2 Alcohol Production from Lactose

Lactose is a sugar which may cause environmental damage as it is a major component of whey (waste from the dairy industry). Thus, its use as a substrate for alcohol production was studied early. As *Saccharomyces* sp. are not able to use this substrate the main yeast species used belongs to the genus *Kluyveromyces*. Marwaha

and Kennedy (1985) described a process for the continuous alcohol production from whey permeate using immobilized cell reactor systems. In this process a bioreactor packed with alginate-entrapped *K. marxianus* NCYC179 was used for continuous fermentation of whey permeate to ethanol. A maximum ethanol productivity of 28 g L^{-1} h^{-1} was attained at a dilution rate of 0.42 h^{-1} and 75% lactose consumption (substrate feed rate in the inflowing medium was 200 g L^{-1} lactose). The immobilized cell bioreactor system was operated continuously at a dilution rate of 0.15 h^{-1} for 562 h without any significant change in the efficiency and viability of the entrapped yeast cells (84–81%). More recently, El-Batal et al. (2000) made experiments on whey fermentation by *Kluyveromces* immobilized cells in copolymer carriers produced by radiation polymerization. In this study, yeast cells were immobilized in hydrogel copolymer carriers composed of PVA with various hydrophilic monomers, using a radiation copolymerization technique. Yeast cells were immobilized through adhesion and multiplication of yeast cells themselves by using batch fermentation; the ethanol production was 32.9 g L^{-1}, which was about 4 times higher than that of cells in the free system. Hydrogel copolymer carriers were used in a packed-bed column reactor for the continuous production of ethanol from lactose at different concentrations (50, 100, 150 g L^{-1}). For all lactose feed concentrations, an increase in dilution rates from 0.1 to 0.3 h^{-1} lowered the ethanol concentration in fermented broth, but the volumetric ethanol productivity and the volumetric lactose uptake rate were improved. The fermentation efficiency was lowered with the increase in dilution rate and also at higher lactose concentration in the feed medium, and a maximum of 70.2% was obtained at the lowest lactose concentration, 50 g L^{-1}. More recently, an industrial-scale pilot plant (11,000 L) using kefir yeast immobilized on delignified cellulosic material was described by Athanasiadis et al. (2003): the system showed good operational stability, exhibiting relatively high ethanol yield and ethanol productivity.

9.3.3 Ethanol Production from Starch

Starch is a very abundant substrate but its direct assimilation by yeasts is generally unlikely and thus some pretreatment is often necessary. To avoid this step it is possible to use a specific yeast such as *S. diastaticus* and a process using such a yeast immobilized on wood chip particles was recently described by Razmovski (2000). But it is also possible to use immobilized cells of a good fermenting yeast (*S. cerevisiae*) and immobilized enzymes (glucoamylase) as done by Chithra and Baradarajan (1992) and Giordano et al. (2000): cells and enzyme may be immobilized in separate particles or together in the same particles. However, it appeared that the productivities of these processes were smaller than those for fermentation of glucose-containing solutions.

9.3.4 Ethanol from Other Substrates

Among the substrates having some interest for the production of alcohol, great interest has been devoted to cellulose, hemicellulose and pentoses (xylose). The yeast *Pichia stipitis* appeared as a good species to ferment xylose solutions or hemicellulose

hydrolysates, (Sanroman et al 1994; Liu et al. 2001). *Candida sheateae* was also investigated (Hinfray et al. 1995) and also *Pachysolen tannophilus* (Amin et al. 1988). Chen and Weyman (1989) described a system able to use cellulose directly. In this process, baker's yeast cells were entrapped on glass fibre disks by means of alginate, and the enzymes cellulase and β-glucosidase were precipitated on the yeast cells by tannin. The disks carrying the yeast–enzyme co-immobilizate were installed in a continuous dynamic immobilized bioreactor. Cellulose was added continuously to the bioreactor. In the first few days, the efficiency of the system was good but decreased over the next 5 days to 40%, likely owing to the negative effect of tannin. On this subject of the direct use of cellulose a comprehensive review was made by Chandrakant and Bisaria (1998).

The alcoholic fermentation at a pilot scale from dried sweet potato was investigated by Yu et al. (1994), while Roukas (1994) was interested in the use of carob pod extract as a substrate for the alcoholic fermentation by immobilized cells of *S. cerevisiae* in alginate beads. An interesting substrate may be the Jerusalem artichoke. In this plant the reserves are made of inulin, a polymer of fructose. This polymer may be directly hydrolysed and fermented by *K. marxianus*, and a process using cells immobilized in calcium alginate beads was proposed by Bajpai and Margaritis (1986): the bioreactor was continuously operated with good results (volumetric ethanol productivity of 118 g L^{-1} h^{-1} at a dilution rate of 2.8 h^{-1} and 87% substrate conversion) and its half life was 105 days.

9.3.5 Immobilized Cells and Processes

Because of its economic importance, ethanol production has initiated a lot of studies dealing with the development of specific processes based on the use of immobilized cells.

In order to improve the efficiency of immobilized cell systems, different processes for the fermentation have been analysed for many years. Feng et al. (1989) analysed a continuous fermentation process using *Schiz. pombe* yeast flocs: a suspended-bed bioreactor utilizing air was employed in which the total yeast particles were retained and was allowed to operate over 3 months without interruption. The yeast cell concentration was held at 40 g L^{-1} (dry weight) and a high productivity of 20–24 g L^{-1} h^{-1} was obtained. These *Schizosaccharomyces* yeast flocs may also be used in an immobilized cell reactor separator (ICRS) as described by Dale et al. (1994): an ICRS with gas-phase ethanol product stripping was operated with both sucrose and molasses feeds continuously over 90 days. The feed concentration range was 300–600 g L^{-1}. Using *Saccharomyces* cells, Del Borghi et al. (1985) described a process called rotating biological surface (RBS): a spongy material was employed to trap yeast cells on the disks. In this way, an ethanol productivity of 7.1 g L^{-1} h^{-1} was achieved in the RBS-1CR at a dilution rate of 0.3 h^{-1}.

Many authors developed some apparatus making possible simultaneous bioreaction and separation by a so-called immobilized yeast membrane reactor. Vasudevan et al. (1987) designed a fermentor in which the microbial cells were sandwiched between an ultrafiltration membrane and a reverse osmosis membrane. The ultrafiltration membrane provided free passage for all nutrients which were supplied under

pressure, eliminating diffusional resistance. The reverse osmosis membrane preferentially allowed passage of the product, improving purity and concentration. Ethanol fermentations with *S. cerevisiae* were carried out for 160 h using this reactor with good performances. However, Woehrer (1989) analysed the continuous ethanol production in a three-stage horizontal tank bioreactor (HTR) by yeast cells entrapped in calcium alginate and concluded that "Compared to other continuous ethanol production processes using entrapped yeast cells, the HTR is among the best", and in the same year Shukla et al. (1989) described a novel microporous hollow fibre membrane-based immobilization technique for whole cells making it possible to reach a productivity of 41 g L^{-1} h^{-1} with an initial glucose concentration of 100 g L^{-1} and a yield of 0.45 g g^{-1}. But it is also possible to associate the immobilized yeast cells and a device allowing the elimination of the alcohol (which is a possible inhibitor for the yeast activity). That was done by Shabtai et al. (1991): they developed a system comprising an immobilized yeast reactor producing ethanol, with a membrane pervaporation module for continuously removing and concentrating the ethanol produced. The combined system consisted of two integrated circulation loops: in one, the sugar-containing medium was fed and circulated through a segmented immobilized yeast reactor (the bead matrix was a cross-linked polyacrylamide hydrazide gel coated with calcium alginate), in the other, ethanol-containing medium was circulated through the membrane pervaporation module. Long-term continuous operation (over 40 days) was achieved with a productivity of 20–30 g L^{-1} h^{-1}. As in some cases a possible limitation or inhibition due to a lack of diffusion or to limited escape of carbon dioxide inside the bed of immobilized cells may affect the efficiency of the process, the use of fluidized-bed bioreactors (Busche et al. 1992) or trickled-bed reactors (Jamuna and Ramakrishna 1992) was studied. Ogbonna et al. (2001) presented a study dealing with the scale-up of fuel ethanol production from sugar beet juice using a loofa sponge immobilized bioreactor. They concluded that "by using external loop bioreactor to immobilize the cells (here a flocculent strain of *S. cerevisiae*) uniformly on the loofa sponge beds, efficient large scale ethanol production systems can be constructed". But to our knowledge none of these systems are running on an industrial scale.

Another interesting approach to overcome some technical problems was to use immobilized systems made of a strain of microorganism and something else, such as another strain or species or an enzyme. A study by Andreoni et al. (1983) suggested the utilization of immobilized β-glucosidase enzyme and immobilized growing yeast cells in the ethanol production from municipal solid wastes, and Amin et al. (1983) conducted experiments on the co-immobilization of *S. bayanus* and *Zymomonas mobilis*. A new immobilized biocatalyst called Maxaferm was described later by Noordam et al. (1995) for the continuous production of ethanol from dextrins: the Maxaferm system has been developed for the co-immobilization of enzymes and microorganisms (in this case amyloglucosidase and *S. cerevisiae*).

Recently, Amutha and Gunasekaran (2001) studied the production of ethanol from liquefied cassava starch using co-immobilized cells of *Z. mobilis* and *S. diastaticus*. They noted that the concentration of ethanol produced by immobilized cells was higher than that by free cells of *S. diastaticus* and *Z. mobilis* in mixed-culture fermentation and that in repeated-batch fermentation using co-immobilized cells, the ethanol

concentration increased to 53.5 g L^{-1}. Also, the co-immobilized gel beads were stable up to seven successive batches. Continuous fermentation using co-immobilized cells in a packed-bed column reactor exhibited an ethanol productivity of 8.9 g L^{-1} h^{-1}. An original use of immobilized yeast cells and free cells of a fungus was recently studied by Farid et al. (2002): in this paper the authors analysed the alcohol production from starch by mixed cultures of *Aspergillus awamori* and immobilized *S. cerevisiae*. They concluded that repeated batch by this co-culture were successfully used 12 times without a significant loss in alcohol production.

But the co-immobilization of different species of yeasts did not always lead to a better efficiency as shown by Lebeau et al. (1996) when investigating the continuous alcoholic fermentation of a mixture of glucose (35 g L^{-1}) and xylose (15 g L^{-1}) by *S. cerevisiae* and *C. shehatae* co-immobilized in a double-chambered bioreactor.

9.4 Brewing

In the brewing process, fermentation is made up of two steps: the first step or main fermentation consists in the conversion of most of the fermentable sugars into ethanol by a growing and abundant yeast population; in the second step or maturation, the main objective is to decrease the concentration of diacetyl below the taste threshold for organoleptic reasons, diacetyl being a secondary product of main fermentation. A continuous process would be the most interesting from the economic point of view because of the shortened fermentation time, but the major difficulty lies in keeping a biological system aseptic for a relatively long period. However continuous fermentation of beer has long been attractive since as early as the end of the nineteenth century (Virkajarvi and Linko 1999). The emergence of immobilization technology in the 1970s boosted research on the continuous processes. Huge numbers of papers and patents on this subject have been published in the last 30 years. Among them very few led to industrial applications except for beer maturation or alcohol-free beer production. Nevertheless the feasibility of many proposed processes has been demonstrated at the laboratory or pilot scale (Virkajarvi 2001). The main supports used in the brewing process were discussed in Sect. 9.1.2.

9.4.1 Examples of Proposed System Configurations

Because of the different characteristics of primary and secondary fermentations most of the processes either concerned only one of the two fermentations or have several serial reactors (multistage processes). The most difficult fermentation to manage is the primary one, which is more vigorous, and many critical points have to been solved technically: removal of excess biomass and CO_2, sustaining yeast viability, optimization of oxygen feeding, prevention of clogging the reactor, high residence time and prevention of microbial contamination in a continuous run.

9.4.1.1 Main Fermentation

For the main fermentation high amounts of fermentative yeasts are needed. Very few studies have dealt with batch fermentation. Hsu and Bernstein (1985) modified a

conventional fermenting vessel with two screens that hold alginate beads containing fermenting yeast in the vessel. The whole process lasted 7 days but the organoleptic characteristics of the beer were slightly different. Other authors proposed recycling the same biocatalyst 20 (Ju et al. 1986) to 42 times (Pardonova et al. 1982) or for 3 months (Nedovic et al. 1993), shortening the fermentation time to 12–16 h.

In order to get high productivity a continuous process is much more interesting at the industrial level. As early as 1966 some attempts were made at continuous main fermentation by mixing diatomaceous earth and yeasts and passing malt wort through a kieselghur filter with a residence time of 2.5 h, but the bioreactor had a lifetime of only 7 days before clogging. This system was then improved by Baker and Kirsop in 1973 (Virkajarvi 2001) by operating in a tubular reactor containing the mixture, resulting in an increased lifetime, but it still remained insufficient. Moreover, to achieve satisfactory flavour formation and organoleptic qualities a plug flow reactor, packed or fluidized bed, seemed to be more suitable (Yamauchi et al. 1995). An alternative should be a series of continuous stirred-tank reactors (Linko et al. 1998). In these conditions, immobilization by entrapment always led to swelling of the carrier, preventing long-term operation, and immobilization by adsorption was preferred.

Studies on packed-bed reactors seem to be more extensive with different kinds of carriers: ceramics, glass beads, calcium alginate beads, DEAE-cellulose or wood chips. Shindo et al. (1994) experimented with chitosan beads in a fluidized-bed reactor and by optimizing the recycling flow rate in the reactor obtained a life time of 900 h at a flow rate of 40 mL h^{-1}. Later (Pajunen et al. 2000) this kind of system was extrapolated at the pilot scale with a flow rate of 28 L day^{-1} for 50 days and a bed volume of 1,000 L. A similar process was investigated with alginate beads (Wang et al. 1989): 40% (v/v) beads were used and the ratio of recirculation was 5, giving a fermentation time of 14 h.

Another possible technology would be a loop bioreactor containing a tubular matrix carrier made of silicon carbide (Meura-Delta process, see later; (Virkarjarvi and Linko 1999) or one layer of metal fibres which had been sintered. Alternatively the carrier may be a sintered silicon carbide carrier in a multichannel loop reactor design or may comprise several tubes placed concentrically around each other (Arnaut et al. 2001). Complete attenuation was then achieved in a continuous two-stage bioreactor with a hold-up vessel arranged in series. The total residence time including the time in the hold-up vessel was 2.5 days (van de Winkel et al. 1993).

To achieve the high yeast concentration and activity needed in the first fermentation optimal oxygen feeding is necessary. This can be realized using an air-lift or bubble column bioreactor. This was suggested in several works using various carriers such as alginate (Leskosek-Cukalovic and Nedovic 2002) κ-carragheenan (Mensour et al. 1996; Pilkington et al. 1999) or calcium pectinate beads (Yamauchi et al. 1995; Smogrovicova et al. 1998), spent malt grains (Branyik et al. 2004) and DEAE-cellulose (Branyik et al. 2001). As an example, Labbat breweries in Canada produced beer in a 50-L gas-lift reactor containing yeasts entrapped in carragheenan beads with air in the proportion of 2–5% and with a residence time of 20 h (Mensour et al. 1996). With yeasts immobilized in pectate beads at a ratio of 25% (v/v) in the air-lift the residence time was 13 h.

Another strategy consists in using a multistep system, only one step being an aerated reactor: continuous stirred tank or air-lift. Several configurations have been tested: a fluidized-bed reactor and air-lift with a residence time of 12 h (Smogrovicova et al. 1997); a two-stage packed-bed system (Kronlof et al. 1996), in this case the first stage was aerated and considered as a prefermentor, giving a global production of 60–130 L day^{-1} with a residence time of 20–40 h; an aerated continuous stirred-tank reactor followed by a fluidized-bed reactor (Yamauchi and Kashihara 1996).

9.4.1.2 Secondary Fermentation (Maturation)

The use of immobilized yeasts allows the duration of this step to be shortened, compensating the low reaction rates by high catalyst concentrations. In the case where the main fermentation is continuous (with free or immobilized yeasts) the secondary fermentation is run in one or two reactors in series. Several processes have been run at the industrial level with 7–50-m^3 reactors allowing the duration to be reduced from several weeks in the traditional process to 2–3 h. Two kinds of carrier were used for adsorption. The first one, since 1990, was DEAE-cellulose (Pajunen 1996) in an installation with an annual capacity of 1×10^8 L. The continuous production cycles varied between 2 and 8 months before regeneration. The second one was porous glass beads in two-stage column reactors (Virkajarvi and Linko 1999).

9.4.1.3 Examples of Integrated Processes at Laboratory, Pilot or Industrial Scale

A very attractive design for the brewing process would be an integrated one making it possible to carry out both fermentations in a multistage continuous system. At the laboratory level a three-stage packed-bed reactor containing yeast adsorbed on glass beads had been proposed (Yamauchi et al. 1994). A two-stage system has been investigated (Smogrovicova et al. 1999): primary wort fermentation was conducted in a gas-lift reactor using calcium alginate (residence time 12 h), secondary fermentation was conduced in two parallel packed-bed systems using calcium alginate (residence time 57 h) or gluten (residence time 61 h) for yeast immobilization. Another two-step process could consist of a short aerobic prefermentation of the wort in a continuous stirred-tank reactor followed by a packed reactor filled with calcium alginate beads containing yeast cells. In such a configuration no further maturation is needed (Nakanishi et al. 1985).

Finally in spite of numerous studies and trials, beer production using immobilized yeasts has kept the brewing world waiting for a breakthrough, mainly owing to difficulties in controlling the hydrodynamic and temperature stability of the reactor for a long time and only three processes have reached industrial development.

The first one is the Kirin process in Japan developed since the mid-1980s and exploited for about 10 years producing 185,000 L per year (Virkajarvi 2001). It consists in a three-stage system (four reactors) with yeast adsorbed on porous glass beads (Fig. 9.2): the first stage is an aerated stirred-tank reactor for free yeast growth; the second step is made of two packed beds in series with immobilized yeast for the main fermentation; the last step after heat-treatment is also a packed bed for green beer maturation.

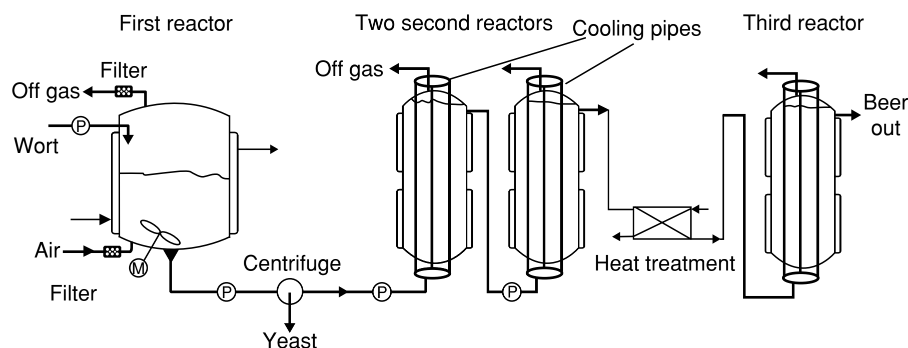

Fig. 9.2. The Kirin process. (Redrawn from Yamauchi et al. 1994)

The second potential industrial process was developed by the VTT technical research centre of Finland (Virkajarvi 2001). It consists of a slightly aerated prefermentor, a buffer tank and a main fermentor (packed-bed reactor containing glass beads); after heat-treatment the green beer enters a second packed-bed reactor for the maturation. The system at a pilot scale was run for 14 months but a problem for longer-term instability and efficiency appeared caused by the decline of yeast viability and plugging. The author proposed solving this problem by substituting glass beads by wood chips. The feasibility at an industrial scale was demonstrated (4×10^6 L per year).

The third semi-industrial process, the Meura-Delta, consists in two loop reactors in series with a matrix of silicon carbide inside for adsorption of yeast (Sect. 9.4.1.1). The first bioreactor is operated at an attenuation of 40% and final attenuation is reached in the second bioreactor. The aeration is arranged by diffusion through plastic tubing in the circulation loop. At least one brewery uses it in Canada with a productivity of 0.5×10^6 –3×10^6 L a year according to the final attenuation.

9.4.2 Alcohol-Free Beer

Traditionally, alcohol-free beers are produced by arrested batch fermentation, vacuum distillation, reverse osmosis, or dialysis. Dealcoholized beers generally lack body and have poor aroma profiles. In order to circumvent these disadvantages, several immobilized yeast cell reactors were developed to produce beer with a final alcohol content below 0.5%. For example, immobilization of yeasts by passive colonization of multichannel silicon carbide membrane carriers with a void volume of 30% and pore sizes ranging from 8 to 100 μm was studied with a view to achieving optimal flavour development (van de Winkel et al. 1991).

Others carriers have been described in the literature. Continuous fermentations with yeasts immobilized on wide-pore sintered glass (Siran) in a fluidized-bed fermentor were carried out at both a laboratory and a pilot plant scale (reactor volumes of 2 and 60 L, respectively) (Aivasidis et al. 1991). Following a colonization phase

at 25–30°C requiring less than 2 weeks, the temperature was lowered to just above 0°C, where the beer fermentation took place. A product containing 0.1–0.3% alcohol (carbohydrate conversion rate of approximately 7%) was obtained after about 2–6 h as compared with 3–4 days with conventional cold/contact fermentation. A system stability of 1 year was observed, with no colonization of wild-type yeast strains from contaminated worts or other sources. Blockages were not observed and yeast or carrier make-ups were not required. The organoleptic properties of the final product corresponded to those of conventionally manufactured low-alcohol beers.

Others processes resort to immobilization on DEAE-cellulose and limited fermentation optimally performed in a packed-bed reactor. This highly controllable system combines short contact times between yeast and wort with the reduction of off-flavours. In some cases this was due to higher activities of hexokinase and pyruvate decarboxylase of immobilized cells compared with those of free cells grown in batch culture (Van Iersel et al. 2000). Using a similar system millions of hectolitres of alcohol-free beer have already been produced. In the reactor a limited fermentation was carried out under strictly anaerobic conditions, very low temperature, relatively high pressure and a short contact time. With the combination of these factors, only a small amount of glucose was metabolized, resulting in a low-alcohol product (less than 0.1% ethanol). In addition, the limited growth under anaerobic conditions stimulated the yeast to restore its redox balance by the reduction of carbonyl compounds (Van Dieren and Bavaria 1996) and simultaneously an increase of ester formation (ethyl acetate and isoamyl acetate) was noted (Van Iersel et al. 1999; Navratil et al. 2002). Nevertheless, in this case, introduction of regular aerobic periods to stimulate yeast growth was recommended to achieve an optimal and constant flavour profile of the alcohol-free beer. A patented method (Lommi et al. 1997) also recommended reactivating the yeast at 2–15°C for 10–30 h. If necessary, the reactor could be regenerated.

During a 12-month pilot-scale project both at Guinness Brewing Worldwide Research Center and at Grolsche Bierbrouwerij Nederland (van de Winkel et al. 1996), a silicon carbide immobilizing carrier system was investigated for the continuous production of alcohol-free beer with 0.05, 0.1 and 0.5% alcohol by volume with a decreased worty flavour and a full beery aroma. The scale-up capability of the immobilized bioreactor system was studied and the operating parameters (dilution rate, fermentation temperature, wort oxygenation, number of bioreactor stages, colonization procedure, cleaning and sterilization procedures) were optimized. From the results it was demonstrated that scaling-up was reproducible and predictable with a single bioreactor stage operating at 10°C with oxygen levels below 1 mg L^{-1} for the production of an acceptable alcohol-free beer.

9.4.3 Miscellaneous

Immobilization of yeasts has been studied for purposes other than conventional fermentation. An oxygen scavenger for beverages has been patented (Edens et al. 1989). It consists of dry yeast immobilized in or on a solid material, e.g. paraffin wax, which allows only very slow penetration by water. Yeast cells were mixed with a slurry of molten paraffin at 95°C. A glass slide was coated with a 0.1-mm layer of

this slurry. The slide was heated at 65°C for 10 min and submerged in air-saturated water. The oxygen concentration of the water was reduced from 7.5 mg L^{-1} to undetectable in 14 days.

For production of diet beer several similar methods have been developed using co-immobilized enzyme (amylase) and yeast (*S. cerevisiae*). This is a way to produce beers with higher attenuation containing less residual sugar. A glucoamylase was bound to living yeast cells, resulting in particle size of approximately 10 μm. The apparent K_m value, the pH and the temperature dependence of the activity and stability of the bound enzyme were significantly different from the characteristics of the free glucoamylase. Compared with the native yeast, the co-immobilizate led to a considerably increased final degree of attenuation when applied for beer wort fermentation or for maturation of beer (Hartmeier and Muecke 1982).

In two others works, brewer's yeast was immobilized in calcium alginate gels. In the first one (Godtfredsen et al. 1981), the immobilized cells were packed in a simple reactor allowing continuous operation. A suitable dextran-coupled amyloglucosidase co-immobilized with brewer's yeast was also applied for production of low-calorie beer in a simple reactor system. In the second work (Juchem et al. 2000), the ability of the beads to reduce the carbohydrate content of the beer was investigated in a repeated batch system and a continuous system using a multistage fluidized-bed bioreactor. In both cases the new technology made possible a significant intensification of the fermentation achieved by a high yeast density coupled with the enzymatic activity.

9.4.4 Influence of Immobilization on the Organoleptic Qualities

Most works report modifications in the minor by-product concentrations for the beer produced by immobilized yeasts. These modifications vary a lot according to the kind of process, batch or continuous (Bardi et al. 1997a), kind of reactor (Yamauchi et al. 1995), fermentation temperature (Bardi et al. 1997a; Bekatourou et al. 2002), dissolved oxygen (Nakanishi et al. 1985; Virkajarvi and Kronlof 1998) and even type of carrier (Smogrovicova and Domeny 1999). Generally succinic acid production was increased (Yamauchi et al. 1995; Shindo et al. 1992, 1993) in connection with an enhanced consumption of isoleucine and acetic acid production was decreased. Most of the time higher alcohols were less concentrated in the final product (Smogrovicova et al. 1998; Smogrovicova and Domeny 1999; Tata et al. 1999) but their concentration could increase for very low temperature (0–7°C) in the batch process (Bardi et al. 1997a); the total nitrogen content was higher (Virkajarvi and Kronlof 1998; Smogrovicova and Domeny 1999), except for immobilization on gluten pellets (Smogrovicova et al. 1999). For production of esters and diacetyl the results are very different according to the studies. It seems that for these metabolites the key parameters, which are the level of yeast activity linked with the sugar flux, the biomass density in the reactor (Smogrovicova et al. 1998) and the redox state of the medium, are strongly dependent on the operating conditions. In fact the organoleptic quality of the beer produced by immobilized yeasts can be controlled by:

- The relative importance of the different stages of a multistage process. For example, yeast metabolism was successfully subdivided into a growth and a

restricted phase through a combination of a continuous stirred-tank reactor and an immobilized yeast packed-bed reactor (Yamauchi et al. 1995). The process control strategy based on the relative intensity of attenuation (proportion of sugar consumption) in the two reactors was optimized with a ratio of 1:2, higher alcohols being mainly produced in the stirred reactor and esters in the packed-bed reactor.
– The level of aeration if the process contains a preaeration step (Virkajarvi and Kronlof 1998).
– The ratio of the biocatalyst volume to the reactor volume (Smogrovicova et al. 1998).

Several authors reported final products with no significant differences from the beer produced in the conventional way (Pardonova et al. 1982; Smogrovicova and Domeny 1999; Umemoto and Mitani 1999; Bekatorou et al. 2002).

9.5 Fruit Wines

As is the case for grape wine, the most frequent technology employed for fruit wine making by immobilized yeasts is entrapment in alginate gel. This method has been studied for:

– The development of a new type of Umeshu (a liqueur made from Japanese apricot fruit, shochu and sugar) (Takatsuji et al. 1992). It was fermented in a reactor containing an immobilized growing yeast strain isolated from Japanese apricot juice. The initial Umeshu juice diluted 2 times with water was circulated through the reactor at 25°C with a dilution rate of 0.13 h^{-1}. Fermented Umeshu with good flavour was manufactured stably by this process for 30 days.
– Fermentation of watermelon juice by a wine yeast (Nakada 1990). The juice with addition of glucose up to 21.1% and adjusted to pH 4 with citric acid flowed through the bioreactor at 0.03 L h^{-1} at 25°C. The alcohol productivity of the bioreactor was 10 g ethanol (L gel)$^{-1}$ h^{-1}. The watermelon wine obtained after 4 days of operation contained 9.3% ethanol and the ratio of isoamyl acetate to isoamyl alcohol (4.47) was high, but formation of ethyl caproate was low compared with the that for wine made by the conventional fermentation method.
– Fermentation of fresh sugar cane juice and fruit juices by mixed yeast strains in a three-stage rhomboid bioreactor. The ethanol concentration in the wine made from cane juice was 9.5% and a mixture of wine yeasts produced a wine containing 10–13% ethanol from mandarin and orange juices at residence times of 6–20 hours for 7 months (Fukushima and Hatakeyama 1983).
– The production of alcoholic beverages from different fruit juices (mango, peach, plum, cherry) containing 8–18% sugar giving 11–12% ethanol (60–84% of the theoretical yield) in batch fermentation by entrapped *S. cerevisiae* (Qureshi and Tamhane 1985).
– Bottle-fermented kiwifruit sparkling wines production by a combination of fermentation using *S. cerevisiae* immobilized in double-layered calcium alginate beads and termination of the fermentation using an antimicrobial substance from paprika seed (PSAS) having strong antimicrobial activity against wine yeasts (Yokotsuka et al. 2004). Secondary alcoholic fermentation in bottles

could be terminated several days after the addition of PSAS to give a concentration of 0.1 g L^{-1} to the fermenting base wine. Beads were easily inserted into the bottles and removed as ice plugs by the conventional disgorging method. The sparkling wines produced had a strong fruity smell and a good balance of sweet and sour flavours.

Entrapment in carragheenan gel has also been proposed for fermentation of ripe Cavendish banana fruit pulp, which contained approximately 126 g L^{-1} total sugars (del Rosario and Pamatong 1985). The volumetric productivity and fermentation efficiency were about 15 g L^{-1} h^{-1} and 94%, respectively. The concentrations of alcohol and residual sugar in the product were 54 and 12.8–14.5 g L^{-1}, respectively.

Adsorption on dicarboxycellulose (Sado et al. 1992) or derivatized cellulose (del Rosario and Pamatong 1985) was tested.

As in grape wine making, the deacidifying yeast *Schiz. pombe* can be useful for malic acid elimination from fruit wines. It has been tested immobilized on oak shavings for apple must amended with red currant must: elimination of 100, 80.7, and 79.8% of initial malic acid, at flow rates of 0.033, 0.079, and 0.092 mL h^{-1}, respectively, was obtained (Czyzycki et al. 1991). Other authors deacidified plum juice by *Schiz. pombe* entrapped in calcium alginate beads (Tachibana et al. 1989) prior to alcoholic fermentation: plum (*Prunus salicina*) juice initially contained 1.2–1.4% malic acid and was batch-treated with 5% of immobilized cells of *Schiz. pombe* (2.1×10^9 cells L^{-1} juice) at 20°C for 3 days to remove 70% of the malic acid. When continuous decomposition of malic acid was carried out using a reactor (2 L) containing 1 L of immobilized cells at 20°C, with a flow rate of 1.5 L day^{-1}, the retention time was 16 h and the concentration of malic acid in the treated juice remained at 27–35 mg L^{-1} for 16 days.

9.6 Cider

For cider production two strategies are possible: the use of yeast alone or the use of co-immobilized yeast and lactic acid bacteria.

For fermentation of pure cultures of yeasts the different methods of immobilization are mainly based on adsorption methods:

- Adsorption on multiple parallel porous ceramic plates. The bioreactors prevented clogging associated with insoluble substances in the sample and gas channelling. Thus, low-fermentation apple juice was possible (Aso et al. 1993).
- Adsorption on an ion-exchange sponge that can have a tailored surface charge was used in high original gravity (1.106) cider fermentation. Continuous circulation of the medium through columns containing weakly basic sponge decreased the batch fermentation time, and increased the final ethanol concentration, possibly aided by sponge-enhanced CO_2 removal from solution (O'Reilly and Scott 1993).
- Adsorption on polyethylene. The use of immobilized yeast at 500×10^6 –600×10^6 cells mL^{-1} for fermentation at 15–20°C improved the quality of fruit wines and decreased the period of fermentation by 3–5 times (Sarishvili et al. 1992).
- Adsorption on foam glass put in a column where the apple juice was circulated with a residence time of 5–6 days. The fermentation was carried out for more

than 3 months at 22°C with no changes in the sensory quality of the product (Bonin and Wzorek 2000).

But entrapment in calcium alginate gel was also carried out for continuous fermentation of apple juice. The average values characterizing the process were as follows: fermentation efficiency, 84.7% of the maximal theoretical yield; ethanol concentration in the mash, 38.9 g L^{-1}; and volumetric productivity, 6.3 g L^{-1} h^{-1} (Dallmann et al. 1987). Alginate gel was shown to be better than pectate gel from an organoleptic point of view (Krasny et al. 1993).

A method for controlling the alcohol and sugar content of cider produced by alginate-entrapped yeast by varying the CO_2 pressure has been patented (Divies and Deschamps 1988). At 0.2 bar of CO_2, the ethanol concentration was 4.6% and the sugar concentration 14.9 g L^{-1} and at 3–5 bar of CO_2, the values were 4.8 and 16.2, respectively. It was possible to produce in the same fermentor a "hard" cider with 3% alcohol and 48 g sugar L^{-1} as well as a "soft" cider with 2% alcohol and 66 g sugar L^{-1}.

For co-immobilization calcium alginate has been proposed for *S. bayanus* and *Leuconostoc oenos* (*Oenococcus oeni*) in a continuous packed-bed bioreactor (Nedovic et al. 2000). The continuous process permitted much faster fermentation compared with the traditional batch process. The flavour formation was also better controlled. By adjusting the flow rate of the feeding substrate through the bioreactor, i.e. its residence time, it was possible to obtain either "soft" or "dry" cider. However, the profile of the volatile compounds in the final product was modified compared with that of the batch process especially for higher alcohols, isoamyl acetate and diacetyl. This modification was due to different physiological states of the yeast in the two processes. Nevertheless, the taste of the cider was acceptable.

A sponge-like material was also used to immobilize both *S. cerevisiae* and *Lactobacillus plantarum* (Scott and O'Reilly 1996). The sponge's open porous network promoted extensive and rapid surface attachment of microorganisms throughout the depth of the material. The matrix surface can also be chemically modified, and basic characteristics enhanced both the initial rate of uptake and also that of final loading (in excess of 10^9 yeast cells g^{-1} sponge and 10^{10} bacterial cells g^{-1} sponge). The flavour of the product was satisfactory.

9.7 Vinegar

For vinegar manufacturing several processes using immobilized yeasts have been proposed using different substrates. Some of them are based on a two-step fermentation, the first one by yeasts and the second one by acetic acid bacteria. For example, continuous production of kiwifruit and persimmon wines in a bioreactor with calcium alginate entrapped yeast cells was studied (Yamashita 2002). When the juice was in the reactor for residence times of 12 and 6 h, the ethanol concentration and the productivity were 11 and 10%, and 7.5 and 13.4 g L^{-1} h^{-1}, respectively. Fermentations of both fruit juices were continued for 50 days without microbial contamination. Continuous production of fruit vinegar using a bioreactor with fixed *Acetobacter aceti* cells on cotton fabrics was then developed. The fabrics were packed into a column and inoculated with *A. aceti*. When the kiwifruit and

persimmon wines fed the column the production rates of acetic acid were 7.4 and 5.2 g L^{-1} h^{-1}, respectively, on the basis of the total column volume with 45 g L^{-1} of the acetic acid in the vinegar. The surface culture using growing cells fixed on the woven cotton fabrics was superior to the submerged culture involving aeration, from the viewpoint of the higher productivity and energy efficiency. Yeast entrapment in calcium alginate beads was also used in view of vinegar production from saccharified rice (Nakajima and Sugiura 1990) or rice flour by repeated batch fermentations (Tamai et al. 1990). When rice flour was used the aim was to reach a high concentration of ethanol (approximately 120 g L^{-1} broth) without residual glucose. This was achieved for a 0.14 L gel L^{-1} packing ratio. The process could be repeated for more than 25 batches with a stable ethanol yield [0.38 g ethanol (g rice flour)$^{-1}$] and cell viability in the gel beads (96%). The ethanol productivity of this system was 3.9 g L^{-1} h^{-1}, which is much higher than that of batch fermentation in vinegar breweries.

A method for manufacturing vinegar from potato comprising (1) liquefaction, (2) saccharification, (3) alcoholic fermentation and (4) acetic acid fermentation was patented (Nagao and Yamamoto 1991). Steps 2 and 3 were combined to reduce microbial contamination in a bioreactor comprising a fermentation chamber packed with beads containing immobilized saccharifying enzymes and yeasts for concomitant reactions.

A process for continuous alcoholic and acetic acid fermentation of onion juice was developed by using yeast and acetic acid bacteria, respectively, immobilized on porous ceramic granules and rings (Takahashi et al. 1993). In continuous vinegar production in multibioreactors, the activities of the immobilized yeast and the acetic acid bacterial cells were not lost during more than 6 months of operation. For acetic acid bacterial cells the method was better than for cells immobilized on calcium alginate, for which a decrease of 50% of initial activity was observed for 3 months of operation. By developing a new cyclic operation, acetic acid yield increased by 16% compared with the yield from steady-state operation. The 4% onion vinegar produced had satisfactory organoleptic properties.

Mixed cultures of immobilized yeasts and another microorganism can be another possible strategy. For this purpose some authors proposed co-immobilizing *Monascus* and *Saccharomyces* in alginate carrier (Wang 1998). Vinegar was prepared by mixed fermentation of glucose mother liquor as the main material. The product ratio of vinegar was 4.5 kg kg^{-1} glucose liquor, and the appearance and flavour were good. Others authors suggested entrapping separately growing yeast cells and *A. aceti* cells in calcium alginate gel as pellets (Sumonpun and Kummun 1989). The immobilized yeast cells converted glucose to ethanol and the immobilized *A. aceti* simultaneously converted ethanol to acetic acid. Preliminary studies showed that a 1:4 ratio of immobilized yeast and immobilized *A. aceti* gave the maximum yield of acetic acid, approximately 3.4% after 22–24 days of cultivation in coconut water medium containing 2% glucose in shake flask culture at 250 rpm.

Finally, immobilized yeasts can also be used for refining fruit vinegar. A method has been patented for apple or apple/Japanese pear (10–30%) vinegar (Panasyuk et al. 1988) which is fed to a packed-bed column containing 300–800 × 10^6 immobilized cells mL^{-1} at a flow rate of 0.02–0.08 h^{-1} for 12–48 h.

9.8 Dairy Products

As far as dairy products are concerned the different utilizations of immobilized cells can be divided into the treatment of whey for its valorization (except for the production of ethanol presented in Sect. 9.3), the treatment of milk and the production of kefir for manufacturing fermented beverages from milk.

The use of salted whey (a liquid by-product from the dairy industry) was investigated (Mostafa 2001) as a substrate for either acetic acid or glycerol production using two yeast strains (*K. fragilis* and another one isolated from waste whey); pH 8.5 and 32°C were the optimum operating conditions for maximal acetic acid production (25.8 g L^{-1}) and supplementation with peptone and pH 7 for glycerol batch production (13.2 g L^{-1}). The experiments in a membrane cell recycle bioreactor gave better results than those obtained for the immobilized cell batch reactors (18.7 g L^{-1} for glycerol production).

The potential of three lactose metabolizing yeasts, *C. pseudotropicalis*, *S. fragilis* and *K. marxianus*, for the removal of biological oxygen demand and chemical oxygen demand from dairy industry wastewater under unsterilized conditions was evaluated (Marwasha et al. 1988). The most efficient was *C. pseudotropicalis*. Using entrapment procedures in an alginate matrix, 40 g (dry weight) cells L^{-1} was the optimum operational cell density. Nitrogen source supplements further improved the ability of immobilized yeast cells to carry out the treatment. For milk or whey treatment two original and similar methods have been investigated.

An immobilized preparation of whole cell-based catalase was obtained by cross-linking the yeast cells permeabilized with toluene in hen egg white using glutaraldehyde for 2 hours at 4°C. Immobilized cells could be reused for the removal of H_2O_2 from milk (Kubal and D'Souza 2004).

Others authors have developed and characterized a new low-cost enzymatic preparation for milk whey saccharification (Gonzalez Siso and Suarez Doval 1994) consisting of β-galactosidase-rich whole cells of the yeast *K. lactis*, previously cultured on milk whey and immobilized by covalent linkage to corn grits (an inexpensive material). Permeabilization of immobilized cells with ethanol increased the intracellular β-galactosidase activity up to 240-fold, and the cells did not further metabolize the glucose and galactose produced. More than 90% milk whey lactose hydrolysis was achieved in a packed-bed bioreactor at 37°C. In another work (Decleire et al. 1985) whey hydrolysis was compared in column reactors containing whole yeast cells immobilized in calcium alginate or in hen egg white in relation to cell β-galactosidase activity, flow rates, temperature and time. With cells having an activity of 1.3 U mg^{-1} (dry weight) immobilized in calcium alginate, 80% hydrolysis was obtained at 4 and 20°C with flow rates of 0.50 and 1.65 bed vol h^{-1}, respectively; the values were 0.2 and 0.4 bed vol h^{-1} with cells entrapped in hen egg white. When the flow rate was expressed as millilitres per hour per gram of wet yeast, no significant difference was observed between either matrix, and 80% hydrolysis was reached with flow rates of 1.7 and 5 mL h^{-1} (g wet yeast)$^{-1}$, respectively, according to the temperature. The best performance was achieved by the yeast egg white reactor. At 4°C, hydrolysis decreased by 10% after 13 days and by 20% after 17 days. Many more applications of purified immobilized β-galactosidase have been studied.

In the field of dairy products many works deal with kefir. In fact, kefir is made from gelatinous white or yellow particles or granules called "grains" formed on cultured milk. These grains contain the lactic acid bacteria/yeast mixture clumped together with casein and a branched polysaccharide composed of glucose and galactose forming an insoluble matrix. They range from the size of a grain of wheat to that of a hazelnut. The grains, then removed after milk fermentation, as well as the fermented beverage are called kefir.

To remove lactose from milk the use of kefir granules obtained by fermentation, containing both bacteria and yeasts, has also been proposed and patented. They were sterilized at a low temperature, inactivated and coated with a semipermeable film-forming material, yielding an immobilized lactase (Snow Brand Milk Products Co. 1982).

A mixture of wheat flour and sour milk was treated according to the method of the traditional Greek fermented food *trahanas*, and was used as a model cereal-based support (starch–gluten–milk matrix) for co-immobilization of lactic bacteria and yeasts for potential use in food production (Plessas et al. 2005). Cell immobilization was proved by microscopy and by the efficiency of the immobilized biocatalyst for alcoholic and lactic repeated fermentations at various temperatures (5–30°C). The stability of the system was always good, revealing suitability for industrial applications. Finally, respectable amounts of lactic acid and volatile by-products were produced, revealing potential application of the immobilized biocatalyst in fermented food production or use as a food additive, to improve nutritional value, flavour formation or preservation time.

The main application of immobilized kefir microorganisms is the production of fermented beverages from milk or whey. For this purpose the same Greek research group has developed different methods (Athanasiadis et al. 2004) They reported a novel whey-based beverage with acceptable organoleptic properties where various treatments were studied. Kefir yeast immobilized on delignified cellulosic materials (DCM) or gluten pellets were proved to accelerate whey fermentation significantly, with the latter support not being so preferable. Kefir granules seemed to achieve similar fermentation times as DCM. The optimal final pH of the product, indicating the amount of fermented lactose, was suggested to be 4.1 since the profile of the volatile by-products was higher than for other final pH values. The addition of fructose seemed to be beneficial for the volatile content of the product, although its acceptability as determined by a preference panel was similar to that of the control.

This delignified cellulosic-supported biocatalyst was also found to be suitable for batch or continuous modified whey fermentation containing 1% raisin extract and molasses (Kourkoutas et al. 2002a). Batch fermentations were carried out at various pH values, and the effect of temperature on the kinetic parameters, in the range 5–30°C, was examined. At pH 4.7 the shortest fermentation time was obtained. The formation of volatiles indicated that the concentration of amyl alcohols (total content of 2-methyl-1-butanol and 3-methyl-1-butanol) was reduced as the temperature became lower. 1-Propanol and isobutyl alcohol formation also dropped significantly below 15°C. The percentage of ethyl acetate increased as the temperature was reduced. At 5°C the content of total volatiles in the product was only 38% of the volatiles formed during fermentation at 30°C.

For the continuous process, ethanol productivities ranged from 3.6 to 8.3 g L^{-1} day^{-1} (Kourkoutas et al. 2002c). The continuous fermentation bioreactor was operated for 39 days, stored for 18 days at 4°C, and operated again for another 15 days without any diminution of the ethanol productivity. The concentrations of higher alcohols (1-propanol, isobutyl alcohol and amyl alcohols) were still low. The main volatile by-products formed in the continuous process were similar to those observed in alcoholic beverages, particularly ethyl acetate, and the fermented whey had a good aroma. The possibility of using such a process for the production of potable alcohol or a novel, low-alcohol content drink was proposed.

Several lactic acid bacteria (*Lactobacillus kefiranofaciens*, *Lactobacillus kefir*, *Lactococcus lactis* subsp. *lactis*), *Enterococcus durans* and yeasts (*S. italicus*, *S. unisporus*), all isolated from kefir-grains from Turkey and Yugoslavia, were immobilized in calcium alginate (Gobbetti and Rossi 1993). A continuous process for the production of a new kefir-like cultured milk was performed at 28°C for 30 days, with dilution rates of 0.03 and 0.06 h^{-1}, respectively, for free and immobilized cells. The pH values were 4.41 and 4.55 and the CO_2 and ethanol concentrations were 0.54 and 0.66 g L^{-1} and 4.50 and 4.58 g.L^{-1}, respectively. The synthesis of aroma compounds (diacetyl and acetoin) was reduced under the conditions of the continuous process, but it increased during storage at 4°C when these compounds reached about the same concentrations as in the traditional kefir. Despite lower values of viability the new cultured milk approached the characteristics of traditional kefir.

9.9 Aroma

In the field of aroma production or aroma enhancement very few applications of immobilized yeasts can be found. They are of two types.

The first one is the production of natural aroma compounds through biocatalysts. 2-Phenylethanol, which has a rose-like odour, can be produced from L-phenylalanine by *S. cerevisiae*. Unfortunately this product inhibits growth even at low concentration. This problem of the inhibitory effect of the product on the yeasts can be tackled by an in situ product removal technique: addition of an ester as a second water-immiscible phase in which partitioning of 2-phenylethanol is very favourable. Thus, the yeast is immobilized in chitosan alginate beads to protect them from the toxic extractant and the production of 2-phenylethanol can be increased compared with production using the conventional method with free cells (Stark et al. 2000).

Another process is based on a simplified double reaction for production of esters which can be added to foods or cosmetics. The first reaction is the production of ethanol by calcium alginate gel immobilized yeast cells and the second reaction is the synthesis of ethyl oleate in presence of oleic acid, in a lipase-catalysed reaction. In this two fluid-phase system the presence of lipase enzyme does not influence the cell growth, the glucose consumption and the concentration of ethanol in the water phase. However, 1 U mL^{-1} lipase in the water phase of the fermentation broth increased threefold the concentration of ethyl oleate in the oleic acid phase (Kiss et al. 1998).

An alternative approach to microbial production of bioflavours, eliminating the need for lengthy product purification, was presented by Kogan and Freeman (1994). It was

based on co-immobilization of precursors for bioflavour generation and microbial cells, traditionally employed for food and beverage processing, within beads made of a food-grade gel matrix. Following incubation under controlled conditions, the bioflavour – or bioflavour mixture – was generated and accumulated within the beads. The flavour-retaining beads might then be employed as a food additive. A feasibility study demonstrated this approach with ethanol production by baker's yeast co-immobilized with glucose medium. Complex bioflavour generation was also demonstrated by baker's yeast co-immobilized with apple juice, generating cider flavours. Beads providing beer taste were also readily made via co-immobilization of brewing yeast with malt.

To efficiently produce a fermented flavoured liquor for improving the taste of bread, a repeated batch fermentation was studied in a bioreactor with the use of immobilized yeast cells (Tamai et al. 1997). It contained about 5% (wt/v) ethanol and ethyl acetate, isoamyl alcohol, isobutyl alcohol as major flavouring component and organic acids with varying concentrations.

The second kind of application is related to the modification of aroma or taste of the product using immobilized yeasts. Several works deal with soy sauce. It can be produced with *Zygosaccharomyces rouxii* and *C. versatilis* immobilized on 4–6-mm porous aluminosilicate glass beads in a two-stage bioreactor (Horitsu et al. 1991). The fermentation time was 6 days. Experiments were also made encapsulating these yeasts in calcium alginate beads, but the fermentation ability was inadequate. A method to produce a soy-sauce-like condiment from a hydrolysed soy sauce raw material by a mixture of immobilized yeasts and immobilized lactic acid bacteria has been patented. *Pediococcus halophilus* and *S. rouxii* were immobilized separately in carragheenan beads (Yamasa Shoyu Co. 1985). The same volume of each kind of bead (60 mL) was then filled in a column (200 mL) and the soy sauce was passed through it in the ascending direction at 28°C for 48 h while flushing with nitrogen gas. A similar process has been tested in order to improve nutritive and organoleptic properties of Worcestershire sauce using immobilized *S. cerevisiae* to increase isoamyl alcohol, ethanol and ethylphenol content, the major aroma components of fermented Worcestershire sauce (Fujimoto et al. 1993).

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Chapter 10

Production of Antioxidants, Aromas, Colours, Flavours, and Vitamins by Yeasts

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10.1 Introduction

The application of yeast and yeast-derived products by the food industry continues to be a rapidly growing area. Recent focus on improving human health through capturing greater value from yeast products has led to a rise in the recognition of the nutraceutical potential of many of the current products and has renewed emphasis on research that demonstrates the efficacy of new and existing products. Historically, yeast products have consisted of whole yeasts that are provided as yeast slurry or yeast paste, dried active yeasts, yeast autolysates, yeast extracts, separated components, such as protein isolates and amino acids, cell wall glucans and mannoproteins, vitamins, sterols, carotenoids, other lipids, enzymes, nucleic acids, polysaccharides, and chemically, physically, or enzymically modified components. Some examples of derivatives of yeast cell components are the chemically modified proteins (acylated, phosphorylated, encapsulated enzymes, immobilized enzymes, etc.), physically modified proteins that are partly denatured or texturized, enzymatically modified proteins that are re-partially digested by acid or enzymatic treatment or enzymatically modified proteins with covalently attached amino acids, nucleotides, and nucleosides, flavouring products and flavour substances, salt replacers, and substances or immobilized enzymes that are encapsulated inside the yeast for use as flavours and pharmaceuticals (Abbas 2001, 2003, 2004; Benítez et al. 1996; Halasz and Lasztity 1991; Peppler 1967, 1979; Reed 1981).

Yeast and yeast-derived products contribute to food flavour and aroma in a number of ways as added ingredients or as biocatalysts that carry out fermentation or biotransformation of food components thereby yielding a variety of products with desirable features. The use of yeast and yeast-derived products as bioflavouring agents and biocatalysts for edible meats, breads, other bakery products, cheeses, margarine flavours, yogurts, kefirs, other fermented dairy products, animal feeds, alcoholic beverages, fragrances, fruity flavours, soya-derived products, fermented cocoa beans, fermented tea, fermented vanilla beans, fermented syrups, pickles, ciders, vinegars, and a great variety of other fermented foods and beverages is well

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documented. Yeasts have also been tapped as sources of colorants, vitamins, antioxidants and as supplements for their nutraceutical or health-promoting attributes.

A number of yeast genera have found uses in the previously mentioned applications. These range from the widely used species and strains belonging to the genus *Saccharomyces*, to other genera such as *Candida*, *Debaryomyces*, *Geotrichum*, *Hansenula*, *Kloeckera*, *Kluyveromyces*, *Pichia*, *Schizosaccharomyces*, *Sporobolomyces*, *Yarrowia* and *Zygosaccharomyces*, to name only a few.

The primary goal of this chapter is to provide a background summary and an update on recent developments in this area with emphasis on the production of aromas, vitamins, antioxidants, colours, and flavours. It should be noted that these products are synthesized by metabolic pathways that parallel the biogenetic relationships of other naturally derived materials from plants and animals as illustrated in Fig. 10.1 (Sanderson 1978).

10.2 Background and Definitions

No serious overview of the topics selected can proceed without delineating its scope by providing the reader with concise definitions that can help assemble the products whenever possible into best-fitting groups. This task is made difficult as there is considerable confusion and overlap in the published literature, particularly when it comes to aromas and flavours produced by yeasts. For example, esters produced by yeasts can contribute to both aroma and flavour in the case of alcoholic and nonalcoholic fermentations. Therefore, in many cases of aromas and favours made by yeasts there is no clear or consistent distinction found in the literature. To a food scientist flavour primarily consists of two elements: (1) taste, which is perceived in the mouth and is mainly due to nonvolatile constituents present in food, and (2) aroma, which is perceived in the nose and is mainly attributed to volatile components

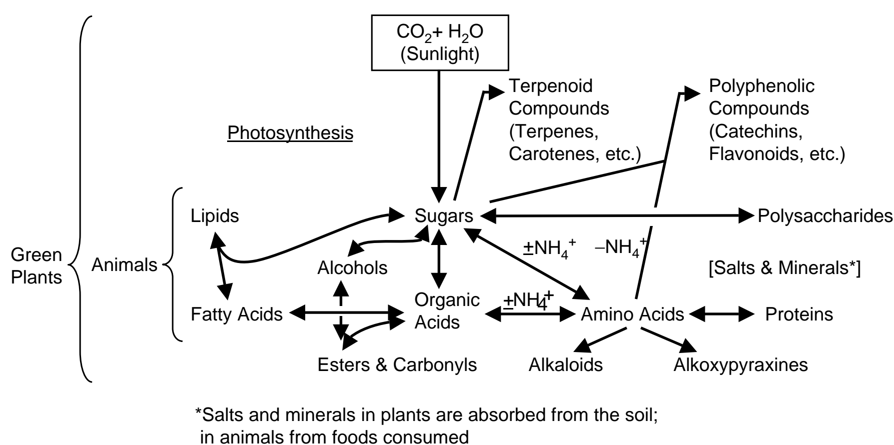

Fig. 10.1. Biogenetic relationship of compounds that comprise living organisms including those that serve as food materials. (Adopted from Sanderson 1978)

(Sanderson 1978). In sensory evaluation, descriptive aroma procedures have been developed which objectively and precisely define aroma as part of flavour (Noble 1978). While the biogeneses of the volatile and nonvolatile components of food are highly interrelated, it is well established that minor variations in a volatile component can lead to a major impact on flavour (Sanderson 1978). For the purpose of this chapter, aromas will be defined as esters, other fusel oil components, and other volatile products that are readily detected by smelling, while flavours will be defined as yeast and yeast-derived products that contribute to organoleptic properties or taste such as yeast extracts, nucleotides, organic acids, and polysaccharides and simple chemical compounds which include polyols such as inositol and glycerol. These products contribute as a whole or in part to the five primary recognized elements of food flavour: sweetness, saltiness, sourness, bitterness, and astringence (Sanderson 1978).

Providing working definitions for yeast-produced colours, vitamins, and antioxidants is a somewhat easier task as long as it is understood that some of the common yeast-derived or yeast-produced pigments may provide in addition to colour enhancement, antioxidant activity as is the case of astaxanthin, a salmon fish feed additive. A similar situation exists for the vitamin B_2, riboflavin, which can be listed as a vitamin as well as a food colorant and as an antioxidant. Keeping the aforementioned in mind, what follows is a summary of the topics under consideration.

10.2.1 Antioxidants

Yeasts and yeast extracts have been recognized as a source of antioxidant compounds for some time (Forbes et al. 1958). In the process outlined by Forbes et al., a 20% suspension of yeast is subjected to a series of organic extractions to yield a white crystalline preparation that can be added to prevent oxidation of food products such as fats, baby foods, whole milk, or milk products. More recently, the increased interest in natural antioxidants has given rise to the screening of microbial sources for compounds to replace the synthetic compounds currently in use as food antioxidants. Natural antioxidants can also be used in nutraceutical applications as supplements (Gazi et al. 2001; Nishino and Ishikawa 1988). Natural oxidants are presumed to be safer for human beings (Gazi et al. 2001). The function of antioxidants and the exact mechanism of their mode of action are still open to conjecture, but it is generally agreed that they act by donating hydrogen protons to substrates, thereby rendering them nonreactive to oxygen-derived free radicals that are referred to as reactive oxygen species or ROS (del Rio et al. 2003; Heath 1981). It is hypothesized that yeast peroxisomes play a similar role to plant peroxisomes. Therefore the response in yeasts to oxygen-derived radicals would involve several enzymes, including catalases, superoxide dismutases (SOD), glutathione (L-γ-glutamyl-L-cysteinylglycine), as well as several NADP-dependent dehydrogenases (del Rio et al. 2003). It is well established that antioxidants are inactivated in the process and that their activity is proportional to the quantity used based on their permitted usage level (Heath 1981).

Yeasts synthesize a number of bioactive compounds which can serve as antioxidants. These have found numerous uses in foods to retard oxidative degeneration of

fatty substances and in nutraceutical supplements to improve health and well-being (Bastin et al. 2002; Doll 2002; Gazi et al. 2001; Ok et al. 2003). They consist of the oxygenated carotenoid torulahodin, both the organic acid and the salt forms of citric acid, coenzyme Q or ubiquinone, glutathione, hydroxymethyl and hydroxylethyl furanone (2*H*), tocotrienol, α-tocopherols (α-TOHs) and other forms of tocopherols, riboflavin (vitamin B_2) and the flavins derived from it, FMN and FAD, and 2,4-hydroxyphenyl ethanol (Cremer et al. 1999; Do et al. 1996; Imai et al. 1989; Kawamukai 2002; Padilla et al. 2004; Penninckx 2002; Sugawara 2001; Suzuki et al. 2003). Other factors that are produced by yeasts and/or that are present in yeast-fermented products or in yeast cell biomass at the end of fermentation that have antioxidant activity or free-radical scavenger activity include several other oxygenated carotenoids, selenium-enriched yeast cells, the wine component resveratrol, octacosanol, yeast-derived cell wall β-glucans, uncharacterized soluble proteins that are produced in yeasts under oxidative stress, sulphur-containing amino acids, cytochrome c, the yeast enzyme Cu,Zn-SOD, and products of SOD genes, CUP1 and SOD1 (An 1996; Archibald 2003; Becker et al. 2003; Combs et al. 2002; Farid and Azar 2001; Forbes et al. 1958; Forman et al. 1983; Imai et al.1989; Lee and Park 1998a, b; Marova et al. 2004; Mast-Gerlach and Stahl 1997; Park 2003; Sakaki et al. 2001, 2002; Shitazawa et al. 2002; Visser et al. 2003).

Glutathione plays an important role as an antioxidant. The depletion of glutathione leads to the accumulation of ROS following the treatment of *Saccharomyces cerevisiae* with the pungent sesquiterpenoid unsaturated dialdehyde, polygodial (Machida et al. 1999). Polygodial exhibited a strong yeasticidal activity against cells of *S. cerevisiae*, in which production of ROS at a significant level could be detected with a fluorescent probe. The production of ROS in polygodial-treated cells was further confirmed by its elimination and the accompanying protection against yeasticidal effects in the presence of antioxidants such as L-ascorbate and α-TOH. Polygodial could accelerate ROS production only in cells of the wild-type strain but not in those of a respiratory-deficient petite mutant (rho0), indicating the role of the mitochondrial electron transport chain in the production of ROS. Unlike antimycin A, which accelerates ROS production by directly targeting the mitochondrial electron flow, polygodial caused depletion of cytoplasmic and mitochondrial glutathione, which functions in eliminating ROS that is generated during aerobic growth. It was hypothesized that the polygodial-mediated depletion of intracellular glutathione was possibly dependent on a direct interaction between its enal moiety and the sulphydryl group of the cysteine in glutathione by a Michael-type reaction. The breeding of high-glutathione-producing strains and their production by fermentation has been described recently (Liu et al. 2003; Sakato 1992; Shimizu et al. 1991; Udeh and Archremowicz 1997; Wei et al. 2003).

Increasing the level of Cu,Zn-SOD in beer was attempted through the genetic engineering of a beer brewing strain of *S. cerevisiae* yeast by the cloning of the Cu,Zn-SOD gene coupled to an α-factor leader (Cremer et al. 1999). The protein product was secreted by the transformed strain (RHS1) and the transformants were active in catalysing the reaction with superoxide. The enzymic activity of SOD was assayed directly and by determining antioxidant activity. The quantity of SOD secreted was insufficient for fermentation processes. Additional expression of the

protein may be achieved by further optimization of the transformation process (e.g. change of the promoter region) and by the use of various recipient cultures.

The antioxidative role of coenzyme Q or ubiquinone in yeasts is well established. Ubiquinone is an essential component of the electron transfer system in both prokaryotes and eukaryotes and is synthesized from chorismate and polyprenyl diphosphate in eight steps. The enzyme *p*-hydroxybenzoate (PHB) polyprenyl diphosphate transferase catalyses the condensation of PHB and polyprenyl diphosphate in ubiquinone biosynthesis. The gene for PHB polyprenyl diphosphate transferase (designated ppt1) was isolated and the gene was disrupted in a strain of the fission yeast *Schizosaccharomyces pombe* (Uchida et al. 2000). This strain could not grow on minimal medium supplemented with glucose. The expression of COQ2 from *S. cerevisiae* in the defective *S. pombe* strain restored growth and enabled the cells to produce ubiquinone-10, indicating that COQ2 and ppt1 are functional homologues. The ppt1-deficient strain required supplementation with antioxidants, such as cysteine, glutathione, and α-TOH to grow on minimal medium. This supports the role of ubiquinone as an antioxidant and the observation that the ppt1-deficient strain is sensitive to H_2O_2 and Cu^{2+}. The ppt1-deficient strain produced a significant amount of H_2S. Thereby the oxidation of sulphide by ubiquinone may be an important pathway for sulphur metabolism in *S. pombe*. Ppt1-green fluorescent protein fusion proteins localized to the mitochondria, indicating that ubiquinone biosynthesis occurs in the mitochondria in *S. pombe*. Thus, analysis of the phenotypes of *S. pombe* strains deficient in ubiquinone production clearly demonstrated that ubiquinone has multiple functions in the cell apart from being an integral component of the electron transfer system.

The formation of several antioxidants can be induced in yeasts grown under stressful conditions or in response to fermentation medium ingredients such as phenolics or additives that are known to be toxic to cells grown aerobically (Cruz et al. 1999; Fung et al. 1985; Larsson et al. 2000; 2001; Millati et al. 2002; Wang et al. 2001). In another variation, synthetic antioxidants are added to fermentation media during the production of fodder yeast to stimulate aerobic growth and to increase cell biomass (Pobedimskii et al. 1998; Larsson et al. 2000). The screening of yeasts for free-radical-scavenging activity is an active area of research (Gazi et al. 2001). The recent publication by Gazi et al. (2001) describes the screening of 25 yeast strains that were cultivated in yeast peptone dextrose broth (YPD) and in yeast malt extract broth (YMB) media under both shaking and stationary conditions. This was followed by measuring the decrease of absorbance at 517 nm of a solution of 1,1-diphenyl-2-picrylhydrazyl after mixing using the supernatant of each cell culture. It was found that all strains tested are capable of producing the activity in at least one condition. Among the tested strains, *Hansenula anomala* (134 units/mL) produced the highest activity during YPD shaking culture. On the other hand, *Rhodotorula glutinis* (199 units/mL) produced the highest activity during YMB stationary culture.

In a recent patent, Nishino and Ishikawa (1998) describe antioxidants that can be used in pharmaceutical, cosmetic, and food applications. These antioxidants comprise whole yeast cells, their cultured products, or their extracts and are selected from a number of genera and yeasts that consist of *Candida gropengiesseri*, *C. parapsilosis*, *C. maltosa*, *C. stellata*, *C. tropicalis*, *Hansenula holstii*, *Hyphopichia*

burtonii, *Pichia membranaefaciens*, *R. glutinis*, *R. minuta*, *R. rubra*, *S. bayanus*, *S. elegans*, *S. unisporus*, *Torulopsis magnoliae*, and *Zygosaccharomyces bisporus*. In this patent, the antioxidant activities of microorganisms were evaluated by adding microorganisms to both Rose Bengal (I) containing medium and in a dye-free medium, culturing yeast under light irradiation, and measuring the viable count or the turbidity of each medium. The antioxidants disclosed inhibit oxidation of low-density-level lipids and are useful as antiarteriosclerotics. A lotion was prepared by combining a 15 mL EtOH extract of *C. parapsilosis* ATCC 6295, 2 g poly(oxyethylene) hydrogenated castor oil, 3 mL 1,3-butylene glycol, 0.2 g perfume, 0.2 g antiseptic, and H_2O to 100 mL.

Another recent publication, by Kakizono et al. (2003), outlines an efficient method for screening antioxidant high-production yeast cells comprising performing a mutation treatment upon a group of cells, reacting the cells with a redox fluorescent indicator capable of generating fluorescence upon being oxidized by ROS, detecting the fluorescence generated from the intracellular fluorescent indicator with an optical detector upon irradiating with excitation light, and isolating the cells possessing the relatively low fluorescence with a fractionation mechanism (Kakizono et al. 2003). This method enables the selection of high-antioxidant-producing cells which appear normally with an extremely low frequency.

A recent patent application describes the isolation and purification of a natural antioxidant compound from natural sources including yeasts [*S. carlsbergenesis*, *S. cerevisiae*, or from a commercially available yeast extract, and saltbush (*Atriplex halimus*)] that can be synthesized chemically, by processes that improve the potency of the product (Mirsky et al. 2001). These antioxidants when used with/without chromium can be formulated for use in animals and humans.

Bio-Catalyzer α-ρ no. 11 (Bio-Normalizer) or BN is a complex natural health food product prepared by yeast fermentation of medicinal plants or unripe papaya fruits which has been reported to possess antioxidant properties (Afanas'ev et al. 2000; Haramaki et al. 1995). The effects of BN have been compared in vivo and in vitro with those of some classical antioxidants to determine their protective properties against free-radical-mediated damage of erythrocytes of thalassemic patients as well as damage to liver, and to peritoneal macrophages of iron-overloaded rats. The principal difference between the protective mechanisms of BN and rutin was observed only in thalassemic cells. Rutin was able neither to remove iron from cells nor to affect haemoglobin oxidation. Thus, rutin's antioxidant effect seems to depend exclusively on its oxygen radical scavenging activity. To better understand the effects of orally administered BN on oxidative damage in the rat heart, the BN-supplemented animals were (1) exposed to ischemia-reperfusion using the Langendorff technique or (2) homogenized and exposed to peroxyl radicals generated from 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN). During reperfusion following 40 min of ischemia, leakage of lactate dehydrogenase from hearts isolated from BN-supplemented rats was significantly lower than from hearts of control animals. Furthermore, lower levels of AMVN-induced accumulation of thiobarbituric acid reactive substances and of protein carbonyl derivatives were detected in homogenates prepared from hearts isolated from BN-supplemented rats than in samples from control animals. The findings confirm an antioxidant action of BN

and show that it protects the heart against ischemia-reperfusion-induced damage. From these results, it is clear that yeasts generally have radical scavenging activity and are a good source of potent natural antioxidants.

10.2.2 Aromas

During fermentation yeasts synthesize a vast number of aroma and flavour compounds (Fig. 10.2; Berry 1995; Suomalainen and Lehtonen 1979). The numerically and quantitatively largest groups of aroma compounds synthesized by yeasts consist of fusel alcohols, fatty acids, and their esters (Suomalainen and Lehtonen 1978, 1979). These are generally compounds with a molecular weight of less than 300 (Noble 1978). It has been shown that these are primarily due to yeast metabolism since significant differences in their production have been demonstrated by the use of different yeast genera, species, and strains. In addition to the choice of yeast, several factors contribute to aroma production. These include changes in fermentation conditions such as temperature, pH, aeration, agitation, and the nature and concentration of the substrate utilized (Suomalainen and Lehtonen 1979). Yeast-derived aromas or odours are primarily products of the application of yeasts for the production of alcoholic and nonalcoholic beverages such as beer, wine, sherry, sake, brandy, spirits such as rum and whisky, and other fermented beverages. Modern aroma research has revealed that the complex aroma of alcoholic beverages involves over 400 different chemical compounds which include acids, esters, carbonyl compounds,

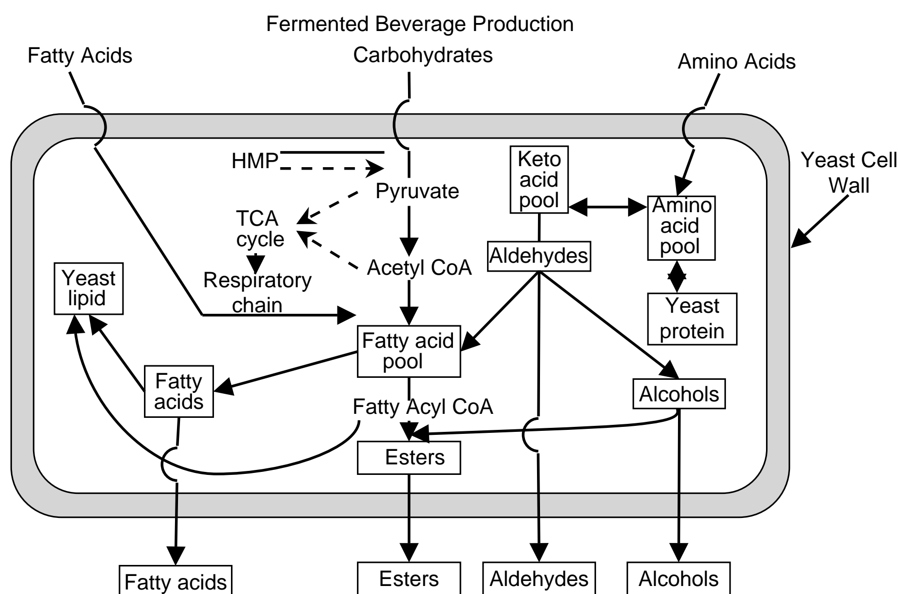

Fig. 10.2. Basic routes by which yeasts form the major flavour groups during fermentation. (Adopted from Ramsay 1982)

acetals, phenols, hydrocarbons, nitrogen compounds, sulphur compounds, lactones, sugars, and a variety of other unclassified compounds that are listed in Table 10.1 in addition to alcohols (Berry 1989, 1995; Dickinson 2003; Garafolo 1992; Suomalainen and Lehtonen 1978, 1979). The unique aromas of the many alcoholic beverages produced by yeasts with some exceptions are frequently the result of a pattern or specific ratios of the previously listed components rather than caused by the presence or absence or a specific concentration of one or a few components (Noble 1978). Yeasts also contribute significantly to the aroma of fermented foods such as breads, cheeses, other dairy products, fermented meat products, vanilla, cocoa, and fermented soy-derived foods. Since many of the aroma compounds that are associated with alcoholic beverages are also present in other fermented foodstuffs, I have chosen to combine all of these into six groups.

10.2.2.1 Fusel Alcohols

Fusel alcohols comprise the largest group of aroma compounds in alcoholic beverages. Their concentration varies considerably in spirits, with rums containing an average of 0.6 g/L, whiskeys about 1.0 g/L and brandies about 1.5 g/L (Dickinson 2003; Suomalainen and Lehtonen 1978, 1979). The main fusel oil synthesized by yeasts is isoamyl alcohol, with *n*-propyl alcohol, isobutyl alcohol, phenethyl alcohol, tryptohol, and optically active amyl alcohol as the other long-chain and complex alcohols (Dickinson 2003; Etschmann et al. 2003; Fabre et al. 1997, 1998; Kunkee et al. 1983; Mo et al. 2003; Pan and Kuo 1993; Suomalainen and Lehtonen 1978, 1979; Ter Schure et al. 1998). Several theories have been proposed to explain the formation of fusel alcohols by yeasts, the oldest of which is Ehrlich's so-called catabolic derivation from exogenous amino acids such as leucine, isoleucine, valine, and threonine (Dickinson 2003; Suomalainen and Lehtonen 1979, 1978; Ter Schure et al. 1998). This theory is

Table 10.1 The number of aroma compounds identified in alcoholic beverages

Compound	Number
Alcohols	38
Acids	80
Esters	118
Carbonyl compounds	41
Acetals	17
Phenols	41
Hydrocarbons	11
Nitrogen compounds	18
Sulphur compounds	11
Lactones	17
Sugars	4
Unclassified compounds	11
Total	407

Adopted from Kahn (1969)

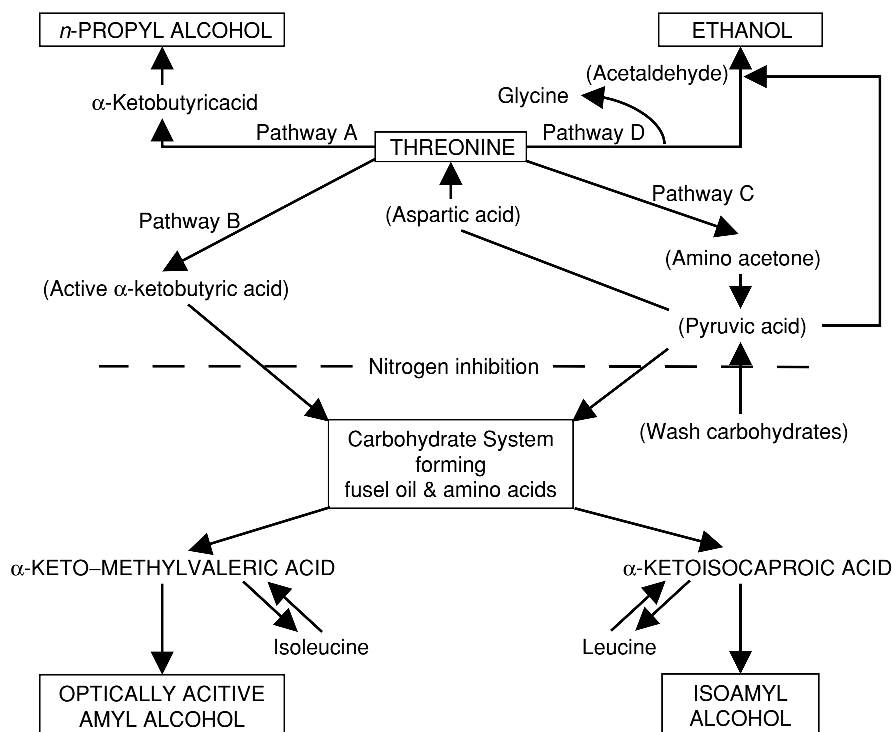

Fig. 10.3. Biochemical relationships of threonine and isoleucine in the formation of fusel alcohols. (Adopted from Reazin et al. 1973)

consistent with more recent work which provides evidence that *S. cerevisiae* grown on isoleucine forms primarily optically active amyl alcohol with significant additional production of *n*-propyl alcohol and isoamyl alcohol when cells are grown on threonine (Suomalainen and Lehtonen 1979). The biochemical pathways describing the production of these alcohols from amino acids is provided in Fig. 10.3 (Suomalainen and Lehtonen 1979). The constitutive expression of the BAP gene which codes for the permease involved in branched amino acid transport has been demonstrated to increase the production of isoamyl alcohol derived from leucine but no comparable increase in isobutyl alcohol derived from valine or amyl alcohol derived from isoleucine was observed (Kodama et al. 2001). The genes for two aminotransferases (ECA39 and ECA40) that actively participate in the transamination of branched amino acids in the mitochondria and cytoplasm have been deleted in order to determine their role in the formation of higher alcohols (Eden et al. 2001). Deletion of the ECA39 and ECA40 genes had little impact on the production of propanol, while the deletion of ECA40 had a drastic impact on the production of isobutyl alcohol and partial impact on the production of active amyl and isoamyl alcohol (Eden et al. 2001). The production of isoamyl acetate in *S. cerevisiae* is regulated at the co-transcriptional level of two genes involved in cytosolic branched-chain amino acid aminotransferase

and L-leucine biosynthesis (Yoshimoto et al. 2002). These findings provide added support to the findings that the catabolic pathway from amino acids fails to explain the formation of fusel alcohols by yeasts grown on carbohydrates with inorganic nitrogen sources or under nitrogen limitation. Therefore, the anabolic formation route from sugars first proposed by Ayräpää provides a plausible alternate explanation (Suomalainen and Lehtonen 1979). Thus, the formation of fusel alcohols in yeasts involves both degradative and synthetic pathways as illustrated in Fig. 10.4 (Suomalainen and Lehtonen 1979). Evidence indicating a high level of specificity of the enzymes involved in the catabolic and anabolic pathways points to the complex operation and regulation of yeast genes involved in the formation of fusel oils as summarized in a recent review by Dickinson (2003). Gaps in the current knowledge of the genetics and biochemical basis for fusel alcohol formation in yeasts will continue to provide the impetus for more research since its delineation will have many practical applications in the production of alcoholic beverages.

10.2.2.2 Fatty Acids

Fatty acids with a carbon chain length ranging from C3 to C16 represent another major group of aroma compounds that are synthesized by yeasts during alcoholic fermentations (Suomalainen and Lehtonen 1979). An important component of alcoholic beverage aroma is provided by the shorter-chain volatile fatty acids such as propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid, caproic

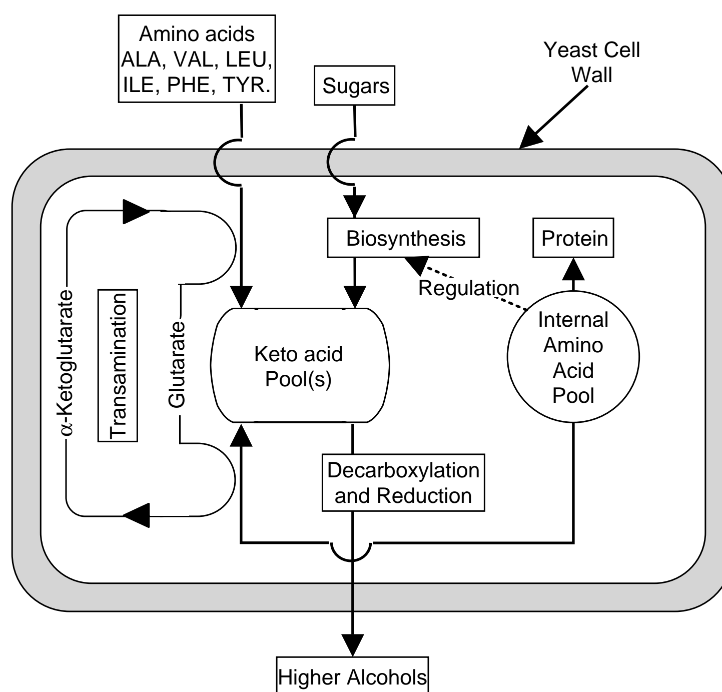

Fig. 10.4. The formation of fusel alcohols. (Adopted from Äyräpää 1973)

or hexanoic acid, caprylic or octanoic acid, and capric acid. These fatty acids are synthesized by yeasts irrespective of the raw material used but with noticeable clear differences in the relative proportions of these acids in the various alcoholic beverages (Edwards et al. 1990; Suomalainen and Lehtonen 1978, 1979). The differences in the relative amounts in the main volatile fatty acids in three spirits are provided in Table 10.2 (Suomalainen and Lehtonen 1979). The acid content of alcoholic beverages varies considerably, with low levels reported for Scotch whisky and cognac, which contain levels in the range 100–200 mg/L, when compared with heavy rum or wines, which tend to have significantly higher levels of volatile acids reported in the range 500–1,000 mg/L (Suomalainen and Lehtonen 1979).

In yeasts, the synthesis of fatty acids begins with acetyl coenzyme A (CoA) formed from the oxidative decarboxylation of pyruvate obtained from glycolysis and involves a multienzyme complex which binds all of the intermediates until the fatty acids are formed (Suomalainen and Lehtonen 1979). This synthesis is illustrated in Fig. 10.5 (Suomalainen and Lehtonen 1979), which provides a scheme for the formation and elongation of fatty acids that leads to even- or odd-numbered chains depending on whether acetyl-CoA or propionyl-CoA is the starting material. The way in which yeasts influence the fatty acid composition of alcoholic beverages is not known. The yeast strain used, the composition of the medium, the temperature, and the aeration employed influence to a great extent the final levels of fatty acids, carbon chain length, and level of saturation (Suomalainen and Lehtonen 1979). At lower temperatures, yeasts synthesize a larger amount of fatty acids when compared with the amounts synthesized at higher temperatures, with more of the unsaturated fatty acids being synthesized under aerobic and semiaerobic conditions. For example, the amount of the fatty acids caproic and caprylic secreted into the fermentation medium at 10°C by *S. cerevisiae* is higher than that secreted at 30°C, whereas

Table 10.2 Relative amounts of the main volatile fatty acids, excluding acetic acid, in the acid fractions of Martinique rum, Scotch whisky and cognac

Acid	Martinique rum (%)	Scotch whisky (%)	Cognac (%)
Propionic	15.7	1.5	2.7
Isobutyric	3.6	4.9	3.6
Butyric	15.3	1.5	3.6
Isovaleric	4.7	5.9	3.3
Valeric	6.5	0.1	Traces
Caproic	5.4	4.2	8.2
Caprylic	14.5	26.7	35.0
Capric	17.5	31.6	30.4
Lauric	6.5	16.2	8.6
Myristic	1.1	2.2	1.6
Palmitic	4.0	1.7	1.1
Palmitoleic	1.0	2.0	0.5
Others	4.2	1.5	1.4

Adopted from Nykanen et al. (1968)

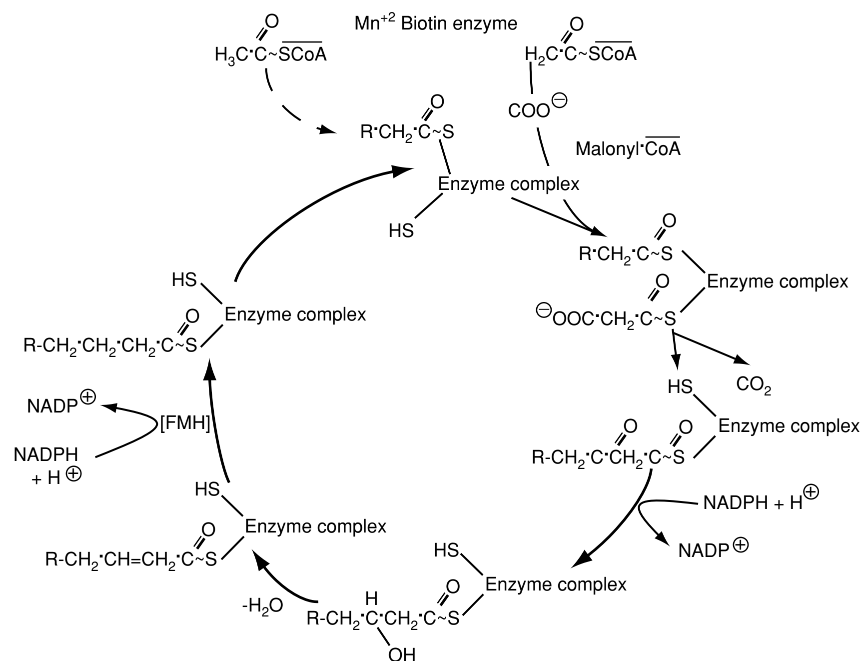

Fig. 10.5. The formation of fatty acids. (Adopted from Lynen 1967)

that of capric and lauric acids is independent of the fermentation temperature (Suomalainen and Lehtonen 1979). When C6–C10 fatty acids are present in beer in levels that exceed 1 mg/L, they contribute to what is known as a "caprylic flavour" characterized by a musty, rancid, or soapy odour (Tressl et al. 1980).

10.2.2.3 Fatty Acid Esters

Fatty acid esters are by far the most prevalent group of aroma compounds produced by yeast fermentation (Buzzini et al. 2003; Cristiani and Monnet 2001; Suomalainen and Lehtonen 1978, 1979; Verstrepen et al. 2003). The ester content of alcoholic beverages is reflected in the perception of their aromas as demonstrated by comparing light rums which have an ester content of 50 mg/L with heavier variety of rums which contain greater than 600 mg/L (Suomalainen and Lehtonen 1979). Fatty acid esters of alcoholic beverages are classified into three major fractions: light, middle, and heavy on the basis of their boiling-point ranges (Suomalainen and Lehtonen 1979). The light fraction consists of fruit esters with pleasant aromas which include all esters that have a boiling point lower than that of isoamyl alcohol such as ethyl, isobutyl and isoamyl esters of short-chain fatty acids (Suomalainen and Lehtonen 1979). The middle fraction comprises ethyl esters that elute during distillation between ethyl caproate and phenethyl alcohol. These include primarily ethyl esters

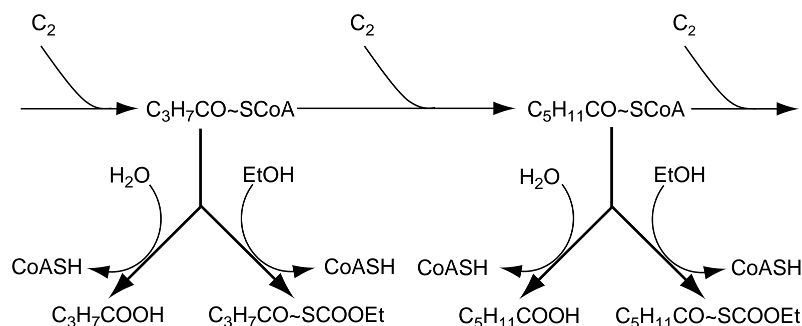

Fig. 10.6. Simplified scheme for the formation of esters and higher fatty acids (Adopted from Nordström 1964)

of caprylic and capric acids. The main components of the heavy distillate fraction are all other esters that elute after phenethyl alcohol (Suomalainen and Lehtonen 1979). The biosynthesis of esters is similar to that of fatty acids and is illustrated in Fig. 10.6 (Suomalainen and Lehtonen 1979). An increase in ethyl esters of caprylic, capric, and palmitoleic acids is noted if the yeast is present during distillation (Suomalainen and Lehtonen 1979; Verstrepen et al. 2003).

During fermentation in yeasts, the formation of esters is carried out by intracellular enzyme-catalysed reactions. The expression level of two genes coding for the yeast alcohol acetyltransferases, ATF1 and ATF2, was shown to influence the production of ethyl acetate and isoamyl acetate (Verstrepen et al. 2003, 2004). Double deletion of the former two genes resulted in partial reduction in the two previously mentioned esters, suggesting the existence of as-yet-unknown other ester synthases (Verstrepen et al. 2003). Ester production by yeasts is dependent to a great extent on the composition of the carbon source and the type of assimiliable nitrogen in the medium used, the fermentation parameters selected, such as pitching rate and top pressure, dissolved oxygen, as well as the genus, species, and strain employed (Verstrepen et al. 2003).

Several fatty acid esters produced by yeasts during alcoholic fermentations have found other applications and an example of this is the use of the short-chain acetate esters of ethyl acetate as a solvent, of isoamyl acetate as a banana food flavour, and of phenyl ethyl as a flowery or rose aroma enhancer in cosmetics and foods (Armstrong 1986; Ashida et al. 1987; Fabre et al.1998; Fukuda et al. 1990a, 1998a, b; Fujii et al. 1996; Furukawa et al. 2003a; Janssens et al. 1987; Quilter et al. 2003; Verstrepen et al. 2003). Screening a selected yeast for 2-phenylethanol production in a molasses-containing medium supplemented with phenylalanine has shown that *Kluyveromyces marxianus* CBS 600 and *K. marxianus* CBS 397 can produce up to 3 g/L at 35°C (Etschmann et al. 2003; Fabre et al. 1997, 1998). The production of the important food and cosmetic aroma compound ethyl oleate can also occur through enzymic catalysis by using immbolized cells of *S. bayanus* to produce ethanol in the presence of oleic acid in a fermentation medium (Kiss et al. 1998). The increased synthesis of ethyl caproate by *S. cerevisiae*, which is an important aroma and flavour compound in sake, has been attributed to inositol limitation

(Arikawa et al. 2000; Furukawa et al. 2003b). Fatty acid ethyl ester synthesis by *S. cerevisiae* is also important in the development of Scotch whisky aroma and flavour (Goss et al. 1999). The release of fatty acids and the production of medium-chain fatty acids and their ethyl esters in the absence of exogenous lipids by yeast strains isolated from musts and wines in the absence of aeration has recently been described (Bardi et al. 1999; Ravaglia and Delfini 1993). Medium-chain fatty acids and their esters are responsible for stuck alcoholic fermentation and their formation is strongly dependent on the yeast strain used and the fermentation medium (Ravaglia and Delfini 1993).

10.2.2.4 Carbonyl, Sulphur, and Phenolic Compounds

Carbonyl, sulphur, and phenolic compounds are other types of yeast fermentation by-products that impact the aroma of alcoholic beverages (Suomalainen and Lehtonen 1979). Owing to their lower sensory thresholds, these compounds are of particular interest as they can contribute to undesirable odours in alcoholic beverages such as beer (Arai 1980; Russell et al. 1983; Suomalainen and Lehtonen 1979; Tressl et al. 1980). The carbonyl compounds of great interest are the aldehydes that are intermediates in the formation of fusel alcohols. Diacetyl and 2,3-pentanedione are formed during fermentation from the decarboxylation of the two α-keto acids, α-acetolactic and α-aceto-α-hydroxybutyric acid, respectively (Suomalainen and Lehtonen 1979). These compounds, illustrated in Fig. 10.7, contribute to a buttery aroma in beer (Tressl et al. 1980). Sulphur compounds derived from the degradation of the amino acids cysteine and methionine lead to the formation of off-flavours in beer, such as the offensive smelling hydrogen sulphide, diethyl sulphide, and dimethyl sulphide (Suomalainen and Lehtonen 1979; Tressl et al. 1980; Van Haecht and Dufour 1995). Sulphites produced by yeasts during alcoholic fermentation can also have a positive effect as they act as antioxidants and flavour stabilizers (Hansen and

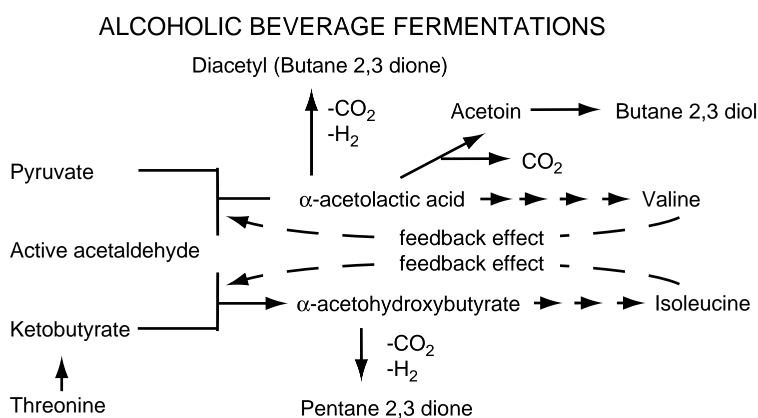

Fig. 10.7. Reduction of the level of diacetyl in wort by reduction of α-acetolactic acid through a feedback mechanism. (Adopted from Berry 1995)

Kielland-Brandt 1996). Inactivation of the MET2 gene which encodes the enzyme homoserine *O*-acetyltransferase increases the level of sulphite in beer (Hansen and Kielland-Brandt 1996). The constitutive expression of MET25 which codes for the enzyme *O*-acetylserine sulphydrylase leads to a reduction in the off-flavour compound hydrogen sulphide (Omura et al. 1995). Phenolic compounds produced during alcoholic yeast fermentations are derived from the catalysis of *p*-coumaric acid, ferulic acid, and vanillin, which are typically of plant origin (Ettayebi et al. 2003; Meaden 1998; Suomalainen and Lehtonen 1979). The products formed by yeast action consist of 4-ethylphenol, 4-ethylguaiacol, and 4-methyl guaiacol as shown in Fig. 10.8 (Dias et al. 2003; Meaden 1998; Suomalainen and Lehtonen 1979). These compounds, while desirable in low levels, can lead to phenolic off-flavours when present in levels in excess of 100 ppb in beer (Tressl et al. 1980). The production of phenolic off-flavours by wine yeast strains of *S. cerevisiae* and other yeast genera such as *Rhodotorula*, *Candida*, *Cryptococcus*, *Pichia*, *Hansenula*, *Dekkera*, and *Brettanomyces* that contribute to phenolic off-flavours in wine products (Coghe et al. 2004; Edlin et al. 1995; Shinohara et al. 2000). Mousy off-flavour production in grape juice and in red and white wines attributed to the yeast *Dekkera* (*D. bruxellensis* and *D. anomala*) and *Brettanomyces* yeasts has been recently described by Grbin and Henschke (2000) and by Dias et al. (2003).

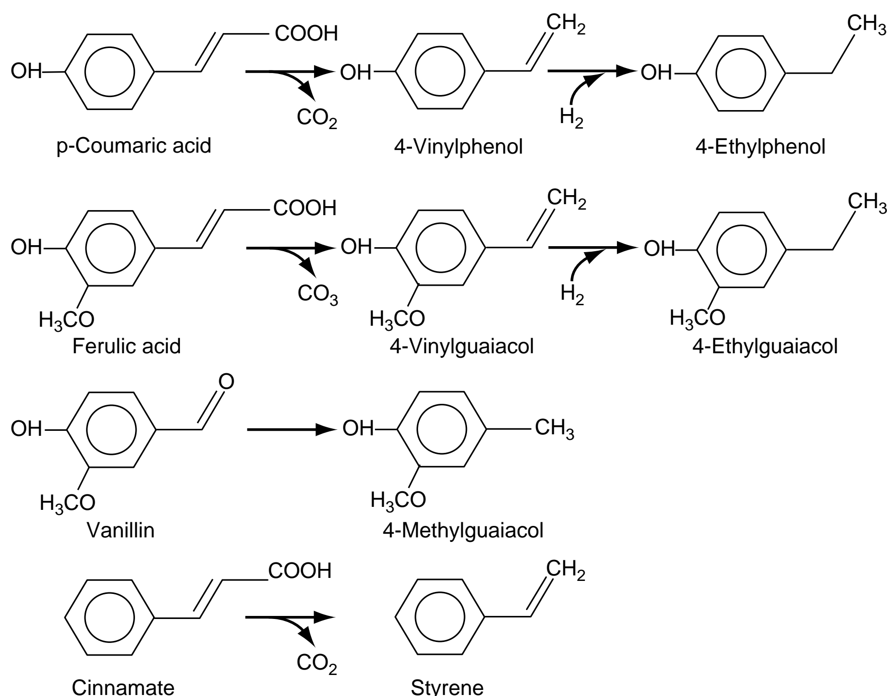

Fig. 10.8. The decarboxlyation of cinnamic acids by *Saccharomyces cerevisiae*

10.2.2.5 Lactones

Lactones are an important group of compounds that are of interest to the food industry as they impart highly pleasant ripened fruit aromas characteristic of peach, apricot, or coconut when added to over 120 foodstuffs (Dufosse et al. 2000, 2002; Endrizzi et al. 1996; Feron 1997; Gatfield et al.1993; Groguenin et al. 2004; Heath 1981; Vandamme 2003; Vanderhaegen et al. 2003; Van Der Schaft et al. 1992; Wache et al. 2003). Lactones are made up of a carbon ring with an oxygen atom and are formed by intramolecular esterification between the hydroxyl and carboxylic group of a hydroxy fatty acid as illustrated in Fig. 10.9. The recognition that the pigmented yeast *Sporobolomyces odorus* can synthesize the lactones responsible for the peach-like odour, γ-decalactone (GC10) and dodecenolactone, has given rise to interest in the de novo microbial production of these compounds from the readily available, natural ricinoleic hydroxy fatty acid (Endrizzi et al. 1996). This hydroxyacid represents over 90% of castor bean oil and thereby provides an economical source as a feedstock which can compete with the chemical synthetic route (Endrizzi et al. 1996). While several yeasts can carry out the aforementioned biotransformation most recent focus has been on using three yeasts *Yarrowia lipolytica*, *Sporidiobolus salmonicolor* (*Sporidiobolus odorus*), and *Sporidiobolus ruinenii*, with reported production of GC10 in the range 0.4–1.2 g/L (Groguenin et al. 2004; Dufosse et al. 2000, 2002; Feron et al. 1997). These levels are below the 5.5 g/L reported earlier for *Y. lipolytica* (Farbood and Willis 1985). A schematic representation for the pathway for the key acyl oxidase enzymes involved in the production of GC10 from ricinoleic acid in *Y. lipolytica* is provided in Fig. 10.10 (Groguenin et al. 2004). The key challenges to the further improvement of industrial processes with the three yeasts are the toxicity, yield, and final concentration and composition of the lactones produced. Several strategies can be employed to overcome these challenges, ranging

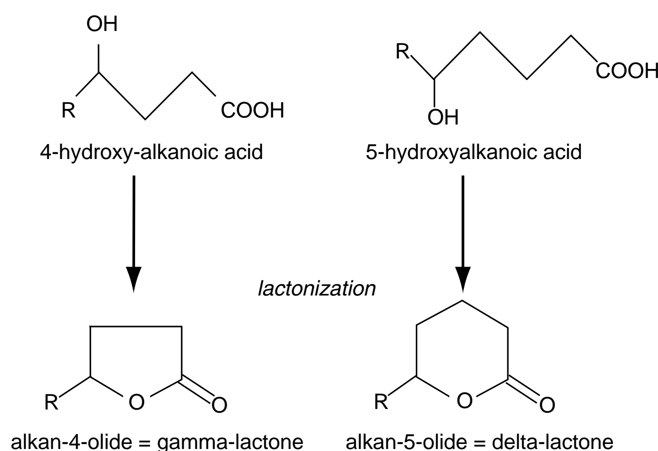

Fig. 10.9. Structure of γ- and δ-lactones and of corresponding hydroxy acids. (Adopted from Groguenin et al. 2004)

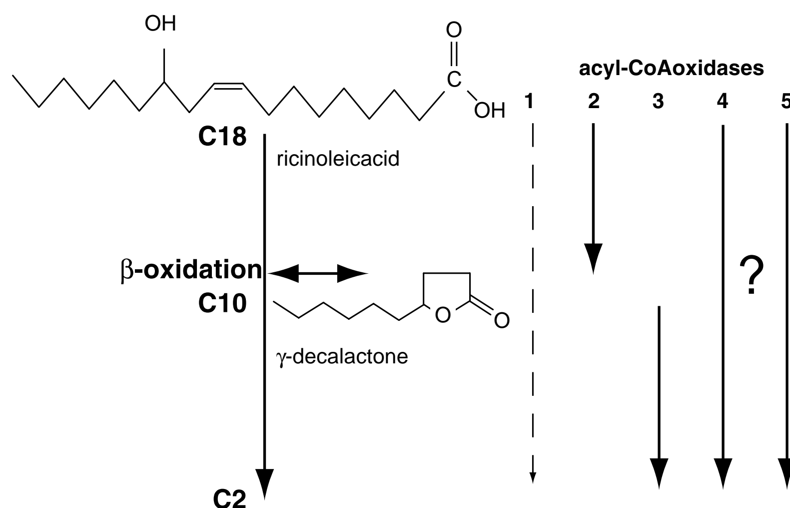

Fig. 10.10. Schematic representation of the pathway from ricinoleic acid to γ-decalactone and activities of the acylcofactor A (*acyl-CoA*) oxidases of *Yarrowia lipolytica*. (Adopted from Endrizzi et al. 1996)

from genetic engineering and/or modification of yeast strains, use of batch vs. fed-batch fermentation, to the continuous extraction of the GC10 decanolide with the use of a membrane based solvent-extraction technique (Akita and Obata 1991).

10.2.2.6 Other Chemical Compounds

Other chemical compounds that contribute to the aroma of yeast-fermented products include breakdown products from sugars such as the furanones, furfural and hydroxymethyl furfuryl, and their alcohol derivatives, production of chiral alcohols from prochiral ketones, volatile organic acids such as acetic acid, which imparts a sour or vinegar smell, pyrazines which are formed from reactions of sugar aldehydes with amino acids which undergo a Strecker degradation reaction followed by self-condensation and oxidation to produce these compounds with burnt or roasted aroma notes, and monoterpene-derived alcohols which are formed from monoterpene hydrocarbons, which are important components of essential oils and convey a fresh fragrance to foodstuffs (Heath 1981; Hecquet et al. 1996; King and Dickinson 2000; Kometani et al. 1995; Maga 1982, 1992; Vandamme 2003).

10.2.3 Colours

Concerns about the use of chemically produced dyes in foodstuffs have resulted in the increased use of naturally produced colorants that are derived from animal, plant, and microbial sources. As a part of this trend, yeasts that are commonly found as part of the food flora and thereby generally recognized as safe (GRAS)

have been tapped as sources of a number of carotenoids for use as food colorants such as astaxanthin, β-carotene and γ-carotene, lycopene, lutein, torulene, torulahordin, and zeaxanthin (An et al. 2001; Bekers et al. 1972; Bhosale and Gadre 2001a–c; Bogdanovskaya 1973; Bogdanovskaya and Zalashko 1982; Bon et al. 1997; Buzzini 2000; Calo et al. 1995; Cang et al. 2002; Cannizzaro et al. 2003; Chen and Liu 1999; Farid and Azar 2001; Fontana et al. 1996a, b, 1997; Frengova et al. 2003, 2004; Guirnovich et al. 1966; Guirnovich and Koroleva 1971; Jacob 1991; Johnson and Schroeder 1995; Johnson 2003; Kilian et al. 1996; Koroleva et al. 1982; Kusdiyantini et al. 1998; Lewis et al. 1990; Liang and Zhang 2003; Martin et al. 1993; Matelli et al. 1990; Misawa 1997, 1999; Misawa et al. 1998; Misawa and Shimada 1998; Miura et al. 1998; Nikolaev et al. 1966; Parajó et al. 1997, 1998a, b; Pshevorskaya and Zabrodskii 1972; Ramirez et al. 2000; Reynders et al. 1996; Sakaki et al.1999, 2001, 2002; Shih and Hang 1996; Shimada et al. 1998; Shitazawa et al. 2002; Simova et al. 2003; Slyusarenko et al. 1976; Squina et al. 2002; Vazquez et al. 1998; Vazquez and Martin 1998; Yang et al. 1993; Zalaskho et al. 1973; Zeile 1972). A number of yeasts are known to produce carotenoids that have been evaluated as food colorants and these include several species of *Rhodotorula* (*R. glutinis*, *R. lactis*, *R. gracilis*, *R. rubra*), *Rhodosporidium*, *Phaffia rhodozyma*, and *Sporobolomyces pararoseus*. Improving the commercial potential for yeast production of the previously mentioned carotenoids has been pursued with improved yeast strains from the aforementioned yeasts and from selected and genetically engineered strains of *S. cerevisiae* (Misawa and Shimada 1998), *C. utilis* (Misawa et al. 1998; Shimada et al. 1998), so-called red yeast, (Fang and Chiou 1996; Yang et al. 1993), and other *Candida* species that have been developed through classical selection and genetic engineering methods (Bekers et al. 1972; Bhosale and Gadre 2001a–c; Bogdanovskaya and Zalashko 1982; Buzzini 2000; Cang et al. 2002; Chen and Liu 1999; Cheng et al. 2004; Frengova et al. 2003, 2004; Girard et al. 1994; Jacob 1991; Koroleva et al. 1982; Martin et al. 1993; Misawa et al. 1998, 1999; Misawa and Shimada 1998; Nikolaev et al. 1966; Pshevorskaya and Zabrodskii 1972; Sakaki et al. 1999; Shih and Hang 1996; Shimada et al.1998; Simova et al. 2003; Slyusarenko et al. 1976; Squina et al. 2002; Zalaskho et al. 1973; Zeile 1972; Yang et al. 1993). The commercial exploitation of one of these yeasts, *Phaffia rhodozyma* or *Xanthophyllomyces dendrorhous*, has resulted in industrial-scale production of the carotenoid astaxanthin (Abbas 2001, 2003, 2004; Jacobson et al. 2000; Palagyi et al. 2001; Kim et al. 2003). Astaxanthin-containing yeasts and astaxanthin extracted from these yeasts has in addition to aquafeed uses as a colorant, other uses as an immunostimulant in poultry, fish, mammalian feed, and food when combined with other antioxidants derived from plant sources such as vitamin E. Other colorants of foods that are produced by yeast fermentation are the vitamin B_2 or riboflavin and caramel colours produced from processing of yeast extracts (Halasz and Lasztity 1991).

10.2.4 Flavours

Flavours can be classified in multiple ways depending on their mode of formation either naturally by biogenetic pathways from known precursors or by processing in which biological, chemical, physical, or a combination of these approaches is

employed (Heath 1981). Natural flavours are mostly metabolites produced in living cells and thereby their formation is strongly dependent on the genetic traits and the environmental conditions under which they are produced (Heath 1981). Flavours produced by processing are generally breakdown products or complex products derived from the interaction of components within a food matrix under the conditions selected (Heath 1981; Chao and Ridgway 1979). Products produced by brewer's yeast that contribute to both aroma and flavour include higher alcohols and their derived esters, carbonyl compounds, and sulphur-containing compounds. Many of these products are derived from amino acid metabolism in yeasts, while others are derived from carbohydrate and lipid metabolism. Yeast-produced flavours can be classified into three very general categories: yeast metabolic products, and this includes products synthesized or derived through yeast biocatalysis, yeast cell mass derived products, which include products prepared through yeast autolysis, and complex products resulting from the interaction of yeast-derived products with other food matrix ingredients. Products representing each category are provided in Table 10.3 and it should be noted that in some cases many of these products have in addition to flavour attributes, aroma, antioxidant, colorant, vitamin, and nutraceutical properties.

10.2.4.1 Yeast Metabolic Products

The metabolic products of yeasts that are used as flavours include a variety of short or long branched or unbranched alcohols, and aromatic alcohols which include in

Table 10.3 Yeast products

Yeast metabolic products	Yeast-derived products	Yeast beverage and food flavour products
Alcohols	Yeast extracts	Baking or bread flavours
Aldehydes	Ribonucleotides	Beer or malt flavours
Amino acids	Cell wall mannoproteins	Cheese and other dairy product flavours
Carbohydrates and other glycosides	Cell wall glucans	Cocoa flavours
Carotenoids	Edible proteins	Distilled spirits flavours
Fatty acids	Choline, glycerol, and inositol	Edible meats flavours
Esters(fragrances)	Mineral-enriched yeast	Roasted flavours
Yeast metabolic products	Yeast-derived products	Yeast beverage and food flavour products
Lactones	Sterols	Soy-derived flavour products
Phenolic compounds flavours	Vitamins	Maple or syrup or caramel
Polyols	Whole cell or single-cell proteins or single-cell oils	Wine, cherry, brandy, and sake flavours
Organic acids	Yeast enzymes	
Terpenes		
Vitamins		

addition to ethyl alcohol, phenyl ethyl alcohol, propyl alcohol, butyl alcohol, octyl alcohol, isoamyl alcohol; amino acids and their breakdown derivatives that are used for their sweet or bitter taste; carbohydrates, glycosides, or polyols such as glycerol, xylitol, and inositol that are used as thickeners, sweeteners, emulsifiers, or for their organoleptic properties; fatty acids and fatty acid esters, and organic acids such as isoamyl acetate, ethyl acetate, ethyl caproate, acetic, formic acid, citric acid, caprylic acid, and lactic acid, which impart a mouth taste of pleasant, sour, or salty notes; and esters, lactones, and aldehydes such as acetaldehyde and GC10 that contribute to a fragrarent, astringent, and sweet taste (Asano et al. 2000; Hecquet et al. 1996; Vandamme 2003; Van den Bremt et al. 2001).

10.2.4.2 Yeast-Derived Products

10.2.4.2.1 Yeast Extracts and Yeast-Hydrolysed Proteins

Yeasts extracts are flavour products that are primarily derived from the soluble fraction of yeasts enriched in amino acids such as glutamic acid in the free acid form or as monosodium glutamate provide a bouillon-like, brothy taste to food (Nagodawithana 1992). These products have found uses by the food industry as lower-cost-flavour protein sources that are competitive with hydrolysed vegetable proteins (HVPs) from soy, wheat, and from other vegetable origins (Cooper and Peppler 1959; Nagodawithana 1992). Yeast extracts are usually derived from yeasts specifically grown under optimized conditions for food application (Chae et al. 2001; Cooper and Peppler 1959; Nagodawithana 1992). In some cases yeast extracts can be derived from spent brewer's yeast, which is readily available from alcohol production facilities in great abundance and thereby represents an inexpensive readily available feedstock (Chae et al. 2001; Nagodawithana 1992). Yeast extracts are provided as powders or pastes and are used at 0.1–0.5% finished dry product basis (Nagodawithana 1992). The manufacturing of yeast extracts employs one of three distinct processes that use autolysis, plasmolysis, or hydrolysis (Chae et al. 2001; Halasz and Lasztity 1991; Lieske and Konrad 1994; Nagodawithana 1992; Peppler 1967, 1970; Reed 1981). The yeast extracts prepared from these processes have different cost structures and product and flavour attributes, and consequently have some tradeoffs. These tradeoffs consist of the cost of using commercial enzymes to autolyse the yeast vs. yeast self-autolysis or the use of high salt, mechanical disruption, or an acid hydrolysis step followed by soluble product recovery (Chae et al. 2001; Lieske and Konrad 1994). Modifying the flavour and colour profile of yeast extract can be accomplished with selected fermentation processes and media as well as by subjecting yeast slurry prior to extraction to O_2 at 60°C for up to 30 min as described in a recent patent (Lieske and Konrad 1994). In this process pasteurized yeast (15% cells by weight) was treated with pure O_2 in a closed reactor at an O_2 partial pressure of 1 atm and pH 6.1, with stirring at 500 rpm. After 15 min, the yeast flavour was modified from very yeasty to mild and the pink colour present initially reduced significantly (Lieske and Konrad 1994). Another mild process for the extraction of yeast was developed that consists of preparing a slurry of a low-moisture active-spray-dried yeast in water at a ratio of 1:5 at 39°C followed by stirring for 30 min and centrifuging the treated material at 2,300 rpm to remove the

soluble fraction which contains trehalose (Cooper and Peppler 1959). The residue after centrifugation was diluted to provide a cream of 16% solids that can be drum-dried. The product derived by this process had a light colour, reduced flavour, and was low in ash but high in protein and nucleic acids when compared with the original composition of the viable yeast. The extract prepared by this process has found many uses in various types of food and feed products. Another yeast extraction process was developed that uses brewer's yeast from a beer factory by combined enzymatic treatments using the enzymes endoprotease, exoprotease, 5′-phosphodiesterase, and adenosine monophosphate (AMP) deaminase (Chae et al. 2001). The effects of enzyme combination, enzyme dosages, and treatment sequence on the recovery of solid and protein, flavour, and compositional characteristics were all investigated (Chae et al. 2001). It was determined that exoprotease dosage strongly affected the recovery of protein and the degree of hydrolysis and sensory characteristics. When the yeast cells were treated using optimal combination of endoprotease and exoprotease (0.6% Protamex and 0.6% Flavourzyme), high solid recovery (48.3–53.1%) and the best flavour profile were obtained. Among various treatment sequences using multiple enzymes, treatment with protease followed by nuclease resulted in the highest 5′-guanosine monophosphate (5′-GMP) content. The optimal concentrations of both 5′-phosphodiesterase and AMP deaminase were found to be 0.03%. After treatments using the optimal combination of enzyme, enzyme dosages, and treatment sequence for four enzymes, a high solid yield of 55.1% and a 5′-nucleotides content of 3.67% was obtained (Chae et al. 2001). A cruder form of yeast extract that is made from both soluble and insoluble fractions by autolysis is employed when a cost-effective product with increased water-binding capability and milder yeast flavour is desired as is the case for meat analogues (Nagodawithana 1992).

Yeast extracts are very powerful savoury flavour ingredients. The sensory quality of yeast extracts is highly influenced by the specific molecular cell composition of the yeast cell. Hydrolysed edible proteins and peptides from yeasts are a good source of the amino acid L-lysine and therefore can be used in food products that need supplementation with this amino acid. The sulphur-containing amino acids cystine, cysteine, and methionine are the limiting amino acids in yeast extracts and therefore attempts have been made to enrich yeast extracts with these amino acids (Reed 1981). Cysteine is a source of sulphur in the generation of meat-like flavours by Maillard chemistry (Hurrell 1982; Stam et al. 2000). Recently, an enriched yeast extract in the amino acid cysteine was produced through the application of genetic engineering to overexpress and overproduce the yeast cysteine-rich protein metallothionein (Stam et al. 2000). This approach can result in the enhancement of the savoury value of yeast extracts prepared by this approach for meat, baked goods, and cheese food applications. Another example of widespread use for yeast proteins and peptides in foodstuffs is as cocoa replacement flavouring components as these can be produced by roasting of yeast extracts in combination with chemical processing (Liggett 1978).

10.2.4.2.2 Nucleotides

The RNA compositions of baker's yeast and of *C. utilis* yeast used to produce single-cell protein have been reported in the range 8–11 and 10–15%, respectively

(Nagodawithana 1992). The ease and cost effectiveness of the methods of production of these two yeasts has resulted in their use to prepare yeast extracts with a high concentration of RNA. The ribonucleotides 5′-inosine monophosphate (5′-IMP) and 5′-GMP are important flavour components of yeast extracts. Ribonucleotide-enriched fractions of yeast extracts are prepared with the nucleotides 5′-GMP and 5′-IMP by enzymic degradation of yeast cellular RNA in hydrolysed yeast extract cell preparations (Chae et al. 2001; Halasz and Lasztity 1991; Nagodawithana 1992; Noordam and Kortes 2004; Patane 2004). A yeast–malt sprout extract was produced by the partial hydrolysis of yeast extract (derived form *S. fragilis* or *C. utilis*) using the sprout portion of malt barley as the source of enzymes, and may be used under the Federal Food, Drug, and Cosmetic Act as a flavour enhancer in food. It contains a maximum of 6% 5′-nucleotides (USA Food and Drug Administration 1973). An improved method for the production of these flavouring agents using liposomes containing 5′-phosphodiesterase that is fused with spheroplasts or protoplast prepared from yeast cells is described in a recent patent application (Patane 2004). The enzymes used are fungal or plant 5′-phosphodiesterases which can release the four free RNA 5′-nucleotides as the final products. Further treatment of the 5′-nucleotide-enriched yeast extract preparations with the enzyme adenyl deaminase from *Aspergillus melleus* converts the 5′-AMP to the flavour-enhancing nucleotide 5′-IMP (Kondo et al. 2001; Nagodawithana 1992; Steensma et al. 2004). The results of the safety evaluation and toxicological data of phosphodiesterases derived from *Penicillium citrinum* and from *Leptographium procerum* are summarized in recent publications (Kondo et al. 2001; Steensma et al. 2004). When used in conjunction with monosodium glutamate or glutamic acid containing peptides in yeast extracts, the aforementioned nucleotides enhance mouthfeel in soups, sauces, marinades, soft drinks, cheese spreads, and seasonings by contributing a meaty flavour with sour, sweet, salty, and/or bitter notes (Noordam and Kortes 2004).

10.2.4.2.3 Yeast Polysaccharides

The major yeast cell wall polymers glucans, mannans, phosphomannans, and mannoproteins have recently been tapped for their potential flavour application as food fibre additives, for their emulsifying properties, and for their nutraceutical potential (Abbas 2003; Halasz and Lasztity 1991; Peppler 1970; Reed 1981; Sucher et al. 1974). These cell wall polymers have been characterized from a number of yeasts which include *S. cerevisiae*, *Pichia holstii*, *Hansensula* sp., a number of *Candida* species, and several *Rhodotorula* sp. Other yeast-produced exopolysaccharides that have gum-like properties that have received recent attention are those produced by *Cryptococcus laurentii* and the yeast-like fungi *Tremella mesenterica* and *T. fuciformis* (De Baets and Vandamme 1999).

Sucher et al. (1974) have described in some detail capturing the greatest value from yeast cell mass from the processing and fractionation of cells to prepare yeast extract proteins, ribonucleotides, and cell wall glucans. The approaches detailed by Sucher et al. in 1974 are still characteristic of current practices employed in the commercial production of yeast-derived products. The process described involved the homogenization of washed yeast cells, followed by alkaline treatment, and centrifugation to remove cell wall constituents. The glucan thus separated was further

washed and dried under vacuum to yield a mild-tasting product which formed a viscous solution upon rehydration. The alkali extract was then acidified to pH 4.5 to produce an isolated yeast protein with a considerable RNA content. The RNA content could be reduced by nuclease treatment and the resultant product used as an amino acid supplement for human consumption. The original RNA contents of 9–14% were typically reduced to less than 3%. After precipitation of the enzyme-treated isolated yeast protein, a yeast extract with a fried-meat flavour is produced with a glutamic acid content of 27–40% of the corresponding remaining protein (Sucher et al. 1974).

10.2.4.2.4 Mineral-Enriched Yeast

Yeasts grown in a medium containing high levels of metallic cations such as chromium, selenium, germanium, or zinc tend to accumulate these metals intracellulary (Halasz and Lasztity 1991; Reed 1981). Owing to their important biological functions as cofactors in enzyme reactions and in other intracellular proteins such as in the case of the chromium-containing glucose tolerance factor (GTF), the use of yeasts cells fortified in these metals as food supplements has gained ground (Halasz and Lasztity 1991; Reed 1981).

10.2.4.2.5 Membrane Lipids and Extracts

Yeast-derived lecithin, inositol, choline, glycerol, and glycolipids can be recovered from the cell membrane lipid extracts or from fermentation broth (Bednarski et al. 2004; Halasz and Lasztity 1991; Harrison 1970). Owing to their physical attributes the use of these components has long been established as flavours in foodstuffs as emulsifiers and surfactants. Owing to their additional health benefits, these components can also be used to fortify foods as food supplements. A commercial process to produce glycerol by fermentation using a strain of *C. glycerinogenes* has been developed and genetically engineered strains of *S. cerevisiae* that can overproduce glycerol or inositol have been reported (Omori et al. 1995; Zhuge et al. 2001).

10.2.4.2.6 Vitamin-Enriched Yeast

Yeast cells are good sources of a number of the B vitamins such as thiamine, pantothenic acid, riboflavin, vitamin B_6, and vitamin B_{12} (Halasz and Lasztity 1991; Harrison 1970; Peppler 1970; Reed 1981). Yeast cells are also good sources of biotin, folic acid, and of ergosterol, which can be converted by UV radiation to vitamin D_2 (Reed 1981). In addition to vitamins, yeasts are also a good source of coenzyme Q.

10.2.4.2.7 Single-Cell Proteins or Oils

A number of other whole-cell yeast products in use consist of use as a single-cell protein or single-cell oil primarily in animal feed applications where yeasts are grown on food processing waste streams from grain, oilseed, candy, brewery, dairy, and wood processing and vegetable oil refinery by-products (Farid and Azar 2001; Ghazal and Azzazy 1996; Halasz and Lasztity 1991; Harrison 1970; Papanikolaou et al. 2002; Peppler 1970, 1979; Reed 1981). Owing to regulatory issues and finished product quality consistency issues, these products have had a limited direct application in foodstuffs.

10.2.4.2.8 Yeast Enzymes

Intracellular yeast enzymes can be prepared from whole yeast cell mass by mechanical disruption and other means. These enzymes have found several food uses (Harrison 1970; Halasz and Lasztity 1991; Mosiashvili et al. 1971; Peppler 1979; Reed 1981). Examples of these are the use of yeast invertase obtained from *S. cerevisiae* and other sucrolytic food yeasts in the confectionary industry to break down sucrose to manufacture liquid-centre candies (Halasz and Lasztity 1991; Reed 1981). Yeast lactases obtained from the GRAS milk sugar fermenting yeast genus of *Kluyveromyces* are used to hydrolyse milk sugar or cheese whey to prepare sweeter sugar slurry. Baker's yeast ribonuclease for is used for RNA digestion during the manufacture of yeast nucleotides. Most recently yeast cytosolic oxidoreductases of brewer's yeast have been used to block Maillard reaction of dicarbonyl intermediates, thereby preventing their decomposition to off-flavour final products (Halasz and Lasztity 1991; Reed 1981; Sanchez et al. 2003).

10.2.4.3 Yeast Beverage and Food Flavour Products

Yeast and yeast metabolic products contribute to the formation of unique distinctive flavours through the formation of complex chemical substances and through other physical interactions with food and beverage matrices in several fermented foods. These reactions involve numerous components that are not fully characterized and contribute to a yeasty character that is frequently used to identify yeast-fermented and/or yeast-containing product.

10.2.4.3.1 Bread and Baked Products

Among these many products are crusty baked breads that are characterized by intense roasted odorant flavours. These flavours are attributed in great part to bread dough composition and preparation methods as nonfermented doughs lack the desired bread taste (Schieberle 1990). It is currently recognized that bread flavour is affected by a great number of compounds produced during fermentation and during baking which include alcohols, diacetyls, esters, organic acids, and carbonyl and other compounds (Annan et al. 2003; Imura et al. 2003; Watanabe et al. 1990). A recent review with 58 references covered many attributes of bread production and included commercial formulations of bakers' yeast, yeast production practices, yeast metabolism and nutrition, application of yeast to bread making, leavening activity, and taste and flavour of yeast-leavened bakery products (El-Dash 1969; Randez-Gil et al. 1999; Van Dam 1986). In this review it is indicated that at least 211 different compounds have been identified in baked breads (Van Dam 1986). Baker's yeast contributes significantly to the formation of these flavour compounds in doughs and breads and these include the alcohols ethanol, propanol, butanol, butan-2-ol, pentanol, pentan-2-ol; the organic acids acetic and lactic; the carbonyl compounds acetaldehyde, propanal, butanal, pentanal, and furfural; and browning reaction products such as melanins and caramels that are concentrated in the bread crust (Van Dam 1986). Several detailed studies have indicated that bakers' yeast is an important source of Maillard-type bread-flavour compounds (Schieberle 1990). The most important odorants present in nonheated

yeast/sucrose and heated homogenates were determined and these consisted in nonheated homogenates, of the odorants 2- and 3-methylbutanoic acid, furaneol, butanoic acid, 2-methylpropanoic acid, 4-vinyl-2-methoxyphenol, and phenylacetaldehyde. After heating the homogenate, 2-acetyl-1-pyrroline (ACPY), methional, GC10, and γ-dodecalactone, followed by 2-acetyltetrahydropyridine (ACTPY), 3-methylbutanol, and 2,5-dimethyl-3-ethylpyrazine became the predominant odorants. A comparison with the primary odorants of wheat bread crust revealed that the yeast is a potent source of the important crust odorants ACPY, methional, ACTPY, and furaneol. Model experiments carried out further to determine the source of these crust odorants showed that ACPY and ACTPY are formed in yeast from the reaction of proline with 2-oxopropanal. The importance of sulphur-containing compounds in enhancing the roasty notes of bakery products is confirmed by the use of the beef broth flavour compound 2-acetyl-2-thiazoline and other sulphur-containing compounds (Bel Rhlid et al. 1999).

The role played by higher alcohols in bread flavour has been demonstrated through the selection of isobutyl and isoamyl alcohols overproducing mutants resistant to 4-aza-DL-leucine from a bakery yeast strain of *S. cerevisiae* (Watanabe et al. 1990). Many mutants that produced more isobutyl alcohol or isoamyl alcohol than the parent strains were obtained. In the evaluation of these mutants, bread containing more isobutyl alcohol was evaluated as giving a favourable characteristic flavour, but bread with more isoamyl alcohol was unfavourable. These mutants were able to ferment dough at similar rates to commercial bakers' yeasts. The mutants overproducing isobutyl alcohol or isoamyl alcohol were released from inhibition of the key enzymes acetohydroxy acid synthase and α-isopropylmalate synthase, respectively, in the pathway of branched-chain amino acid synthesis (Fukuda et al. 1990b; Watanabe et al. 1990).

Baker's yeast has also been demonstrated to contain chemoenzymatic synthetic capability through the synthesis of the aroma active 5,6-dihydro- and tetrahydropyrazines from the aliphatic acyloins that it produces (Kurniadi et al. 2003). The published work described the generation of 25 acyloins by biotransformation of aliphatic aldehydes and 2-ketocarboxylic acids using whole cells of baker's yeast as a catalyst. Six of these acyloins were synthesized and tentatively characterized for the first time. Subsequent chemical reaction with 1,2-propanediamine under mild conditions resulted in the formation of 13 5,6-dihydropyrazines and six tetrahydropyrazines. Their odour qualities were evaluated, and their odour thresholds were established. Among these pyrazine derivatives, 2-ethyl-3,5-dimethyl-5,6-dihydropyrazine (roasted, nutty, 0.002 ng/L air), 2,3-diethyl-5-methyl-5,6-dihydropyrazine (roasted, 0.004 ng/L air), and 2-ethyl-3,5-dimethyltetrahydropyrazine (bread crust-like, 1.9 ng/L air) were the most intensive-smelling aroma-active compounds (Kurniadi et al. 2003). While bread making is one of the oldest food-manufacturing processes, it is only in the past few years that recombinant-DNA technology has led to dramatic changes in formulation, ingredients, or processing conditions. New strains of baker's yeast that produce CO_2 more rapidly, are more resistant to stress, or produce proteins or metabolites that can modify bread flavour, dough rheology or shelf life are now available (Randez-Gil et al. 1999).

10.2.4.3.2 Beer Flavouring

The liquid substances recovered following ethanol removal from beers produced with malts and other grains have found many uses in foods and alcoholic beverages where natural flavours or bioflavours are sought (Vanderhaegen et al. 2003; Ziegler 1972). Recent growth in the use of bioflavouring agents has been fuelled by the increased demand of consumers for natural products that do not pose health or environmental disposal risks (Vanderhaegen et al. 2003). These bioflavouring substances can be produced from beer refermentation using nonconventional yeasts or genetically engineered yeasts that impart unique or additional flavours. In another variation, spent yeast cells from stillage from the production of spirits are reslurried and combined in an aqueous malty fermentation medium that has been treated with enzymes to saccharify the sugars (Vanderhaegen et al. 2003). Where a colourless flavoured malt beverage is desired, enzymes are added first to malt water slurry to convert the nonfermentable sugars in malt (Word et al. 1994). The mash is heated and the liquid extracted and combined with a fermentable carbohydrate to yield a mixture which is boiled and inoculated with yeast. The resulting yeast-fermented product is decolorized to produce a clear and colourless base, which is combined afterwards with a sweetener, tartaric acid, a buffer, and a flavouring agent, followed by carbonation, until the product contains CO_2 in the range 0.48–0.57% by weight. The final product is clear and colourless, has a finished alcohol-to-extract weight percent ratio of 1:0.4–1.5, and is relatively low caloric (8–15 cal/oz). The selected parameters serve to minimize consumer sensations of fullness and excess tartness, sweetness, and astringency, while producing desirable taste characteristics and an attractive appearance.

10.2.4.3.3 Cheeses

When freshly made, most cheeses have little flavour and are often bland (Heath 1981; Moskowitz 1980). It is during ripening that distinctive flavours develop in cheese from the degradation of carbohydrates, proteins, and fats (Heath 1981). These reactions proceed until equilibrium is reached under the conditions of aging employed (Heath 1981). Cheese flavour compounds include a number of hydrocarbons, alcohols, carbonyls, acids, esters, lactones, sulphur-containing compounds, amines, and other miscellaneous flavour components. Yeast flora of dairy products, yeasts included in starter cultures with bacteria and/or moulds, and yeast-derived extracts all contribute to the flavour of a variety and types of cheeses and other fermented dairy products. The yeast flora of dairy products is diverse and includes *Debaryomyces hansenii*, *Geotrichum candidum*, *Y. lipolytica*, *K. lactis*, *C. zeylanoides*, *C. lipolytica*, *C. mycoderma*, *D. kloeckeri*, and *C. lambica* (Anderson and Day 1966; Arfi et al. 2003; Chang et al. 1972; Fadda et al. 2004; Hosono and Tokita 1970; Kalle and Deshpande 1977; Martin et al. 2001; Petersen et al. 2002). The yeast floras of artisanal Fiore Sardo cheese, of a cheese curd, a processed cheese, cheeses produced primarily with yeast, of Limburger cheese, and blue and Roquefort cheese provide examples of the contribution of yeasts to cheese flavour development during ripening (Anderson and Day 1966; Chang et al. 1972; Fadda et al. 2004; Hosono and Tokita 1970; Kalle and Deshpande 1977; Martin et al. 2001). *K. lactis* produced large amounts of alcohols, aldehydes, esters, and terpenes when cultured alone or in association with the mould *G. candidum* and especially *G. candidum* strain G3, and

generated the largest amount of sulphides when cultured alone or in association in cheese curd (Martin et al. 2001). Some of the yeasts tested were shown to metabolize the milk sugar lactose, are tolerant to 3–7% NaCl, grow on lactic acid, a product of bacteria used in starter cultures, and are frequently proteolytic and/or lipolytic (Anderson and Day 1966; Chang et al. 1972; Fadda et al. 2004; Hosono and Tokita 1970; Kalle and Deshpande 1977; Martin et al. 2001). Cheese ripened mainly with *K. fragilis* contained upon analysis four aldehydes, four methyl ketones, seven alcohols, five esters and dimethyl sulphide after 3 weeks (Chang et al. 1972). The amounts of carbonyl compounds were rather small when compared with those for various cheeses of other types but the production of methyl ketones, alcohols, and esters was enhanced by the use of this yeast. The concentrations of ethanol, isoamyl and active amyl alcohol, and ethyl acetate were especially high and these compounds were considered to be responsible for the characteristic flavour of yeast-ripened cheese. *C. mycoderma* and *D. kloeckeri* grew in the presence of 3 and 7% NaCl, respectively, in Limburger cheese and produced relatively high proteolytic activities (Hosono and Tokita 1970). H_2S, volatile fatty acids, and volatile carbonyl compounds were produced by both *C. mycoderma* and *D. kloeckeri*, although the amounts of these volatiles differed. In comparison with the volatiles produced by *Brevibacterium linens*, the kinds and amounts of the volatiles produced by these yeasts were generally limited. These yeasts appear to play an important role in the development of flavour in Limburger cheese (Hosono and Tokita 1970). Yeasts associated with Blue cheese are capable of reducing methyl ketones to secondary alcohols and may play a role in flavour development by producing ethanol, other alcohols, and certain esters. Methyl and ethyl esters and 2-phenylethanol produced by yeasts are important in Blue cheese flavour. Quantitative data on the C3, C5, C7, C9, and C11 methyl ketones in Blue and Roquefort cheese showed considerable variation among samples, but no consistent differences between Blue and Roquefort cheese. This is in contrast to the selective conversion of the C8:0 fatty acids and, to a lesser extent, the 6:0 and 10:0 fatty acids to methyl ketones during cheese curing. The C5, C7, and C9 secondary alcohols were measured in Blue cheese by gas chromatography using the methyl ketones as internal standards. The alcohols were present in lower concentrations than the ketones. Ribonucleotides have been shown to contribute to the taste and/or the aroma and/or the mouthfeel of a low fat spread cheese that resembles the taste and/or the aroma and/or the mouthfeel of the full fat spread cheese (Noordam and Kortes 2004).

10.2.4.3.4 Cocoa Flavours

The yeast flora associated with the fermentation of cocoa bean pods in the fields of the countries of origin contributes to the early development of cocoa flavours (Schwan and Wheals 2004). The first stage of chocolate production consists of a natural, 7-day microbial fermentation of the pectinaceous pulp surrounding beans of the tree *Theobroma cacao*. There is a microbial succession of a wide range of yeasts, lactic acid bacteria, and acetic acid bacteria that carry out a fermentation during which high temperatures of up to 50°C are reached. The physiological roles of the predominant microorganisms are now reasonably well understood and the crucial importance of a well-ordered microbial succession in cocoa aroma has been

established (Schwan and Wheals 2004). During the fermentation process in which the pulp is broken down to release the cocoa beans, the microbial flora produces a number of enzymes which include oxidases, a number of glycohydrolyases, peptidases and proteases, lipases and other esterases, and compounds such as alcohols, the volatile organic acid acetic acid, lactic acid, ketones, amino acids, fatty acids and fatty acid esters, and hydrocarbons that alter the colour of the beans to brownish red and contribute to the elimination of the bitter taste associated with the tannins (Heath 1981). Most of these compounds are removed in the drying step which follows which inactivates all microbial and cocoa bean enzymes and removes all gumminess contributed by polysaccharides, thereby resulting in a stable, dry, brittle product which is traded as a commodity worldwide (Heath 1981). At the processing facilities the cocoa beans are heated to produce the roasted aroma characteristic of cocoa powders and chocolate that is attributed to the aldehyde products from oxidative deamination of amino acids and from the formation of pyrazines, which are the primary Maillard reaction products (Heath 1981; Rasmussen and Bach 1996; Maga 1982, 1992). The roasting step is followed by dehulling and removing of the cocoa bean shell (Heath 1981; Rasmussen and Bach 1996). The nibs recovered are ground and subjected to heating in alkali to form a rich liquor high in cocoa butter. In the following step, the cocoa butter is removed by high-pressure presses and the pressed cakes are recovered, and ground to form cocoa powders that are dried and blended to customer-desired colour and taste specifications.

Ingredient cost consideration and limited availability of cocoa beans has generated interest in cocoa powder extenders and replacers. Blends of spray-dried malted barley and roasted barley or other roasted grains are used in some cases at a rate of 35–50% as a replacement for cocoa powders (Heath 1981). In other cases, the stillage and yeast recovered from malt spirits is recovered following distillation of ethanol and other fusel oils and concentrated by evaporation followed by spray-drying to produce cocoa powder replacements. Cocoa-substitute compounds can also be produced from a number of yeast species such as *S. cerevisiae*, *S. carlsbergensis*, *C. utilis*, *C. tropicalis*, or *Brettanomyces* genera that are preferably propagated on hop-containing or non-hop-containing media and combined with a reducing sugar in an aqueous slurry and heated to high temperature under pressure. Alternative processes that use low-moisture yeast cell preparations that are obtained following spray-drying are heated to elevated temperatures with dry heat to over 250°C in an oven to produce a desired product with a roasted flavour (Liggett 1978). More recently, genetic engineering approaches have been used to clone and produce cocoa-flavour precursor peptides in yeasts or bacteria that when mixed with amino acids and saccharides in the fermentation production medium and heated from 100 to 200 ‘C for 1–60 min can result in the production of cocoa flavours that can be used in food, pharmaceutical, or cosmetic products (Rasmussen and Bach 1996). The lipid extracts from oleaginous yeast have been analysed and evaluated for use as cocoa butter equivalent. The commercial use of oleaginous yeast to produce cocoa butter equivalent while attractive remains untapped (Ratledge 1997).

10.2.4.3.5 Fermented Edible Meats and Edible Meat Flavours

The production of meat flavours from products prepared from yeast extracts has been covered extensively (Van Pottelsberghe de la Potterie 1972) in patents and in a

number of publications (Halasz and Lasztity 1991; Huynh-Ba et al. 2003; Nagodawithana 1992; Peppler 1979; Reed 1981). The use of yeasts in the starter cultures to prepare fermented yeast products, yeast extracts for their flavours, or to support growth of microbial flora used to ferment meats such as sausages or products derived thereof, or other whole-cell or cell wall yeast has been described (Bolumar et al. 2003; Durá et al. 2002; Encinas et al. 2000; Samelis et al. 1994). Meaty flavour or notes can be achieved in non-meat-derived products by processing of aqueous yeast or yeast hydrolysed or non-hydrolysed extracts by treatments that utilize heat in the presence of sugars – monosaccharide or oligosaccharide (preferably xylose or lactose) – with/without other amino acids such as methionine, cysteine, or cystine. The process described produces an improved, durable flavour (Van Pottelsberghe de la Potterie 1972). The sources of carbohydrate can be varied and consist of coffee wastes or other ground plants, oats, rye, or barley. The aqueous medium may also contain a carboxylic acid (lactic, malic, succinic, palmitic, stearic, oleic, or their mixture). After the reaction the medium is evaporated to a paste or converted into a powder. The final formulation consists of hydrolysed plant protein free of cysteine, sodium guanylate, malic acid (or a mixture of lactic, palmitic, and succinic acids), xylose, and water with pH set at 2.5–3.0. This mixture is refluxed for approximately 100 h and concentrated to a paste or to a powder under vacuum. The products possess excellent roast beef aroma, and are suitable for use as food additives.

A number of yeasts contribute significantly to the flavour of fermented meat products and meat-flavoured products and these frequently consist of strains of *D. hansenii* (teleomorph of *C. famata*), *D. kloeckeri*, *Y. lipolytica* (perfect form of *C. lipolytica*), *Citeromyces matritensis* (teleomorph of *C. globosa*), *Trichosporon ovoides* (formerly *T. beigelii*) and several other species of *Candida* (*C. intermedia* or *C. curvata*, *C. parapilosis*, *C. zeylanoides*), *Pichia*, *Cryptococcus*, and *Rhodotorula* (Bolumar et al. 2003; Encinas et al. 2000; Ingram and Simonsen 1980). These yeasts are known to secrete lipases and/or proteases which contribute to flavour by offsetting and modifying the acidic pH produced by mixed bacterial starter culture activities through the degradation of lipids to produce free fatty acids and glyerol and the breakdown of nitrogenous compounds to amino acids with release of ammonia. Several recently published reports describe the isolation and characterization of several such enzymes from *D. hansenii* (Bolumar et al. 2003; Durá et al. 2002). In one of these reports, Bolumar et al. (2003) described the first isolation and characterization of a yeast prolyl and of an arginyl aminopeptidase from *D. hansenii*. In a second report, Durá et al. (2002) described the production of a glutaminase by the same yeast. This yeast is typically dominant in meat fermented products at all stages of sausage manufacturing and is known for its high salt tolerance and its production in addition to proteases, lipases and peroxidase activity. The isolation, characterization, and overproduction of the previously mentoned two groups of enzymes have also been reported for *Y. lipolytica* (Nicaud et al. 2002). Both of these yeasts also contribute to significant flavour development of fermented dairy products such as cheeses.

10.2.4.3.6 Soy-Derived Flavour Products

Soybean products are characterized by a mealy and fatty flavour that is bland (Heath 1981; Kinsella and Damodaran 1980). By comparison yeast-fermented soy

products such as miso prepared from soybean paste or other fermented miso products from rice or barley, soy sauce (shoyu), and other fermented soybean protein hydrolysates have complex flavour and aroma profiles (Hamada et al. 1991; Komai et al. 1987; Kumari and Singh 1990; Sarkar et al. 1994; Sasaki 1996). These flavour and aroma profiles vary with the methods used to prepare the raw materials and other ingredients included, microbial flora used, and the ripening or aging process employed. In the production of soy sauce the high concentration of sodium chloride (more than 12% w/v) used favours the growth of salt-tolerant yeast, such as *Z. rouxii*, *Torulopsis etchellsii*, and *C. versatilis* or *Torulopsis versatilis* (Chien 1974; Dahlen et al. 2001; Halasz and Lasztity 1991; Oro 2001; Sugawara 2001). Other less tolerant yeast genera can be used provided that the initial NaCl level is reduced (below 5% w/v) and these include *S. cerevisiae*, *Hansenula* sp., and *Pichia* sp. (Kayahara et al. 1980; Suezawa et al. 2003; Taiyoji and Watanabe 2002). Industrial yeast strains with improved soy sauce aroma and flavour have been developed and used commercially (Kusumegi et al. 1992; Lee and Kim 1993). The salt-tolerant yeasts used in soy sauce production elaborate a number of flavour compounds which includ: 4-hydroxy-2 (or 5)-ethyl-5 (or 2)-methyl-3 (2*H*)-furanone (HEMF), 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone (DMHF), 3-methyl-1-butanol, and volatile alkyl phenolics such as 2-phenylethanol and 4-ethyl guaiacol (4-EG). HEMF has a strong sweet cake-like aroma with a threshold value of less than 0.04 ppb (Sugawara 2001). HEMF is a strong antioxidant that has been shown to exert an anticarcinogenic effect on benzo[α]pyrene-induced mouse forestomach neoplasia (Sugawara 2001). It is also effective in preventing radiation hazards and has an important physiological function as well as being an aroma component (Sugawara 2001). HEMF was formed during the cultivation of yeast by using a precursor of HEMF which may have been produced by the amino-carbonyl reaction of pentose with amino acids during heating (Sugawara 2001). DMHF is found in many fruits such as strawberries and pineapple (Dahlen et al. 2001). While dilute solutions of DMHF exhibit a strawberry or pineapple-like flavour, in the concentrated form DMHF solutions have a caramel-like aroma (Dahlen et al. 2001). DMHF is a secondary metabolite that is produced by the soy-sauce-fermenting yeast *Z. rouxii* after the addition of D-fructose-1, 6-diphosphate to YPD nutrient media (Dahlen et al. 2001). DMHF concentrations in the range 5–10 g/L have been shown to partially and completely inhibit the growth of *Z. rouxii* cells (Dahlen et al. 2001). While 4-EG is an important aroma in soy sauce, when present in excess over several milligrams per litre it gives an off-odour (Oro 2001). A process for continuous production of 4-EG by the salt-tolerant *C. versatilis* in an airlift reactor has been reported (Hamada et al. 1990, 1991). In this process, large amounts of 4-EG(more than 20 ppm) were produced by immobilized yeast cells for up to 40 days with 1–3 ppm of 4-EG, which is the optimal level in conventional soy sauce, produced within 0.5 h (Hamada et al. 1990). Good soy sauce flavour profiles were reported recently with the salt-tolerant yeasts *C. versatilis* and *Z. rouxii* immobilized on a poly(ethylene oxide) resin in a continuous stirred reactor (Van der Sluis et al. 2001). The production of a soy sauce with quality comparable to that produced by the conventional method was demonstrated in a controlled fermentation process using a genetically modified strain of the red yeast *C. versatilis* in which an inducible ferulic acid

decarboxylase was deleted and replaced with a gene coding for a constitutive enzyme (Suezawa et al. 2003).

The selection and use of improved strains of *Z. rouxii* for production of miso from barley, soybean, wheat, or non-salted rice has been reported (Kasumegi et al.1997, 2001; Kayahara et al. 1980; Matsuda and Yamamoto 1999; Taiyoji and Watanabe 2002; Yoshikawa et al. 1990). Mutants of *Z. rouxii* produced more than twofold aromas of higher alcohols such as isoamyl alcohol, propyl alcohol, isobutyl alcohol, butyl alcohol, and/or β-phenethyl alcohol (Kasumegi et al. 1997, 2001, Kayahara et al. 1980; Matsuda and Yamamoto 1999). The use of wine yeast strains that produce ethanol in the presence of high levels of sodium chloride, yielded miso flavour profiles that are characteristic of the wine yeast used (Kawamura and Kawano 1999). The effect of ethanol addition on the formation of fatty acid esters from the degradation of glycerides present in raw materials that are usually formed during miso fermentation was investigated (Ohnishi 1983). It was found that the ratio of ethyl fatty acid esters formed was determined by the level of ethanol present. This suggests that the production of ethanol and other higher alcohols by yeasts is not only responsible for the production of aroma substances but also for the rate at which lipids are metabolized and for flavour development during miso fermentation (Ohnishi 1983). At reduced salt concentration during the production of non-salted rice miso by *Z. rouxii*, ethanol addition (4%) at 45°C leads to increased sugar and protein metabolism, resulting in bitter flavour and light colour development (Taiyoji and Watanabe 2002).

10.2.4.3.7 Other Fermented Foods and Beverages

Yeasts are also responsible for the complex flavours of many other raw materials, foods, and beverages which include a great number of distilled spirits, other fermented fruit and cereal drinks such as wines, ciders, and sake (Fukuda et al. 1998a, b; Furukawa et al. 2003a, b; Lambrechts and Pretorius 2000; Nykanen 1986; Romano et al. 1999), fermented syrups, and caramel or almond flavours such as salicyl flavour aldehyde produced by the methylotrophic yeast *C. methanolovescens* (Van den Bremt et al. 2000, 2001), fermented dairy products such as kefir and yogurt, and other foods characterized by roasted and fragrant flavours which include tea, vanilla beans, and coffee. Some of the yeasts used in some of these applications are listed with the food type in Table 10.4.

10.2.5 Vitamins

Owing to their ability to incorporate ingredients present in fermentation media, yeast cells are an important source of proteins, vitamins, and minerals (Halasz and Lasztity 1991; Mosiashvili et al. 1971). Among the vitamins and other enzyme cofactors that are accumulated and/or synthesized by yeast are thiamine (vitamin B_1), nicotinic acid containing enzyme cofactors NAD and NADP and their reduced forms, pyridoxine (vitamin B_6), pantothenic acid, or CoA precursor (Peppler 1967), cyanocobalamin or vitamin B_{12}, biotin, folic acid (pteroylglutamic acid) or folacin, and riboflavin or vitamin B_2 (Drewek and Czarnocka-Roczniakowa 1986; Halasz and Lasztity 1991; Oura and Suimalainen 1982). Examples of these are the yeast *Kloeckera apiculatas*, which

Table 10.4 Yeast used in main fermented foods

Fermented food	Yeast employed in process
Alcoholic beverages	*Saccharomyces cerevisiae* and other species; *Schizosaccharomyces* (rum)
Breads and cakes	*S. cerevisiae* and *S. exigus*; *Candida krusei* and *C. tropicalis*; *Pichia* and *Hansenula anomala*
Beers	*S. cerevisiae* and *S. uvarum* (ex *S. carlsbergensis*)
Ciders	*S. cerevisiae* and *S. uvarum*; *Hansenisapora valbyensis*; *Metschnikowia pulcherrima*
Coffee	*S. cerevisiae*
Cocoa	*C. krusei*, *C. famata* and *C. holmii*;
Fermented food	Yeast employed in process *P. membranaefaciens*, *S. chevalieri*
Fermented milks	*S. cerevisiae* and *C. kefir* (for kefir)
Fresh, semihard, pressed, or mould cheeses	*Kluyveromyces* sp., *Debaryomyces hansenii*, *S. unisporus*, *Candida* sp., *Pichia* sp., *Yarrowia lipolytica*, *Clavispora lusitaniae*, *Trichosporoninkin*, *Torulospora delbrueckii*
Fermented meat products	*D. hansenii*, *Candida* sp., *Rhodotorula rubra*
Wines	*S. cerevisiae*

Modified from Cristiani and Monnet (2001)

is known to accumulate thiamine up to a tenth of its dry weight, whereas some strains of the yeasts *S. cerevisiae* and *S. uvarum* (*carlsbergensis*) during ethanol production can release thiamine following treatment with UV light (Halasz and Lasztity 1991). *S. cerevisiae* is also the principal source of CoA and the first important commercial preparations of this coenzyme were made from this yeast (Peppler 1967). Some strains of *S. carlsbergenesis* have also been shown to contain vitamin D_2 and vitamin D_3 as well as 25 hydroxy forms of these two vitamins (Halasz and Lasztity 1991). Ergosterol, a precursor to the aforementioned forms of vitamin D, is an important constituent of yeast cell membrane lipids (Fazekas and Sebok 1959; Tanaka et al. 1971; Xue et al. 2002). Increasing yield of ergosterol in yeast cultures was shown to be influenced by the addition of vitamin B_1 and thioglycolic acid to enhance cell mass in aerated cultures (Fazekas and Sebok 1959). It was concluded that the combined addition of vitamin B_1 and thioglycolic acid enhances sterol production in the cells, most likely by the conversion of pyruvic acid to sterols (Fazekas and Sebok 1959). The cultural conditions for ergosterol production by yeasts were also investigated by using an *n*-alkane mixture as the sole carbon and energy source (Tanaka et al. 1971). Among the yeasts tested, several strains of *C. tropicalis* were shown to produce a relatively large amount of ergosterol (Tanaka et al. 1971). *n*-Alkanes with carbon numbers that range from C10 to C14 and from C17 to C18 were good substrates for ergosterol production by this yeast. The addition of a nonionic detergent (0.02%) and a natural nutrient (0.1%) was also effective. Aeration and the pH of the medium also affected the production of ergosterol. Under the optimal conditions employed, a strain of *C. tropicalis* designated as pK 233 produced ergosterol in a yield of approximately 71 mg/L of broth or

5.8 mg/g of dry cells after 5-day cultivation in a medium containing a mixture of *n*-decane to *n*-tridecane as the sole carbon sources. More recently, a method for transforming ergosterol into vitamin D_2 in yeast was described by Xue et al. (2002). In this method UV irradiation of ergosterol containing yeast grown under optimal conditions with molasses as the carbon source resulted in cells that contain 15,000 IU/g (Xue et al. 2002). Clinical observations showed that the yeast cells generated have good preventive and curative effects for rickets (Xue et al. 2002). Other forms of lipid-soluble vitamins and their precursors that are present in yeast are the vitamin E tocopherols, and vitamin A and its precursor, β-carotene.

In addition to the B vitamins consisting of B_1, B_6, and B_{12}, yeasts are also important sources of flavins derived from riboflavin or vitamin B_2, the water-soluble vitamin C or ascorbic acid (Hancock et al. 2001), and multiple forms of coenzyme Q. The production of yeast cell mass with a predetermined level of vitamin B_1 potency was demonstrated by the incorporation and assimilation of vitamin B_1 from a water-clear vegetable extract with a sugar source provided as the carbon source and used as a growth medium (Gorcia and Levine 1942). Improvement of the biological synthesis of the vitamin B_1 in yeast was shown through the incorporation of vitamin B_1 precursors into the fermentation medium (Harrison 1942). The addition of a suitable thiazole derivative, such as 4-methyl-5-β-hydroxyethylthiazole, and a pyrimidine derivative containing a cyano radical, such as 2-methyl-5-cyano-6-aminopyrimidine, in equimolar quantities to the fermenting mash, preferably toward the end of the process under vigorous aeration at 28–30°C at a pH of 5.5–6.0, resulted in the formation of a methylene linkage between the pyrimidine ring and the nitrogen of the thiazole. Other ethylthiazole derivatives can be used in the aforementioned reaction that yielded vitamin B_1 as a product (Harrison 1942). The production of vitamin B_1 for use in beverages and in pharmaceutical applications was described by Silhankova (1978). In this patent, a vitamin B_1 producing strain of *S. cerevisiae* designated DBM 159 was shown to produce normal levels of ethanol while producing an elevated level of vitamin B_1 (approximately 10 mg/L) in a molasses-containing medium (Silhankova 1978). The formation of vitamin B_6 by several genera of yeasts was examined in hydrocarbon-containing media with vigorous aeration by Tanaka and Fukui (1967). Among the genera studied of *Candida*, *Rhodotorula*, and *Hansenula*, a strain of *C. albicans* exhibited the excellent vitamin B_6 producing ability (300–400 μg/L) in a synthetic medium containing hexadecane as the sole carbon source. The addition of corn steep liquor and an appropriate nonionic detergent, such as Tween 85 or Span 60, stimulated yeast growth and vitamin B_6 production (Tanaka and Fukui 1967). The cell and vitamin yields increased with the rate of aeration. In a medium containing glucose as the carbon source, the vitamin B_6 formed was secreted into the fermentation broth prior to achieving maximum cell density. In cells grown with hydrocarbons as the sole source of carbon, vitamin B_6, mainly in the form of pyridoxal phosphate, accumulated inside the cells (Tanaka and Fukui 1967).

The formation of coenzyme Q, cytochrome c, and flavins by yeasts grown on mixed hydrocarbons was studied using several strains of *Candida* and *Pichia* by Teranishi et al. (1971). These strains included a strain of *C tropicalis* designated as pK 233. The time-course changes in the coenzyme Q, cytochrome c, and flavine contents in the yeast cells were studied during growth on hydrocarbons. The effects of medium

constituents, additional purines, and detergents on flavine production and the type of flavines produced were also investigated. *C. tropicalis* pK 233 was shown to grow well on C9–C14 alkanes under the conditions employed. Undecane was most suitable for cell growth and flavine production. A hydrocarbon mixture of four alkanes which was enriched in *n*-undecane was also suitable as the sole carbon source. Several nitrogen sources, namely NH_4NO_3, $(NH_4)_2SO_4$, and $NH_4H_2PO_4$ were all shown to be effective for flavine production by this yeast. The amount of flavines produced by *C. tropicalis* pK 233 was about 5 mg/L of broth under the conditions employed. The time-course study suggested an interesting correlation between the intracellular contents of coenzyme Q, cytochrome c, and flavines during growth (Teranishi et al. 1971).

The production of protein–vitamin concentrates with yeasts in media containing lipids as the carbon source was described by Szechenyi et al. (1973). Media containing lipids such as grease, lard, rancid and waste fats, or tallow, that are emulsifiable at 30–40°C, were fermented with yeasts (*C. guilliermondii*, *C. utilis*, *S. fragilis*, etc.) in the presence of NH_4^+ salts and (or) urea at pH 4.5–5.0. The cell mass recovered from the fermentation broth had high protein (more than 50%) and vitamin content, which included vitamins B_2, B_6, and B_{12}, nicotinic acid, and pantothenic acid. The cell mass was useful as a food or feed additive without considerable purification. Thus, a medium containing animal-derived lipids or their by-products was shown to yield a 75% dry yeast (based on the lipid consumed) that contained greater than 50% protein, 7% lipids, and 6% ash, as well as vitamin B_1 greater than or equal to 3 mg/100 g, vitamin B_2 greater than or equal to 5 mg/100 g, pantothenic acid greater than or equal to 20 mg/100 g, vitamin B_6 greater than or equal to 1.5 mg/100 g, and ergosterol at a concentration greater than or equal to 3 mg/100 g.

The increased demand for vitamins and enzyme cofactors as food additives and supplements has stimulated research to increase the content of these important components in yeasts intracellularly and in some cases as secreted extracellular products. The development of yeast strains with increased vitamin and enzyme cofactors using classical strain selection and/or genetic engineering has resulted in the development of commercial processes for the production of vitamin B_2, riboflavin (Dmytruk et al. 2004; Lim et al. 2001; Protchenki et al 2000; Stahmann et al. 2000; Voronovsky et al. 2002, 2004). The *C. famata* (*D. hansenii*) strains described are among the most flavinogenic microorganisms developed so far and unlike strains of *Pichia* (*Candida*) *guilliermondii* which concentrate vitamin B_2 intracellularly secrete riboflavin into the growth medium.

The genetic engineering of strains of *S. cerevisiae* that can synthesize the fat-soluble vitamins E and A and its precursor, β-carotene, has been described in great detail in a recent patent application (Millis et al. 2000). The cited patent describes the production of the previously mentioned vitamins by fermentative biosynthesis of intermediates using genetically engineered microorganisms followed by chemical synthesis (Millis et al. 2000). The invention provides methods of producing vitamin E (α-TOH and γ-tocopheryl esters), vitamin A (retinol), or β-carotene. The methods comprise using a biological system with enhanced synthesis of farnesol or geranylgeraniol intermediates to shift microbial metabolism away from sterol biosynthesis via genetic inactivation of the squalene synthase ERG9 gene or by inactivation of squalene synthase by zaragozic acid in a strain with a functional ERG9 gene.

Geranylgeraniol biosynthesis is further enhanced in strains overexpressing any of four different cloned geranylgeranyl pyrophosphate synthase genes from several microbial and plant sources, and these include (1) BTS1 gene from *S. cerevisiae*, (2) crtE gene from *Erwinia uredovora*, (3) a1-3 gene from *Neurospora crassa*, and (4) ggs gene from *Gibberella fujikuroi*. The overexpression of the hydroxymethyl-CoA reductase and/or the ERG20 gene which encodes farnesyl pyrophosphate synthase in *S. cerevisiae* also leads to enhanced biosynthesis of fermentative intermediates. The overexpression of multiple isoprenoid pathway genes or an alternative pathway (Rohmer pathway) was investigated in strains that have an erg9 mutation and elevated levels of hydroxymethylglutaryl-CoA reductase. Further chemical conversion of the fermentation products, farnesol or geranylgeraniol, into α-TOH, γ-tocopheryl ester, vitamin A, or β-carotene was described in some detail (Millis et al. 2000).

The genetic engineering of yeast strains that can synthesize the water-soluble vitamin C or ascorbic acid is described in another recent patent application (Berry et al. 1999). The synthesis of vitamins D_2 and D_3 from ergosterol by *S. cerevisiae* and *C. tropicalis* has long been described in some detail in a number of publications (Fazekas and Sebok 1959; Rao and Raghuntha 1942; Subbotin et al. 1974; Tanaka et al. 1971; Xue et al. 2002). The accumulation and secretion of thiamine or vitamin B_1 in beer wort following treatment of *S. cerevisiae* and *S. carlsbergenesis* has been confirmed at the laboratory and pilot-plant scale and the production of thiamine from its precursors by these two yeasts has been recognized for some time (Bakhchevanska et al. 1984; Popova et al. 1982; Silhankova 1978, 1985). The potential use of several yeasts for production of the vitamin B_{12} has been assessed and *Z. bailii* and fodder yeast were recognized as promising yeasts for its production on industrial media and agricultural residues (Bykhovskii et al. 1972; Mosiashvilli et al. 1971; Popova et al. 1971; Trofimenko and Cheban 1970). The production of vitamin B_6 or pyridoxine has been demonstrated on hydrocarbon-containing media with several yeasts, which include several *Candida*, *Rhodotorula*, and *Hansensula* (Tanaka and Fukui 1967).

10.3 Concluding Remarks and Future Outlook

The economic importance of yeasts to the food and beverage industries continues to outweigh all other commercial uses of yeasts. The revived interest in naturally derived products and the increased concerns about products from animal sources has generated renewed interest in food and nutraceutical uses of yeasts. Among the many advantages of yeasts are their ease of production and for several yeast genera the favourable regulatory environment. New strains of yeasts enhanced through genetic breeding to improve production of food ingredients and for use as supplements provide added promise to biotechnologists and consumers alike (Maraz 2002). It is without doubt that the full potential of the commercial value of yeasts has not been fully realized. With new nutraceutical applications under development the 2–4 % projected annual growth in sales for all yeast products will result in markets that exceed US $20 billion by the end of this decade. The continued expansion in the use of *Saccharomyces* and other genera of so-called nonconventional yeasts represents the rise of yeast biorefineries that aim to capture the full potential of these important biocatalysts.

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Chapter 11

Food and Beverage Spoilage Yeasts

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This chapter is dedicated to all those who work in food QA, Quality Assurance laboratories, at the sharp end of yeast spoilage.

11.1 Introduction

Most people have very little knowledge or experience of yeasts; what experience they have is largely confined to the use of yeasts in bread-making or in alcoholic beverages, perhaps through home-brewing. It would surprise many people to realize that they also consume yeast cells daily, by the thousand, particularly in fresh fruit and vegetables. These yeasts are not likely to be the brewing or baking yeast, *Saccharomyces cerevisiae*, but are distributed widely across the 800 known yeast species (Barnett et al. 2000). Consumption of yeast cells in such numbers is not injurious to human health but part of a normal, healthy human diet. But if foods are not consumed but left to incubate at warm temperature, some of these yeasts will grow over time and cause a perceptible alteration in the food. Yeasts of such species may be termed food and beverage spoilage yeasts.

Many excellent reviews of yeast spoilage have previously been published, including those of Ingram (1958), Walker and Ayres (1970), Davenport (1981), Fleet (1992), Tudor and Board (1993), Thomas (1993), Deak and Beuchat (1996) and Pitt and Hocking (1997). In these reviews, the literature of yeast spoilage is examined food by food, listing the yeasts found in and causing spoilage in each individual foodstuff, for example yeast spoilage in cereals; fruit juices and meat. There would therefore seem little need to recapitulate the information already published, in a further review. However, a number of recent technical innovations and social trends in food purchase and consumption made it timely to re-evaluate yeast spoilage in food.

Firstly, DNA sequencing methods applied to yeast identification are revolutionizing the taxonomy and phylogeny of spoilage yeasts (James et al. 1996; Kurtzman and Robnett 1998, 2003). This has meant that very few of the species names associated with spoilage 50 years ago are currently recognized. Sequencing has also enabled a major reassessment of the relationships and phylogeny of many spoilage strains. This has resulted in amalgamation of certain genera and re-naming of others (Kurtzman

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2003). This will doubtless cause much confusion at first, and difficulties in matching the old names in the literature with current nomenclature. It is hoped that the proposed new sequence-based structure of yeast taxonomy will not be subject to further changes and that the relationships between different spoilage yeasts shown by the phylogenetic tree will enable prediction of spoilage behaviour in related yeast species.

A second consequence of DNA sequence-based yeast identification has been the revelation as to the extent of misidentification of strains by older taxonomic methods. Many yeast strains, even in recognized culture collections, have been found to have been misidentified. Furthermore, many new species of spoilage yeasts have been found, previously unrecognized. Such species may not be distinguishable by traditional methods of identification and tended to be force-fitted into other genera. The genus *Candida* in particular had become a ragbag assortment of white non-spore-forming yeasts, with no unifying, clearly defined character. It could be speculated that in the older literature of yeast spoilage, where traditional taxonomic tests were used by inexpert researchers, the level of misidentification may exceed 50%.

The third change influencing yeast spoilage is the increased degree of processing and storage of foods. In human prehistory, foods were obtained fresh and consumed immediately. More recently, a proportion of foods were preserved using traditional methods, such as drying, smoking or salting. In modern times throughout the developed world, many foods are sold as pre-prepared meals, minimizing the time spent by consumers in preparing food. Foods are therefore subject to a far greater degree of factory processing than previously. Such foods are also packaged in a very different way. Traditionally, foods were sold with no packaging, allowing easy access to yeast infection in the domestic environment. Processed ready-meals, soft drinks and even fresh fruit/vegetables are now sold packaged in plastic, minimizing microbial contamination, but altering the concentrations of gases within foods. In the future it is likely that much greater attention will be paid to the opportunities available for spoilage yeasts in factory-prepared processed foods.

In the present review, yeast spoilage will be examined largely from the point of view of the "preservation systems" applied to different foods. Knowledge of which yeast species are resistant to which preservation systems will enable prediction of patterns of yeast spoilage in any new foods preserved using these systems.

11.2 Definitions

What is yeast spoilage in foods or beverages? This is a question to which many people can immediately envisage the answer but would find it much more difficult to define precisely. Extreme examples of yeast spoilage include "blown cans" of soft drinks (Fig. 11.1), cloudy re-fermented wine, pink or red slime dripping from refrigerated meat, white yeast colonies on food, and tainted fruit juices. A spoilage yeast species is one with the ability to cause spoilage. As such, yeasts merely isolated from foods are not necessarily spoilage yeasts. Taking a leaf from the medical textbooks of infective agents, a spoilage yeast is one that can be isolated from a spoiled product, and if re-inoculated back into sterile food of the same variety will grow and cause identical symptoms of spoilage. Yeasts are often isolated from spoiled or symptomless foods and should not be termed spoilage yeasts without further tests.

Fig. 11.1. "Blown" packages of food or beverages represent the most obvious sign of yeast spoilage. This soft drink was inoculated with *Zygosaccharomyces bailii* before capping. Similar cans of beverage distended, ruptured or exploded between 1 month and 2 years

However, does the mere presence of a few yeast cells in a food constitute spoilage? and if not, how much can a yeast grow before the food is defined as spoiled? The answers to these more difficult questions will vary with the food type and the individual yeast species involved. Foods frequently contain yeasts, even spoilage yeasts, at low level without perceptible spoilage. An inoculum of 100 cells/ml is only attained with reasonable care in fruit juices (Lloyd 1975). The metabolic activity of yeasts at a cell concentration up to 10,000/ml is insufficient to make any appreciable difference to the food. Since detectable spoilage requires greater numbers of yeasts than this, approximately 1×10^5–1×10^6/ml (Ingram 1949), and very high concentrations of yeasts are not likely to be found through the initial inoculation, it therefore follows that yeast spoilage requires growth of the yeast population.

To our good fortune, yeasts are almost entirely non-pathogenic. To a human in a reasonable state of health, consumption of moderate numbers of viable yeasts of most of the 800 recognized yeast species (Barnett et al. 2000) is not likely to cause

harm (Fleet 1992). Minor gastrointestinal disorders have been attributed to consumption of beverages spoiled by *Saccharomyces* and *Zygosaccharomyces* yeasts but these have not been confirmed (Todd 1983; Muzikar 1984). It has been suggested that such effects may be due to yeast metabolites rather than to live yeasts per se (Thomas 1993). Similarly, yeasts do not form toxins injurious to human health (unlike mycotoxin-forming moulds). Yeasts of many species do form toxins, "killer toxins" (Philliskirk and Young 1975; Young 1987), but these appear targeted primarily at other strains and species of yeasts. The use of killer strains in breweries has even been proposed, to prevent contamination of brewing strains by wild yeasts (Young 1983; Hammond and Ekersley 1984).

The results of yeast spoilage of foods are diverse, but all are the result of large populations of yeast growing in the food and consequent metabolic activity. In spite of this, yeast growth in foods is unlikely to cause significant deterioration in the nutritional value of the food. Yeast growth is likely to remove only a small proportion of the sugars, for example in a fruit juice. Yeast growth may even increase the nutritional value of foods through addition of yeast protein and vitamins.

However, despite not causing a safety hazard or loss of nutritional quality, the results of yeast spoilage are noticeable by any customer. A consumer is unlikely to realize that it is yeast spoilage; a consumer will simply realize that something is wrong or different about the food, *and will reject it*. A common theme of yeast spoilage is therefore that all spoilage symptoms are noticeable and objectionable to the customer and all will cause complaint by any customer purchasing the food.

Since (1) the presence of yeasts in foods is not a safety issue, (2) yeasts do not form toxins adverse to humans or (3) yeasts do not cause significant nutritional loss, food spoilage by yeasts is dependent entirely on what the customer/consumer notices or objects to. Yeast spoilage of foods or beverages can therefore be defined as "Growth of yeast in a food, sufficient to cause an alteration in that food, perceptible to a consumer, and liable to cause dissatisfaction, complaint, or rejection of that food by the customer".

11.3 Which Foods are Prone to Yeast Spoilage?

Yeast and human biochemistry and metabolism are essentially similar. This being so, it is obvious that yeasts and humans are also likely to have a high level of similarity in their nutrition. In a simplified form (Table 11.1), human nutrition is based around carbohydrates (carbon source), proteins (nitrogen source), fats, vitamins and mineral salts. Yeast nutrition also requires carbon and nitrogen sources, vitamins and minerals. It is therefore not surprising that many human foods can fulfil a role as excellent yeast growth media.

However, human and yeast nutrition vary in two important aspects. Firstly, in a yeast growth medium, all of the nutrients must be present simultaneously, whereas in human nutrition, different parts may be found in different foods, for example vitamins in one food and proteins in another. Human foods not containing a complete balanced yeast diet are less likely to be substrates for spoilage yeasts. Secondly, yeast nutrition is strongly oriented towards small molecules rather than giant polymers. Complex polymers of carbohydrate or protein cannot be transported easily through

Table 11.1 The similarity between human and yeast nutrition lends many human foods prone to spoilage by yeasts. Complex polymers predominate in human nutrition, while yeasts almost exclusively utilize monomers. Yeast nutrition is fully discussed in Jennings (1995)

	Human nutrition	Yeast nutrition
Carbon/energy sources	Carbohydrates, sugars, fats	Simple sugars (fats by lipolytic yeasts)
Nitrogen source	Proteins	Amino acids, ammonium ions, (nitrate and nitrite)
Minerals	Iron, calcium, magnesium, phosphate, sodium, potassium	Magnesium, potassium, phosphate
Trace elements	Iodine, sulphur, copper, cobalt, manganese	Iron, sulphur, copper, cobalt, manganese, zinc, (molybdenum)
Vitamins	Vitamins A–K, including B-group vitamins	B-group vitamins by some species

the yeast cell wall (de Nobel and Barnett 1991) and require extracellular degradation by secreted enzymes. Extracellular degradation is common in the filamentous fungi (moulds) but is rare in single celled fungi (yeasts). Preferred carbon sources for yeast assimilation are simple sugars, and to a lesser extent sugar alcohols, or organic acids. Relatively few yeasts can utilize complex carbohydrates such as starch. Where yeast species have the ability to assimilate starch, growth is usually slow or delayed (Barnett et al. 2000). Some yeasts show lipolytic activity, for example *Yarrowia lipolytica*, and are able to utilize fats (Barth and Gaillardin 1996), but again this is slow compared with growth on glucose. Similarly most yeasts have virtually no proteolytic activity (Ingram 1958) and are therefore unable to grow on proteins as a nitrogen source. An exception to this may be the degradation of casein in milk by red yeasts leading to spoilage of milk products (Ingram 1958). Preferred yeast nitrogen sources are again small molecules, notably amino acids, ammonium ions, and nitrate or nitrite for a few yeast species. Nitrogen compounds are generally much more important to bacteria, especially to those that use them as energy sources, leading to the observation that bacterial spoilage is favoured in foods with a high N/C content (Tilbury 1980a). The practical upshot of this is that bacteria form the predominant spoilage flora in high-protein meat-type foods, while fungi assume greater importance in spoilage of foods of vegetable or fruit origin.

The importance of simple sugars to yeast spoilage cannot be overemphasized (including lactose in milk). This is reflected in the naming of the first yeast genus *Saccharomyces*, which means sugar fungus (Mayen 1837 ex Reed and Peppler 1973); one that contains several important spoilage species. As an illustration of this, the writer was once involved in production of a herbal soft drink, prepared with and without sugar. A variety of 150 yeast and mould species were individually inoculated, and in due course spoilage was observed caused by a selection of the yeast and mould species. Unexpectedly, while every spoilage mould species grew equally well

with and without sugar, every single yeast species failed to grow in the formulation lacking sugar. Ingram (1958) stated "with general experience ... it is sugary substrates, above all, which are likely to be spoiled by yeasts".

Foods containing sugars, amino acids, minerals and vitamins are thus likely to be prime media for yeast growth, but such foods may also be colonized by bacteria or filamentous moulds. Fresh foods are heavily contaminated with microorganisms, yeasts, moulds and bacteria. Over time, if the food is not consumed, many of these microbes will grow and spoil the food or cause illness in consumers (food poisoning). Yeasts, moulds or bacteria can cause spoilage, but in foods liable to growth of pathogenic bacteria such as *Clostridium botulinum*, the food is commonly sterilized by heat to kill bacterial spores ("the Botulism Cook"). Such measures will also completely eliminate any fungal contamination in the food. Yeast and mould spoilage of foods is therefore largely restricted to foods that will not support the growth of or toxin production by bacterial pathogens.

Bacterial growth is eliminated to a very large extent by acidity, bacterial spores will not germinate in foods with a pH less than 4.5 and vegetative cells of pathogenic bacteria will not grow at a pH less than 4.0 (Smelt et al. 1982), low temperature or high osmotic strength in foods. Such foods are not heavily heat-treated. Spoilage by yeasts is therefore largely restricted to those foods with low pH, low water activity a_w (caused either by high salt or high sugar), low temperatures or that contain antibacterial agents such as sulphite.

11.4 Symptoms of Yeast Spoilage

As previously defined, yeast spoilage of food is primarily that which the customer or consumer notices and finds objectionable. The symptoms of yeast spoilage are many and varied; not all may be obvious to all consumers and the food may be eaten. Other minor symptoms may be noticed but may go unreported. Fleet (1992) highlighted the problem of estimating the true significance of yeast spoilage, unless very obvious physical effects accompanied yeast growth.

11.4.1 Gas Production

What then are the obvious signs of yeast spoilage? The most visible sign of yeast spoilage is the production of excess gas (Fig. 11.1), leading to swollen containers or Tetra Paks, "blown" cans, or in extreme examples, exploding glass bottles, leading to physical injury (Grinbaum et al. 1994). It is likely that the great majority of spoilage incidents involving exploding containers will result in a consumer complaint, leading to a distortion of spoilage data in favour of yeasts producing excessive gas pressure.

It is not commonly appreciated by the public that not all yeast species produce gas. Excess gas production by yeasts is a result of fermentation of sugars, normally detected in the laboratory by the appearance of gas in an inverted Durham tube, submerged in broth media. Many yeast species do not ferment, and are termed respiring species. Of the yeasts that do ferment, the range of sugar substrates fermented varies considerably (Barnett et al. 2000). Furthermore, the volume of gas produced, and hence the pressure formed, varies between yeast species. The few

yeast species capable of forming sufficient gas pressure by fermentation to explode bottles include *Zygosaccharomyces bailii*, *Saccharomyces cerevisiae*, *Dekkera bruxellensis* and *Saccharomycodes ludwigii*. Tests carried out in the laboratory by the author have demonstrated fermentation pressures formed by these species between 6 and 10 atm overpressure (Fig. 11.2), far in excess of that formed in the manufacture of bottle-fermented wines, such as Champagne. A quick glance at the literature will reveal that these species include the most famous (or notorious) spoilage species, not because they are widespread or are a particular problem, but simply because they have drawn themselves to the attention of the public in such a spectacular way.

Behind these highly fermentative species are a number of other spoilage species that ferment, but to a much lesser extent. These include *Candida parapsilosis* and *Candida pseudointermedia*, which can generate up to 2 atm overpressure (Fig. 11.2), which is insufficient to explode containers but may lead to consumer complaints such as "sparkling" or "tastes like poor Champagne". The lower severity of symptoms in this instance almost certainly has led to a reduced complaint level and underreporting.

11.4.2 Visible Symptoms of Yeast Spoilage

Perhaps the second most obvious sign of yeast spoilage is the visible appearance of yeast cells themselves. In solid foods this can be shown through the appearance of yeast colonies on the food surface, surface discolouration, films or mucous slimes, particularly in yeasts forming extracellular polysaccharides. How obvious this is to

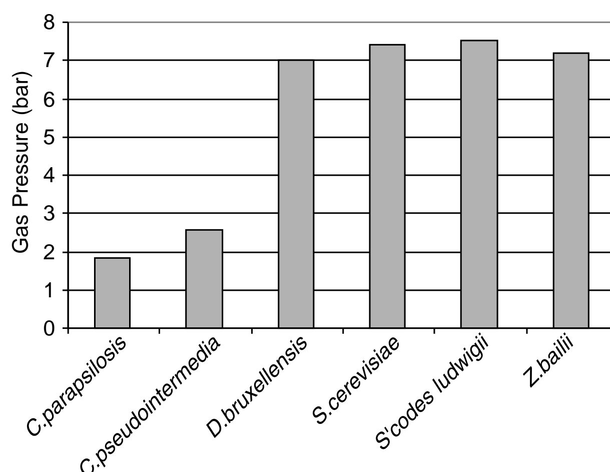

Fig. 11.2. Gas formation by fermentative spoilage yeasts, measured after 2 weeks of growth in a soft drink containing 1 M glucose. The yeast strains were *Z. bailii* NCYC°1766, *Saccharomyces cerevisiae* NCYC°366, *Dekkera bruxellensis* NCYC°823, *Saccharomycodes ludwigii* NCYC°732, *Candida parapsilosis* CMCC°3397 and *C. pseudointermedia* NCYC°2610. All had been identified by D1/D2 26S ribosomal DNA (*rDNA*) sequencing

a customer depends very much on the yeast species and the appearance of the food itself. White yeast colonies are immediately obvious on the surface of tomato soup (Fig. 11.3), but the same yeast colonies might pass unnoticed on the surface of white yoghurt. On most solid foods, pink or red yeasts are more likely to be reported than white or cream yeast colonies, perhaps leading to the regard for *Sporobolomyces* or *Rhodotorula* spp. in yeast spoilage by Pitt and Hocking (1997).

In beverages, liquid yeast media, spoilage yeasts can be seen as hazes, clouds, particulates, surface films or colonies and sediments in undisturbed bottles. In a clear liquid, yeasts are visible as a faint haze at 10^5 cells/ml, but can grow to dense clouds at 10^7–10^8 cells/ml, particularly if bottles are opened and oxygen is permitted to enter. *Dekkera* and *Brettanomyces* spp. can form particularly dense clouds in soft drinks.

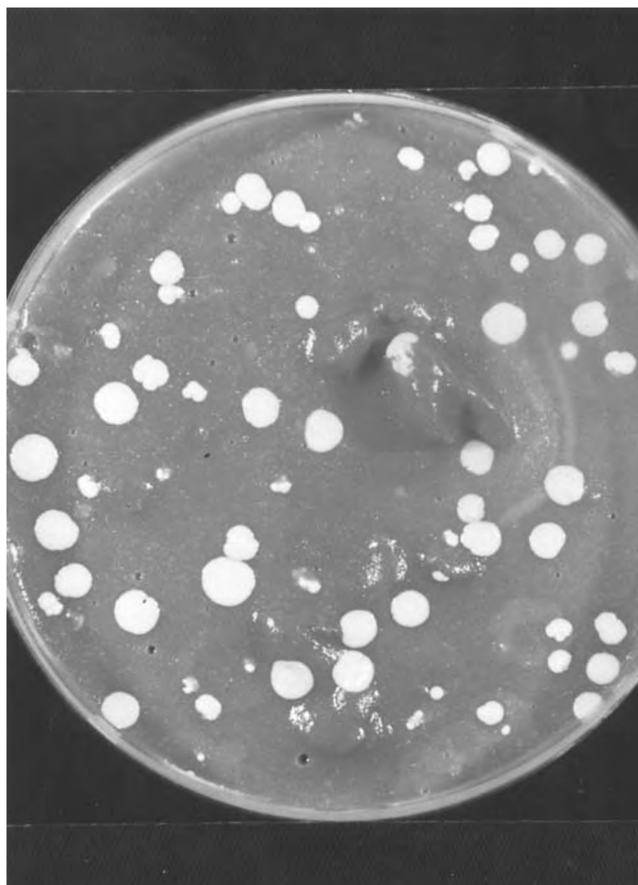

Fig. 11.3. Visible signs of yeast spoilage include white yeast colonies on a sugar-containing tomato soup. Such colonies might pass unnoticed on a white food, such as cheese or yoghurt

Amongst spoilage microorganisms, yeast cells are unusually dense, owing to their thick cell wall, and will sediment rapidly in liquid media. In water, yeast cells fall at 4–5 mm/h (Stratford 1992). As most beverage containers are only 300–400 mm in height, the majority of yeast cells will have formed a sediment within a few days and subsequent yeast growth forms a thick sediment, or a few visible colonies if the inoculum is small. A yeast sediment may be easily visible to the consumer, but the total volume of yeast if dispersed through the beverage would not be.

Yeasts may also aggregate to form more consumer-obvious structures, such as floccs, particulates or surface films. Flocculation has been reported in spoilage yeasts such as *Saccharomyces cerevisiae* and *Z. bailii* (Stratford 1992; Suzzi et al. 1992), but flocculation is most often characteristic of spoilage by *Saccharomycodes ludwigii* (Ribereau-Gayon et al. 1975; Beech and Carr 1977), forming “snowflake” particles in bottled cider.

Surface film formation is characteristic of a number of the less well-known spoilage yeast species, usually in three genera. Film formation is most frequently caused by strains of *Candida boidinii*, *Candida intermedia*, *Candida pseudointermedia*, *Candida pseudolambica* and *Candida tropicalis*, *Issatchenkia occidentalis* and *I. orientalis*, *Pichia anomala*, *P. fluxuum*, *P. fermentati*, *P. galeiformis* and *P. membranifaciens*, but can include spoilage yeasts such as *Dekkera* and *Brettanomyces* spp. (Vollekova et al. 1996). Interestingly, while *P. membranifaciens* is a frequently cited film-forming spoilage in the literature, *P. galeiformis* is a far more frequent cause of spoilage (M. Stratford and H. Steels, unpublished data), suggesting a high level of misidentification in the past.

Surface film formation is characteristic of *all* spoilage yeasts in high-sugar syrups. In sugar syrups with a sugar concentration greater than 35% w/w, the buoyant density of the sugar syrup is sufficiently great to cause yeast cells to float, rather than sediment. In spoilage of high-sugar syrups, it is normal to perceive yeast spoilage as a slick of yeast cells floating on the surface. On the industrial scale, sugar syrups or syrups of fruit juices are frequently spoiled by osmotolerant yeasts floating undetected as surface films on syrups stored in metal tanks. This may be aided by changes in temperature causing condensation on the metal above the headspace, diluting the surface layer and aiding faster yeast growth. Such yeast spoilage cannot be detected in the bulk of the syrup, and only emerges as a heavily contaminated layer when the tank is drained.

11.4.3 Off-Flavours and Off-Odours

To the public, off-tastes and smells in food are probably the least noticeable consequence of yeast spoilage, depending of the perspicacity of the tasters and their familiarity with what the food should normally taste like. One suspects that in a great number of instances, consumers do not notice the altered taste, or if they do, they think that perhaps the food should taste like that. Spoilage from altered taste is probably one of the least reported cases, and the true extent of spoilage from yeasts altering taste but not forming visible changes or gas can only be speculated upon. As an illustration of this fact, there is a story dating from many years ago concerning production of a fruit juice in cardboard packets to be drunk through a plastic straw.

There were many complaints by the public about the plastic straws, because they kept closing or blocking up. After much investigation into the plastic straws, the problem was traced to a mould spoilage problem in the fruit juice. Lumps of mould were blocking the plastic straws. Owing to the opaque cardboard packaging, the public could not see the mould, and not one complaint was ever received concerning the mouldy off-flavours in the spoiled fruit juice.

In theory, yeast growth and metabolism can influence the flavour and smell of food in one of two ways: either by removal of flavoursome food components; or by production of off-flavours or off-odours. In practice, yeast spoilage is nearly always accompanied by the latter, production of small but organoleptically powerful molecules through primary or secondary metabolism. Such molecules are usually volatile and hence give off-odours as well as off-tastes.

The off-taste produced in the greatest quantity is of course that of ethanol. Fermentative spoilage yeasts will generate ethanol in equimolar quantities with carbon dioxide. Ethanol has an unusual slightly sweet taste, and a spoiled fruit juice has a distinct sweet flat note. Comments from consumers reflect their recollections of fermented beverages, "like bad beer" or "like bad wine flavour". Other volatile off-flavours formed by yeasts include acetaldehyde – pungent apples; acetic acid – vinegar; ethyl acetate – sweet pineapple note; diacetal and acetoin – sweet butter.

Spoilage due to certain yeast species may be characterized by distinct off-flavours. *Brettanomyces intermedius* (*Dekkera bruxellensis*) may have a distinct mouse flavour (Beech and Carr 1977; Lafon-Lafourcade 1983), described as leathery, sweaty, farmyard or "Brett" in red wine (Parker 1988). *Pichia* species, notably *P. membranifaciens*, have a yeasty aldehyde off-flavour, forming high levels of acetic acid, acetaldehyde (Rankine 1966; Lafon-Lafourcade 1986) and esters, including ethyl acetate (Lanciotti et al. 1998). *Saccharomycodes ludwigii* forms high levels of acetoin and acetaldehyde (Ribereau-Gayon et al. 1975; Bravo-Abad and Redondo-Cuenca 1985; Romano et al. 1999), while *Kloeckera apiculata* off-flavours in cider comprise high levels of esters and volatile acids (Reed and Peppler 1973; Beech and Carr 1977). The growth of lipolytic yeasts such as *Y. lipolytica*, on fat-rich substrates such as cheese or meat, may result in "free fatty acid rancidity" (Ingram 1958). In an unexpected twist, addition of preservatives to foods may make them more susceptible to spoilage by off-flavours, if the yeasts in question are resistant, and proceed to degrade the preservative. Sorbic acid can be degraded by *Z. rouxii* and *Debaryomyces hansenii* to 1,3-pentadiene, giving a petroleum-like off-odour (Casas et al. 1999).

11.5 Economic Effects of Yeast Spoilage

Any discussion on the economics of food spoilage should first address the problem of estimating the worldwide scale of losses due to yeasts. The following questions require answers. How much spoiled food is eaten, not noticed by the consumer? How much yeast-spoiled food is thrown away without publicity? Either industrially or domestically? To what degree is yeast spoilage complained about?

Frankly, it is impossible to make any true estimation of the levels of yeast spoilage, but the costs must run into millions, possibly billions of euros per year. While any fermentative incidents may be largely reported, visible yeast spoilage is likely to be only par-

tially reported, and spoilage due to off-flavours is likely to be grossly underreported. In addition, there are probably a multitude of instances of minor growth of yeasts causing slight or no customer-perceptible effects that are never reported. By the definition of yeast spoilage used here – being perceptible to the customer– these may not even be classified as food spoilage. Furthermore, as yeast spoilage does not concern public safety, food production companies are not required to publicize any spoilage incidents, and relatively few are ever reported, in order to protect the company brands involved. All that can be said with certainty is that the true scale of yeast spoilage is orders of magnitude greater than the published data, particularly amongst the less obtrusive spoilage species.

Foods in general are "open" or "closed". Open foods can be the non-packaged traditional foods, or packaged foods that have been opened by the consumer. Either way, open foods are freely accessible to infection by any yeast species present. Open foods can be regarded as having an "open shelf life", usually of short duration. Instructions to the customer may be, for example, "refrigerate and consume within 3 days of opening". Such instructions are designed to be fail-safe, allowing a big yeast infection and allowing considerable leeway in temperature. Yeast spoilage of open foods, either domestic or industrial, is very often a result of foods kept for too long or at a very abused temperature.

"Closed shelf life" indicates the stability of a food packaged in a factory and not accessible to yeast infection. Any yeast cells within the food have therefore been derived from the raw materials or from the site of production. Such packages are usually given an antimicrobial treatment to kill or prevent growth of any microbes in the package. Ideally, the antimicrobial measures in place should be sufficient to prevent all microbial growth and completely eliminate food spoilage. In practice, this is often not possible for a variety of reasons; these include legal issues, taste and food quality, public perception and cost. For example, soft drinks may be made immune to spoilage by addition of high levels of sorbic acid, 1,000 ppm at pH 3.4. However, sorbic acid has a legal limit in Europe of 300 ppm (Anon 1989), a taste threshold of approximately 150 ppm and a distinct adverse taste above 300 ppm. Furthermore, the European public is averse to preservatives such as sorbic acid, regarding them as chemical additives. Even when pasteurization is employed, the heat treatment given is always insufficient to kill all microbes every time, occasionally one will survive. As a result, most systems are designed to prevent the growth of most, but not all microbes, and to minimize, but not prevent spoilage. A modicum of good factory hygiene and good manufacturing practice (GMP) is therefore required to fill any gaps left in the preservation system.

When spoilage in foods is reported, the company involved may take one of several courses of action. Major incidents, particularly involving highly fermentative yeasts, may require a public recall of all products involved. This may even extend to television advertising and requests to customers to return products to the shop. Such incidents are rare, but highly damaging to the brand image of the companies concerned. Minor incidents may be on a smaller scale, or may only involve spoilage to a lesser degree. Minor incidents often require a silent recall of all products on shop shelves and in the supply chain. The cost of an incident of food spoilage can be considerable. For example, a soft drinks factory may become infected with a spoilage yeast. Soft drinks are infected, but it may take a month for the yeast to grow and spoilage to become visible. Soft drinks lines run at 30,000 bottles per hour, for perhaps

16 h a day in summer (yeast spoilage is always more prevalent in late summer). Recalling bottles from the infected line could run to more than ten million items. Direct costs of spoilage include the cost of the products, costs of recall and costs of disposal. The indirect cost, the damage done to the company name and the brand image, can be much severer, even to the extent of complete closure and withdrawal of the brand.

11.6 Which Yeasts Cause Spoilage and What are the Properties of the Successful Spoilage Yeasts?

Imagine for a moment a nutritious food, prepared as a hot-water extract of germinated wheat or barley grains, prepared in shallow dishes and exposed to the environment. It is likely that almost all of the 800 yeast species described by Barnett et al. (2000) would grow on this food, and almost all could thus be described as spoilage yeasts (of malt extract). In practice, however, relatively few yeast species are responsible for the majority of instances of food spoilage by yeasts. Pitt and Hocking (1997) noted that while over 110 species from 30 genera were associated with foods, in their experience only about 10–12 species were responsible for spoilage of foods which had been processed and packaged according to normal standards of GMP (Table 11.2). A near identical list of yeast species was presented by Tudor and Board (1993) as the most commonly encountered spoilage yeasts, and Stratford et al. (2000), while only considering spoilage of fruit juices and soft drinks, listed eleven of the most significant spoilage yeasts (Table 11.2). Davenport (1996, 1997, 1998), in

Table 11.2 The most significant and commonly reported food spoilage yeast species

Pitt and Hocking (1997)	Tudor and Board (1993)	Davenport group 1 in Stratford et al. (2000)
Brettanomyces bruxellensis	*B. intermedius*	*B. anomalus*
Candida krusei	*Candida holmii*	*B. bruxellensis*
Candida parapsilosis	*Candida krusei*	*B. naardenensis*
Debaryomyces hansenii	*Debaryomyces hansenii*	*Hanseniaspora uvarum*
Kloeckera apiculata	*Kloeckera apiculata*	*Saccharomyces bayanus*
Pichia membranifaciens	*P. membranifaciens*	*Saccharomyces cerevisiae*
Rhodotorula mucilaginosa	*Rhodotorula* spp.	*Schizosaccharomyces pombe*
Saccharomyces cerevisiae	*Saccharomyces cerevisiae*	*Saccharomyces exiguus*
Schizosaccharomyces pombe	*Schizosaccharomyces pombe*	*Torulaspora delbrueckii*
Torulopsis holmii	*Zygosaccharomyces bailii*	*Z. bailii*
Z. bailii	*Z. bisporus*	*Z. bisporus*
Z. bisporus	*Z. rouxii*	*Z. microellipsoides*
Z. rouxii	–	*Z. rouxii*

Note recent changes in nomenclature (Barnett et al. 2000; Kurtzman 2003); *B. intermedius = B. bruxellensis = Dekkera bruxellensis*; *B. anomalus = Dekkera anomala; Candida krusei = Issatchenkia orientalis; Candida holmii = Torulopsis holmii = Saccharomyces exiguus = Kazachstania exigua*; *Kloeckera apiculata = H. uvarum*; *Z. microellipsoides = Torulaspora microellipsoides*

his "forensic approach " to yeast spoilage considered that it was the properties of the spoilage yeasts that were most significant, not merely the taxonomic names. He considered that the most dangerous spoilage yeasts (group 1) included *Z. bailii*, and abnormal strains of *Saccharomyces cerevisiae* all shared a number of physiological properties (Davenport 1996). Clearly there is general consensus as to which yeasts constitute the greatest threat to foods.

However, there are, in addition, a far larger number of yeast species that also cause food spoilage on a lesser scale. Tudor and Board (1993) referred to these as the "second division yeasts" and listed 19 species. Davenport (1996) also referred to lesser spoilage yeasts, calling them group 2 (spoilage and hygiene yeasts), opportunist species that would only cause spoilage following an error in manufacturing. These were distinct from group 3 (hygiene yeasts) that would not cause spoilage even if inoculated onto a food. Group 3 yeasts could be used as indicator species of factory hygiene.

Having established that the majority of yeast species grow well on a medium like malt extract, why is it that so few yeasts are considered dangerous spoilage species? What extraordinary properties do these species possess? Are these the commonest yeast species? Or do they proliferate more quickly? Data presented by Deak and Beuchat (1996) showed that 99 yeasts species were isolated from all foods, and of these *Z. bailii* only comprised 3.05%, *Z. rouxii* 2.06%, *Saccharomyces exiguus* 1.76% and *B. bruxellensis* 0.14%. The most commonly isolated yeast species from foods were *Candida* and *Pichia* spp. Clearly frequency of isolation does not account for the fame of a few yeasts as dangerous spoilage species. Similarly the growth rate of these yeast species is not exceptional, indeed growth of *Zygosaccharomyces* spp. is often slower than for the majority of yeast species. *Z. bailii* doubles in approximately 2.5 h, *Schizosaccharomyces pombe* doubles in approximately 4 h, while the generation time of *Z. lentus* is near 12 h (Stratford et al. 2000; Steels et al. 1998).

Observations made by Davenport as to the physiological properties of group 1 spoilage yeasts appear relevant here. Group 1 yeasts, comprising the most dangerous spoilage yeasts, were observed to be preservative-resistant, osmotolerant, highly-fermentative and vitamin-requiring (Davenport 1996, 1997). Is it not possible that the highly fermentative property may cause "blown" packages and draw the attention of consumers to spoilage? Similarly the properties of preservative resistance and osmotolerance may enable the spoilage species to proliferate in food environments inimical to the great majority of yeast species. Indeed, does not the list of the most dangerous spoilage species indicate a list of extremeophiles, the only yeast species able to tolerate the high osmotic pressures or elevated concentrations of acidic preservatives used in foods? The individual factors involved in food preservation will now be examined in isolation, and the yeast species most resistant to that preservation factor identified.

11.7 Factors Comprising Preservation Systems

The microbial threat to any foodstuff will be resisted by the "preservation system" used with that food. A preservation system can be regarded as a composite of factors intrinsic to the food, physical and chemical preservation, hygiene and packaging measures. In the food itself this may encompass low pH, low water activity, lack of

nutrients or presence of essential oils. Physical preservative measures include heat and pasteurization, chilled storage, carbonation or modified atmospheres, and chemical preservatives include sorbic, acetic and benzoic acids. Very few foods are protected by a single antimicrobial factor, for example a can of pasteurized soft drink is protected by heat, the acidity of the soft drink, and the packaging restricting oxygen ingress.

11.7.1 Heat

Heat is one of the commonest and most effective methods of preserving foods and beverages. Foods can be very effectively sterilized by retorting sufficiently to kill bacterial spores, or alternatively given a milder heat treatment, pasteurization, that is designed to eliminate vegetative cells. Heat may be applied to the complete package, of food and container, as in tunnel pasteurization, or liquids may be pasteurized "in line" by passage through a plate pasteurizer. The liquid food/beverage can then be hot-filled at 90°C, thus sterilizing the container, or aseptically cold-filled into sterile packaging.

Yeasts are, without exception, relatively sensitive to heat. Temperatures between 45 and 53°C, depending on the species, rapidly kill almost all yeasts. The *D* value indicates the time required to kill 1log (90%) of the inoculum. Yeast *D* values rarely exceed 1 min at 55°C (Corry 1973; Splittstoesser 1996). In comparison, vegetative cells of bacteria such as *E. coli* can also be killed at 52°C but with a *D* value of 18 min (Splittstoesser et al. 1995). The yeast species most resistant to heat include *Saccharomyces cerevisiae*, *P. membranifaciens*, *P. anomala* and *P. galeiformis* (Put et al. 1976; Beuchat 1981; Tchango Tchango et al. 1997) and also *Candida albicans*, *Clavispora lusitania*, *I. orientalis* and *Schizosaccharomyces pombe*. Yeast species showing unusual sensitivity to heat include *Debaryomyces hansenii*, *Z. rouxii* (Beuchat 1981; Samson et al. 1981) and *Cryptococcus magnus* (M. Stratford and H. Steels, unpublished data), which are killed at temperatures as low as 40°C.

Very little yeast spoilage is therefore expected in properly pasteurized foods, except where the yeast cell inoculum is very large. A few survivors might be expected from a yeast population of 10^5 cells/ml heated for 20 min at 60°C. The heat resistance of yeasts may be increased marginally by the presence of high concentrations of sugars or similar solutes (Gibson 1973; Corry 1976), or by cells being in the stationary phase of growth (Iwahashi et al. 1995). The presence of yeast ascospores may also increase heat resistance to a greater degree (Put et al. 1976; Tilbury 1980a), but not to the level achieved by mould genera such as *Byssochlamys* (Tournas 1994) that can survive 25 min at 90°C. The heat resistance of yeast ascospores is also species-dependent, with *Z. bailii* ascospores being less heat resistant than those of *Saccharomyces cerevisiae* (Thomas and Davenport 1985; Raso et al. 1998). As a general rule, yeast ascospores are 10°C more resistant than are their vegetative cell counterparts (Lund 1951; Davenport 1980a).

11.7.2 Acidity and Acetic Acid

In general terms, the pH range of foods and beverages extends from pH 2.5 to pH 7.5. Acidic foods are particularly associated with yeast spoilage, ranging from the

low-pH cola-type beverages, approximately pH 2.5, cider, wine and soft drinks, approximately pH 2.8–3.4, to fruit juices ranging from pH 3.0 in unripe lemon or grapefruit to pH 4.0-4.5 in ripe apple or tomato juices (Hicks 1990). Acidic taste is usually balanced by addition of sugar, with foods of lower pH and higher concentrations of acids, for example citric acid, requiring greater levels of sweetening. This, of course, greatly increases the risk of yeast spoilage in such foods.

The pH range of yeasts, however, extends from below pH 1.0 to pH 10.0 (Aono 1990; Stratford et al. 2000; Steels et al. 2002b). Figure 11.4 shows that the majority of spoilage yeast species are able to grow at pH 2.0 (Pitt 1974) and up to pH 8.5. Furthermore, examination of the growth of yeasts shows that only when the pH falls below 3.0 are yeasts low-pH-stressed, as indicated by slower growth or reduced yield. Acidity in foods per se is therefore of no hindrance to spoilage yeasts, and low pH alone does not contribute directly to protection of foods from yeasts or moulds. However, low pH does have a dramatic effect on food preservation in the presence of weak-acid preservatives, such as acetic acid.

Acetic acid, in small quantities, is a natural component of oils of citrus fruits (Burdock 1995) and strawberries (Willhalm et al. 1966). It can be formed by fermentation in yeasts, notably *Dekkera* and *Brettanomyces* spp. (Geros et al. 2000), but is usually associated with acetic acid bacteria (*Acetobacter* spp.) metabolizing the ethanol in yeast-fermented beverages. Acetic acid is recognized in

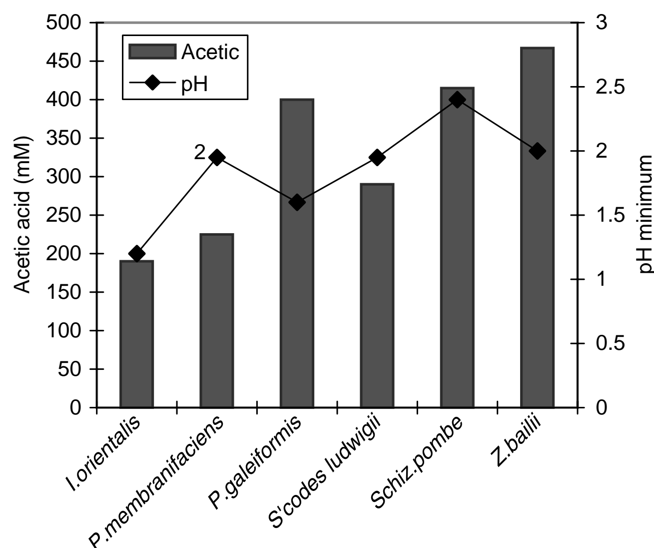

Fig. 11.4. Extreme acetic acid resistance at pH 4.0 shown by certain spoilage yeast species does not correlate with resistance to low pH. The yeast strains were *Z. bailii* NCYC°1766, *Saccharomycodes ludwigii* NCYC°732, *Pichia membranifaciens* 210, *P. galeiformis* 917aH, *Issatchenkia orientalis* CMCC°2528 and *Schizosaccharomyces pombe* NCYC°2722. All had been identified by D1/D2 26S rDNA sequencing

Europe as an acidulant, a food additive that may be used *quantum satis* (Anon 1989). Foods containing acetic acid are easily distinguished by their pungent vinegary taste and smell. These include tomato ketchup, pickles, mayonnaises, salad dressings and kombucha (acetic-fermented tea).

The antimicrobial action of acetic acid is strongly influenced by pH. Acetic acid is a weak acid, forming a dynamic equilibrium in aqueous solution between undissociated acetic acid molecules and acetate anions. The undissociated acid predominates at low pH and appears solely responsible for the antimicrobial activity (Maesen and Lako 1952; Ingram et al. 1956). Undissociated acetic acid is a small, uncharged molecule that is able to dissolve in the hydrophobic lipid plasma membranes of microbes, and thus rapidly pass by diffusion into the cytoplasm (Conway and Downey 1950a, b; Suomalainen and Oura 1955; Walter and Gutknecht 1984; Casal et al. 1998). Once in the cytoplasm, acetic acid dissociates rapidly into acetate ions and protons, causing a severe drop in the pH of the cytoplasm (Neal et al. 1965; Carmelo et al. 1997; Guldfeldt and Arneborg 1998), and inhibiting or killing the microbe.

Yeast species vary widely in their resistance to acetic acid (Fig. 11.4), a fact utilized as a taxonomic test (growth in 1% acetic acid; Barnett et al. 2000). Yeast species most resistant to acetic acid include *I. orientalis* and *I. occidentalis*, *P. galeiformis* and *P. membranifaciens*, *Saccharomycodes ludwigii*, *Schizosaccharomyces pombe*, *Z. bailii*, *Z. bisporus*, *Z. lentus* and *Z. kombuchaensis* (Pitt 1974; Warth 1989a; Kalathenos et al. 1995; Malfeito Ferreira et al. 1997; Saeki 1989; Steels et al. 2002a). *Z. rouxii* strains are unexpectedly sensitive to acetic acid (Giudici 1990). The yeast spoilage flora of any food preserved with acetic acid is therefore predominated by resistant species such as *Z. bailii* and *P. membranifaciens* (Dennis and Buhagiar 1980). Given the only moderate acetic acid resistance of *P. membranifaciens* and the strong acetic resistance of *P. galeiformis* (Barnett et al. 2000) it is likely that many acetic acid resistant *P. galeiformis* strains have been misidentified in the past as *P. membranifaciens*.

11.7.3 High Sugar and Osmotolerance

Yeasts able to grow in high sugar concentrations are often referred to as osmotolerant or osmophilic; however, these names are often also applied to salt-tolerant yeasts, high sugar and high salt being regarded as synonymous in the bacterial field and termed low water activity or a_w. However, research has shown that for yeasts, salt and sugar act inhibit by very different mechanisms (Watanabe et al. 1995) and will therefore be treated separately in this chapter.

High-sugar foods include honey, jams and marmalade, nougat, toffee and caramel, sugar syrups and molasses, marzipan, confectionary and crystallized fruit, fruit cordials and juice syrups. Sugar at a concentration ofmore than 30% w/w has been described as having an antimicrobial effect (Tarkow et al. 1942), yet most yeast species grow well at up to 45% w/w. However high-sugar foods can contain more than 67% sugar w/w and are prone to spoilage only by osmophilic yeasts, amongst which are some of the most xerotolerant organisms known (Tilbury 1980a, b).

Osmotolerant yeasts have been defined as those able to grow at 50% w/w sugar, 0.88 a_w (Sand 1973) while osmophilic species are those able to grow at 60% sugar w/w (Davenport 1975). In addition to causing spoilage, growth of osmophilic yeasts in sugar and syrups can represent a major source of infection of spoilage yeasts, in foods prepared using the sugar as a raw material (Ingram 1949; Scharf 1960).

Detection or enumeration of yeasts from high-sugar foods can be difficult. Plating out yeasts from sugar syrups on normal low osmotic strength media will cause an osmotic down-shock, and prevent yeast growth for several days. It is recommended that samples from high-sugar foods be diluted in 40% sugar to avoid this problem (Beuchat 1998) and grown on high-sugar media containing half the sugar level of the original food.

The antimicrobial action of sugar appears to be primarily via osmosis (Martinez de Maranon et al. 1996, 1997) with the water content of the cytoplasm rapidly removed from cells placed in concentrated sugar. Resistance to high sugar is largely based around accumulation of high concentrations of compatible solutes in the cytoplasm, such as glycerol or arabitol (Tokuoka 1993). Surviving cells grow slowly in high sugar concentrations and are consequentially much reduced in volume.

Yeast species able to grow in high sugar concentrations include *Debaryomyces hansenii*, *Schizosaccharomyces pombe*, *P. ohmeri*, *Z. bailii*, *Z. bisporus*, *Z. lentus* and most notably *Z. rouxii* and *Z. mellis* (von Schelhorn 1950; Scarr 1951, 1968; Sand 1973; Tilbury 1980a, b; Lafon-Lafourcade 1983; Jermini and Schmitt-Lorenz 1987; Tokuoka 1993; Giudici 1990). Spoilage of high-sugar foods is, however, dominated by *Z. bailii*, *Z. bisporus* and *Z. rouxii* (Tokuola 1993).

In addition, there are a family of closely related osmophilic yeast species all associated with spoilage of sugar foods and with insects, such as bees and wasps (Steels et al. 2002b; Stratford and James 2003). These species generally do not form sexual spores and are so termed *Candida*, with the exception of one species that has been sporulated and was given the generic name *Starmerella* (Rosa and Lachance 1998). These are relatively unusual spoilage yeasts and it has been proposed that this group of bee-/wasp-associated yeasts are able to cause spoilage, only following infection from bees/wasps attracted to sugary foods. This group of yeasts includes *Candida bombi*, *Candida apicola*, *Candida etchellsii*, *Starmerella bombicola*, *Candida lactis-condensii*, *Candida davenportii*, *Candida stellata* and *Candida magnoliae*.

11.7.4 High Salt and Halotolerance

Salt toxicity in yeasts is recognized to be primarily due to the toxicity of the sodium ion, Na^+, rather than to the osmotic effects imposed by high-salt concentrations. Resistance to salt is conferred through active sodium efflux pumps or sequestration of sodium ions (Watanabe et al. 1995; Ferrando et al. 1995; Nass et al. 1997; Alepuz et al. 1997; Rios et al. 1997). Salt shock can cause expression of heat shock proteins (Lewis et al. 1995) and accumulation of glycerol (Onishi 1963; Ohshiro and Yagi 1996; Lages et al. 1999). The pH window for optimal yeast growth has been observed to be far narrower in the presence of high salt concentrations (Onishi 1963). Foods containing substantial concentrations of salt include salted meats such as sausage

and bacon, brines and pickled vegetables, and salted fermented foods such as sauerkraut, soy sauce, shoyu and miso.

The majority of yeast species are salt-tolerant microbes and are able to grow in media containing up to 1.5–2 M sodium chloride (8.5–11.5% w/v). The most salt-resistant yeast species able to grow in 20% w/v salt include *Debaryomyces hansenii* and *Debaryomyces tamarii*, *Z. rouxii*, and to a lesser extent, *Z. mellis* (Kurtzman 1998), *Candida etchellsii*, *Candida sorbosivorans* and *Candida parapsilosis* (Onishi 1963; Corry 1973; Tokuoka 1993; Prista et al. 1997; Thome-Ortiz et al. 1998; Betts et al. 1999; Lages et al. 1999). Other yeasts moderately resistant to salt include *P. guilliermondii*, *P. membranifaciens* and *P. subpelliculosa*, *Torulaspora delbrueckii* and *I. orientalis*. Lages et al. (1999) reported *Z. bailii* to be salt-sensitive, on the basis of tests on the atypical type strain: most strains of *Z. bailii* are in fact moderately resistant to salt. Spoilage of foods containing high concentrations of salt is dominated by strains of *Debaryomyces hansenii*, sometimes called *Candida famata*, and *Z. rouxii* (Kato 1981; Fleet 1992; Tokuoka 1993).

11.7.5 Ethanol and Alcoholic Beverages

Ethanol is present in alcoholic beverages fermented by the brewing and winemaking yeast *Saccharomyces cerevisiae* at concentrations up to 20.3% v/v (Peres and Laluce 1998). Ethanol has also been considered as a means of preservation in packaged foods, such as bakery products (Kalathenos and Russell 2003). The degree of protection from spoilage conferred by ethanol depends upon the concentration of ethanol and the species of yeast involved, but overall spoilage is minimized by ethanol in excess of 15% v/v and is eliminated at concentrations greater than 22% v/v. *Z. bisporus* has been isolated from sherries containing 22% v/v alcohol (Hammond 1976; quoted by Thomas 1993). In general terms, fortified wines (17.5% v/v) are spoiled very infrequently, wines (10–15% v/v) are spoiled occasionally, and beers, ciders and alcoholic soft drinks (3–8% v/v) are sufficiently unstable as to require a short shelf life or protection by pasteurization or use of preservatives.

The mechanism of action of ethanol against yeasts is centred upon the integrity of the plasma membrane, ethanol causing leakage and dissipation of the proton motive force, and affecting transport processes (Sa-Correia and van Uden 1986; Cartwright et al. 1986; Jirku et al. 1991; Stevens and Servaas Hofmeyer 1993). Ethanol also affects mitochondria and induces "petites" (Ibeas and Jimenez 1997).

The yeast species most resistant to ethanol are not surprisingly those fermentative species that produce ethanol at the highest concentrations. These include *Dekkera anomala* and *Dekkera bruxellensis*, *I. orientalis*, *P. anomala* and *P. galeiformis*, *Saccharomyces cerevisiae* and *Saccharomyces bayanus*, *Saccharomycodes ludwigii* and *Z. bailii*. Spoilage yeasts of high-alcohol beverages such as wine are also predominated by *Saccharomyces* spp., *Zygosaccharomyes bailii*, *Saccharomycodes ludwigii* and *Dekkera/Brettanomyces* spp. (Peynaud and Domercq 1959; Kunkee and Goswell 1977; Thomas and Davenport 1985; Thomas 1993; Baleiras Couto and Huis in't Veld 1995; Kalathenos et al. 1995; du Toit and Pretorius 2000).

11.7.6 Sulphur Dioxide (Sulphite)

Sulphites are traditional preservatives that have been used in the production of wine, cider and beer for centuries. Flowers of sulphur were burned in wooden barrels, which were then filled, ensuring sterilization of the barrel and thepresence of sulphites, as dissolved SO_2 in the beverage (de Keersmaecker 1996). Sulphites are currently permitted preservatives in Europe (Anon 1989) for use in wine and cider.

Like acetic acid, SO_2 is a weak acid, existing in solution as a pH-dependent equilibrium between molecular SO_2 at low pH and bisulphite and sulphite ions at higher pH (King et al. 1981). Only molecular SO_2 appears to exert an antimicrobial action. Molecular SO_2 passes into cells rapidly, by diffusion through the plasma membrane (Stratford and Rose 1986), dissociates to bisulphite ions and causes a catastrophic fall in cytoplasmic pH (Pilkington and Rose 1988). Membrane transport processes are consequentially inhibited (Freese et al. 1973) and ATP levels fall rapidly (Schimz and Holzer 1979). Sulphites are highly reactive molecules and may also denature a variety of other cytoplasmic targets (Gould et al. 1983).

Resistance of yeasts to sulphites is not always found in all strains of an individual species. However, highly resistant strains (Fig. 11.5) are most likely to be found within the following species: *Saccharomycodes ludwigii*, *Saccharomyces cerevisiae*, *Saccharomyces bayanus* and *Saccharomyces exiguus; Z. bailii* and *Z. lentus* (Jakubowska 1963; Hammond and Carr 1976; Minarik and Navara 1977; Goto 1980; Usseglio Tomasset et al. 1981; Stratford et al. 1987; Rose 1987; Thomas 1993). Consequentially spoilage in beverages where sulphites are commonly used is

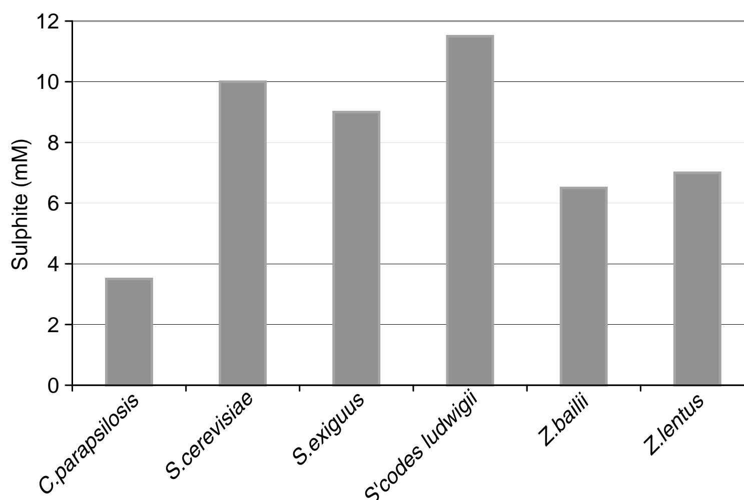

Fig. 11.5. Extreme SO_2, sulphite, resistance at pH 4.0 is shown by certain strains of spoilage yeasts. The yeast strains were *Z. bailii* NCYC°1766, *Saccharomycodes ludwigii* NCYC°730, *Saccharomyces cerevisiae* 292, *Saccharomyces exiguus* 152, *C. parapsilosis* CMCC°3397 and *Z. lentus* NCYC°1601. All had been identified by D1/D2 26S rDNA sequencing

dominated by *Saccharomyces cerevisiae*, *Saccharomycodes ludwigii* and *Z. bailii* (Peynaud and Domercq 1959; Beech 1961; Amerine and Kunkee 1968; Sandu-Ville 1977; Thomas 1993).

11.7.7 Preservatives (Sorbic Acid and Benzoic Acid)

Sorbic acid and benzoic acid are permitted preservatives in Europe, but may be only added up to specified levels in specified foods (Anon 1989). In the USA, however, sorbic acid and benzoic acid have GRAS status and may be found at higher concentrations. The use of these preservatives is widespread in foods as diverse as spreads, fruit syrups, confectionary, bakery products, wine and soft drinks, spent malt grains and almond paste (Sand 1973; Rankine and Pilone 1974; Faid et al. 1995; Jager 1997; Stratford and Eklund 2003). It is also permitted, and fairly common, to encounter mixtures of sorbic acid and benzoic acid in foods, both at reduced concentrations (Taylor 1998). It has been suggested that sorbic acid is more inhibitory to yeasts than benzoic acid (Pitt 1974) but also that benzoic acid is better than sorbic acid at controlling spoilage at pH 3 (Sand and Kolfschoten 1969). As a rule of thumb, sorbic acid and benzoic acid are roughly equal and additive in their anti-yeast effects on a parts per million, milligrams per litre basis.

Sorbic acid is a six-carbon fatty acid, unsaturated in positions 2 and 4, while benzoic acid comprises a carboxylic acid substituted into a benzene ring. Both acids have the great advantage of having relatively little taste and a proven record of safe human consumption and lack of genotoxicity (Ferrand et al. 2000). Benzoic acid is a natural constituent of cranberries and cloves (Swartz and Medrek 1968; Chichester and Tanner 1972). The free acids of both are difficult to dissolve and these preservatives are normally added to foods as soluble salts, sodium benzoate and potassium sorbate.

Both sorbic acid and benzoic acid are weak acids in aqueous solution, and as can be predicted inhibit microbes only in the undissociated acid form that predominates at low pH (Ingram et al. 1956; Azukas et al. 1961; York and Vaughn 1964). Uptake of the undissociated acid molecules is extremely rapid, probably by simple diffusion of these lipophilic acids through the plasma membrane (Macris 1975; Reinhard and Radler 1981b; Walter and Gutknecht 1984; Warth 1989b). Within the cytoplasm, weak acid molecules dissociate and may lower the cytoplasmic pH (Krebs et al. 1983; Holyoak et al. 1996), as has been demonstrated for acetic acid and sulphite.

However, it has been pointed out that the concentrations of protons released by inhibitory concentrations of sorbic acid are insufficient to cause significant pH change (Stratford and Anslow 1998). Alternative sites of action for sorbic and benzoic acids include the membrane (Reinhard and Radler 1981a; Stratford and Anslow 1998), respiration pathways (Palleroni and de Pritz 1960) and glycolysis metabolism (Azukas et al. 1961; Burlini et al. 1993). The effect of subinhibitory concentrations of preservatives is to cause slower growth of yeasts, smaller cell size, and much reduced cell yields (Cole and Keenan 1987; Neves et al. 1994; Stratford and Anslow 1996).

Resistance of certain yeast species to preservatives is a significant threat to the stability of preserved foods. This threat is further exacerbated by the ability of yeasts to adapt to preservatives, enabling growth and spoilage at much higher preservative

concentrations (Ingram 1960; Warth 1977, 1988; Malfeito Ferreira et al. 1997). It is envisaged that foods or beverages containing preservatives may be spilled in production facilities, diluted as they are washed into the drains, and that spoilage yeasts can grow on dilute preservatives, adapting to their presence and thus gain the ability to spoil the preserved foods.

The yeast species most notorious for resistance to preservatives (Fig. 11.6) is *Z. bailii*, together with its close relatives *Z. bisporus* and *Z. lentus* (Ingram 1960; Jermini and Schmidt-Lorenz 1987; Warth 1989a, c; Neves et al. 1994; Steels et al. 1999). These species can grow in soft drinks containing sorbic or benzoic acids at well in excess of the legal limit in Europe. A strain of *Z. bailii* has even been reported that *requires* benzoic acid for growth (Anon 1998). Other less resistant species include *Y. lipolytica* (Rodrigues and Pais 2000) *Candida lambica*, *Candida pseudolambica*, *I. orientalis* and *I. occidentalis*, and abnormal strains of *Saccharomyces cerevisiae* and *Saccharomyces bayanus* (Davenport 1996; M. Stratford and H. Steels, unpublished data). Several yeast species only show resistance to single preservatives, such as *Brettanomyces/Dekkera* spp., which are sorbic acid resistant but benzoic acid sensitive, or *P. membranifaciens*, *P. galeiformis* or *Schizosaccharomyces pombe*, which are sorbic acid sensitive and benzoic acid resistant (Fig. 11.6).

Spoilage in sorbic acid or benzoic acid preserved foods and beverages is consequentially dominated by these same species, *Z. bailii*, *Z. bisporus*, *I. orientalis* (as *Candida krusei*) and abnormal strains of *Saccharomyces cerevisiae* (Ingram 1960; Lloyd 1975; Thomas and Davenport 1985; Davenport 1996, 1997, 1998)

Much research has been carried out into the mechanisms of yeast resistance to sorbic acid and benzoic acid. Degradation of preservatives by metabolism was

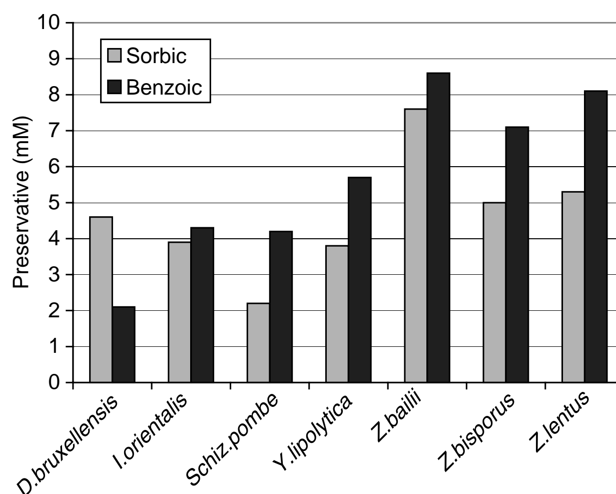

Fig. 11.6. Resistance to sorbic and benzoic acids at pH 4.0 is limited to the spoilage yeasts *Z. bailii* NCYC°1766, *Z. bisporus* NCYC°1555, *Z. lentus* NCYC°1601, *I. orientalis* CMCC°2528, *Yarrowia lipolytica* 474, *D. bruxellensis* 148 and *Schizosaccharomyces pombe* NCYC°2722. All had been identified by D1/D2 26S rDNA sequencing

shown by Warth (1977) not to be a significant factor in resistance. A weak-acid preservative efflux pump was proposed (Warth 1977, 1989b; Henriques et al. 1977) where preservatives were pumped out from the cytoplasm at the expense of ATP. The gene *PDR12* with homology to mammalian multiple-resistance drug pumps was shown to be important in preservative resistance in *Saccharomyces cerevisiae* (Piper et al. 1998). It was proposed that *PDR12* mediated the efflux of preservative anions from the cytosol (Holyoak et al. 1999). However, the work of Cole and Keenan (1987) demonstrated such rapid pH-dependent movements of weak-acid molecules by simple diffusion through the membrane, which must call into question any role for *PDR12* as an anion pump.

11.7.8 Carbonation (CO_2)

Carbonation is the process of dissolving carbon dioxide into beverages under pressure, forming the characteristic taste of sparkling beverages. These include colas, sparkling fruit drinks, mixers such as tonic or ginger ale, cream sodas, lemonades, and sparkling wines such as Cava and Champagne. Carbonation is measured in units of volumes bunsen, the CO_2 volume, at 0°C and atmospheric pressure, dissolved per volume of liquid, or in grams CO_2 dissolved per litre (1.96g/l = 1 vol; Mitchell 1990). Carbonation in soft drinks is typically around 3 vol, ranging from 1.5 vol in sparkling fruit juices to 5 vol in soda water or bottle-fermented wines (Sand 1976b; Mitchell 1990).

It is not often appreciated that carbonation has a considerable antimicrobial effect (Schmidt 1995; Monch et al. 1995), particularly at the higher concentrations permitted by increased pressure. The antimicrobial nature of highly carbonated low-pH soft drinks has enabled successful production of beverages such as "Codds Wallop" in relatively unhygienic conditions since 1870 (Taylor 1998; de Thouars 1999). Inhibition is not caused by pressure per se; yeasts are inhibited by pressurized CO_2, not by nitrogen (Lumsden et al. 1987). The degree of carbonation required to inhibit the growth of most yeasts species is of the order of 2 bar, although yeasts can be killed at 30 bar (Schmitthenner 1949; Amerine 1958; Kunkee and Ough 1966; Eyton-Jones 1987; van der Aar et al. 1993).

CO_2 is also a weak acid in aqueous solution, with a pK_a of 6.3 (Dixon and Kell 1989). Bicarbonate ions predominate at a pH above 6.3, while at more acidic pH, molecular CO_2 is dissolved in solution. The physiological effects of CO_2 at sublethal concentrations may include inhibition of cell division (Lumsden et al. 1987), inhibition of amino acid uptake (Knatchbull and Slaughter 1987), perturbation of cytoplasmic buffering (Sigler et al. 1981), induction of sporulation (Ohkuni et al. 1998) and membrane disruption (Dixon and Kell 1989). An action by CO_2 in lowering cytoplasmic pH is also possible. CO_2 is known to cross membranes so fast by diffusion that concentrations rarely differ across a membrane (Thomas 1995). With a pK_a of 6.3 and a near neutral cytoplasmic pH, CO_2 would certainly dissociate into bicarbonate and protons, forcing the cytoplasmic pH down. Recent work on ultrahigh pressure has shown a substantially greater microbial kill if pressure is applied in the presence of CO_2, for example supercritical CO_2 at 200 bar (Spilimbergo et al. 2002). It has been suggested that the lethality of CO_2 at high

pressure was probably a double effect due to a decline in cytoplasmic pH and cytoplasmic membrane modification.

The yeasts most resistant to carbonation are *Dekkera anomala*, *Dekkera naardenensis* and *Dekkera bruxellensis*, *Saccharomycodes ludwigii*, *Saccharomyces cerevisiae*, *Saccharomyces bayanus*, *Saccharomyces pastorianus* and *Saccharomyces exiguus* (Kunkee and Ough 1966; Reed and Peppler 1973; Ison and Gutteridge 1987; Dixon and Kell 1989; M. Stratford and H. Steels, unpublished data). These yeasts can grow in beverages containing up to 5–6 vol carbonation. Other moderately carbonation-resistant yeasts include *I. orientalis*, *P. fluxuum*, *Candida boidinii*, *Schizosaccharomyces pombe*, *T. delbrueckii*, *Z. bailii*, *Z. cidri*, *Z. microellipsoides* and *Z. fermentati* (Goswell 1986; Ison and Gutteridge 1987; M. Stratford and H. Steels, unpublished data).

Yeasts largely responsible for spoilage of carbonated beverages (some lightly carbonated) include *Dekkera anomala* and *Dekkera bruxellensis*, *Z. bailii*, *T. delbrueckii* and *Saccharomyces cerevisiae* (Pitt and Richardson 1973; Smith and van Grinsven 1984).

11.7.9 Low Oxygen Concentration

It is not often appreciated that low oxygen in a food or beverage is a major factor in preventing microbial spoilage (Sand 1976a, b). Low oxygen concentration is frequently assumed to be responsible for the antimicrobial effects of CO_2 described previously. In reality, these are two separate effects. If CO_2 is dissolved in a beverage, it does not displace the oxygen already there. This will only occur if CO_2 is bubbled through a beverage for some time; CO_2 being a heavy gas will form a layer above the beverage surface. Since oxygen in solution is in equilibrium with the gas in the headspace, there will, in this instance, be a progressive loss of oxygen from the beverage.

The concentration of oxygen in foods depends largely on the diffusion of oxygen from the atmosphere, the food surface being plentifully supplied with oxygen, while deeper into the food, the levels drops sharply. It is no coincidence that the majority of yeasts on fresh fruit and vegetables are found on the surface. Even in an open bottle of liquid, such as a soft drink, the level of oxygen is sufficiently reduced even under a few centimetres of liquid, to greatly inhibit the growth of respiring yeasts. Many processed foods also contain antioxidants, such as vitamin C, to maintain the freshness of the food, which have the effect of lowering the oxygen concentration within the food. Furthermore, most processed foods are contained in a package or bottle. Some packaging is completely oxygen impermeable, such as glass bottles or metal cans. This results in heat-processed bottled or canned foods being under greatly reduced oxygen tension, and only susceptible to spoilage by a limited number of microorganisms (Beuchat and Rice 1979). Different plastics vary considerably in their permeability by oxygen (Rodriguez et al. 1992), which can have a dramatic effect on the microbial species capable of spoiling within the package. It is well known that moulds or *Gluconobacter* spp. in fruit juices are greatly inhibited by oxygen-impermeable packaging (Follstad 1966; Sand 1971a, 1976a; Wyatt et al. 1995).

The benefits of oxygen to yeast growth are generally known, but not completely understood. Primarily, oxygen is required for respiration, as a terminal electron acceptor of the mitochondrial electron transport chain. For respiration, oxygen is required

in large quantities, and in a respiring yeast, respiration may be directly proportional to the oxygen concentration (Johnson 1967). Any yeast totally dependent on respiration will be greatly inhibited in a low-oxygen environment. This includes many very common yeast species in the genera *Rhodotorula*, *Sporidiobolus*, *Aureobasidium* or *Cryptococcus*.

Yeast species capable of fermentation are therefore immediately at an advantage in a low-oxygen environment. However, even fermenting yeasts may require oxygen for purposes other than respiration. Oxygen may be required in some yeast species for active sugar uptake, the Kluyver effect (Barnett and Sims 1982). It has been shown that fermentative brewing yeasts benefit from aeration (Grutzmacher 1991). The explanation for this is that *Saccharomyces cerevisiae* requires low concentrations of oxygen in order to synthesize unsaturated fatty acids and sterols as membrane components. In the complete absence of oxygen, growth of *S. cerevisiae* ceases unless exogenous unsaturated fatty acids and sterols are added (Andreasen and Stier 1953, 1954).

The micro-oxygen requirements of other yeast species have been little researched, but the knowledge available shows that they differ from those of *Saccharomyces cerevisiae*. *Z. bailii* and *Z. bisporus*, despite being fermentative species, are known to require oxygen (van Esch 1987) and would not grow anaerobically in synthetic media, unsupplemented by an unidentified factor present in yeast extract–peptone–dextrose (YEPD) (Rodrigues et al. 2001). Other fermentative spoilage species, *Debaryomyces hansenii* and *Z. microellipsoides*, also grow poorly without oxygen (Tilbury 1976; van Esch 1987).

It appears that spoilage in anaerobic or low-oxygen foods, even by fermentative species, may depend on the presence of as yet unidentified micronutrients.

11.7.10 Lack of Nutrients

Some years ago, a synthetic soft drink was successfully produced and marketed. The manufacturers then improved this by the addition of real fruit juice (Pitt and Hocking 1997). Following juice addition, the levels of spoilage increased to such an extent that the soft drink had to be removed from the market. Fruit juices were adding key nutrients that encouraged proliferation of spoilage yeasts. This illustrates the principle that microbial spoilage may be limited in certain instances by lack of nutrients in a food or beverage.

The nutrients required for growth of microorganisms have been investigated since the early work of Pasteur on fermentation of yeasts. By 1930 numerous studies had been carried out on the nutritional requirements of bacteria and yeasts with the very limited methods available. As a result of these studies, it became apparent that most of the microbial growth-promoting substances were from sources recognized as rich in water-soluble vitamin B, and attention was then centred upon this vitamin. Yeast species differ from each other in their requirements for vitamins. Growth with or without various vitamins has been used as characteristic diagnostic tests in the identification of yeasts (Wickerham 1951; Barnett et al. 2000). B-group vitamins are essential for yeast metabolism but some yeasts can synthesize some or all of their requirements, for example *P. anomala* can synthesize all vitamins, other yeasts can

only synthesize some; hence the remainder must be supplied by the medium (Davenport 1998).

In his "forensic" method of yeast classification, Davenport (1996) divided yeasts into three types: group 1 – spoilage yeasts; group 2 – potential spoilage and hygiene yeasts; group 3 – hygiene yeasts that will not cause spoilage. Davenport (1998) stated that as a defining characteristic, group 1 spoilage yeasts would not grow in the absence of one or more of the B-group vitamins. Spoilage yeasts known to require vitamins include *Z. bailii*, *Z. bisporus* and *Z. mellis* (Barnett et al. 2000), *Dekkera bruxellensis*, *B. naardenensis*, *Saccharomyces exiguus* and *Saccharomycodes ludwigii*. This may form the explanation for the observations of van Esch (1987) that *Zygosaccharomyces* spp. yeasts only occurred in fruit-juice-containing beverages.

In addition to vitamins, yeasts require several metallic ions for growth, including magnesium, potassium, zinc, iron, copper and manganese. In media or foods lacking these metal ions, yeast growth will be prevented. Soft drinks prepared with distilled water were observed to support very little yeast growth (Turtura and Samaja 1975). Many foods also contain acidulants such as citric acid or malic acid, which effectively remove metal ions by chelation. Certain foods may also contain the chelating agent EDTA in the USA, where EDTA has GRAS status. Metal-ion/acid-chelator complexes can be very stable, particularly where acids with multiple carboxyl groups, such as citric acid or EDTA, are complexed with multiply charged transition metal ions (Stratford 1999). Removal of metals by ion-exchange resins has been proposed for prevention of yeast spoilage in grape juice (Feng et al. 1997). Yeasts most resistant to the presence of chelating agents include *Dekkera bruxellensis*, *Dekkera anomala*, *Candida parapsilosis* and *I. orientalis* (M. Stratford and H. Steels, unpublished results). It is possible that resistance to citric acid, together with the ability to utilize nitrate, may enhance the ability of *Dekkera/Brettanomyces* spp. to spoil low-nutrient soft drinks (Smith and van Grimsven 1984; van Esch 1987).

11.7.11 Low Temperature

Low temperature in this context means spoilage at chill, at 5°C or less, and includes spoilage of frozen foods below 0°C. The usual definitions of temperature preference as applied to yeasts are psychrophilic, upper temperature limit 20°C, mesophilic, 0–48°C, and thermophilic, 20–50°C (Watson 1987). These definitions do not assist in defining low-temperature-spoilage yeasts as they could be either psychrophiles or mesophiles. A much more practical definition was proposed by Davenport (1980b) – "cold-tolerant yeasts", those capable of growth at 5°C or lower.

Cold temperatures form a hostile environment for the majority of typical spoilage yeasts and foods stored at low temperatures are also not typical substrates for yeast spoilage at moderate temperature. These include frozen vegetables such as peas, chilled and frozen fish and meat. The usual degradation of these foods by bacteria is arrested by the temperature, and there is an opportunity for spoilage by the slower-growing yeasts.

A number of general observations of cold-tolerant yeasts were made by Davenport (1980b). These were (1) basidiomycete yeasts predominated at low temperature;

(2) most were non-fermentative species; (3) an unusually high proportion, 50%, were nitrate positive. Very few of the typical spoilage yeasts are cold-tolerant. Yeasts that will not grow at 5°C or lower include *Z. bailii*, *Z. bisporus*, *Z. mellis* and *Z. rouxii*, *Dekkera anomala* and *Dekkera bruxellensis*, *Candida parapsilosis* and *Candida tropicalis*, *I. orientalis* and *I. occidentalis* (M. Stratford and H. Steels, unpublished results).

Spoilage at low temperature is therefore rarely accompanied by gas production, and spoilage is usually apparent as a result of surface growth. Yeasts reported at low temperatures are often in the basidiomycete genera *Rhodotorula* and *Cryptococcus* (Margesin et al. 2002) and include *Cryptococcus albidus* and *Cryptococcus magnus*, *Rhodotorula graminis*, *R. mucilaginosa* and *R. nothofagi* (M. Stratford and H. Steels, unpublished results), together with *Y. lipolytica*, *T. delbrueckii* and *Debaryomyces hansenii*. The minimum growth temperatures quoted by Davenport (1980b) are –12.5°C for *Debaryomyces hansenii*, –12°C for *Cryptococcus albidus* and –2°C for *R. glutinis*.

A recent development in low-temperature spoilage concerns the recent discovery of two new species in the genus *Zygosaccharomyces*, *Z. lentus* (Steels et al. 1998, 1999) and *Z. kombuchaensis* (Kurtzman et al. 2001). These species are closely related to *Z. bailii* and *Z. bisporus*, but appear to be a low-temperature branch of the family (Steels et al. 2002a). The new species are intolerant of high temperatures but grow well at 4°C. *Z. lentus* has been isolated from a variety of spoiled foods and is preservative-resistant (Steels et al. 1999). It would appear that *Z. lentus* and *Z. kombuchaensis* are capable of fermentative spoilage of refrigerated foods, immune to spoilage by *Z. bailii* and *Z. bisporus*.

11.8 Spoilage Yeast Ecology

The ecology of the great majority of yeast species so far discovered has been relatively little studied, and is consequently poorly understood. Even for a species as well studied as the brewing/baking yeast *Saccharomyces cerevisiae*, the ecology and natural life cycle are still being researched (Naumov et al. 1998; Sniegowski et al. 2002). For most yeast species our knowledge of their ecology is limited to a series of observations as to the locations of isolation of each species; the most notable exceptions to this being the recent revelations as to the interrelationships between yeast and insect ecology (Lachance et al. 1995).

In the yeast spoilage field, there exist extensive lists of yeasts isolated from different foods, which may or may not have been correctly identified and may or may not have been the cause of spoilage in that food. Very little has been written as to where such yeasts originate, and the ecology of these yeasts in the natural environment, in the food production factory or in the home or domestic environment.

11.8.1 The Global Yeast Community

All of the major spoilage yeasts listed by Pitt and Hocking (1997), Tudor and Board (1993) and Stratford (2000), and the group 1 spoilage yeasts listed by Davenport (1996, 1997), are found throughout the world, with the possible exception of Polar or Antarctic regions. Containers of food or beverages may suffer spoilage by *Z. bailii* and related

yeast species at any location from the Orient to the USA, from Russia to South Africa. The lesser-known spoilage yeasts, the "second division yeasts" (Tudor and Board 1993) such as *Candida boidinii*, *Candida parapsilosis* or *Y. lipolytica*, also appear to be universally distributed, and form part of a global community of spoilage yeasts. A possible exception to the universal distribution of spoilage yeast species may be *Z. lentus* and *Z. kombuchaenisis*. These recently discovered species share many of the characteristics of *Z. bailii*, but are notable for their intolerance of heat (Steels et al. 2002). These species fail to grow at temperatures above 30°C and are killed by temperatures above 40°C. It is therefore unlikely that these species are commonly found in tropical countries.

Many of the lesser-known yeasts have indications of geographic location in their specific names. Such distinctions have little or no true validity. The writer of this chapter has isolated *I. occidentalis* in the Orient (Moscow and Thailand) and *I. orientalis* in western Europe. *Candida wyomingensis* has been found in central Russia, *Candida natalensis* in South America, *Cryptococcus uzbekistanensis* in Brazil and *Pseudozyma antarctica* in Thailand. While it is possible that many of the recently discovered non-spoilage yeast species may have unique habitats, such as within beetle intestines and in specific geographic locations, the great majority of well-known spoilage yeasts appear to be universally distributed.

11.8.2 Sources of Infection

Most of the major food spoilage yeasts (Tudor and Board 1993; Davenport 1996; 1997; Pitt and Hocking 1997; Stratford 2000) could be termed extremophiles. This is, of course, a natural consequence of their ability to thrive in foods designed or treated to kill microbes. *Z. rouxii* is an extreme osmophilic yeast; *Debaryomyces hansenii* is a halotolerant species, *Dekkera bruxellensis* and *Saccharomyces cerevisiae* are CO_2- and ethanol-tolerant, *Saccharomycodes ludwigii* is sulphite tolerant, while *Z. bailii* shows extreme resistance to a variety of weak-acid preservatives (Table 11.3). It therefore follows that for a food to be spoiled, it must be infected with yeasts of the right species. Are these extremophilic species common? Particularly in factories where foods are processed and packaged, or do they require a specific mechanism of infection?

The most likely sources of infection of spoilage yeasts are from fruits (and vegetables), sugar and syrups, water and air, insects and dirty, contaminated equipment (Ingram 1958). Fruit, at all stages of development, represent a major natural habitat for many species of yeasts (Do Carmo-Sousa 1969). Dried, shrivelled and mummified fruit form an excellent environment for osmophilic yeasts, including *Z. bailii* and *Z. rouxii* (Beech and Davenport 1970; Davenport 1975; Tilbury 1980a, b). Sugar, either granulated or in the form of high Brix syrup, is also a potential source of infection of osmophilic yeasts (Ingram 1949; Scharf 1960; Schmidt 1995). GMP can eliminate the risks of yeast infection from sugar to a large extent, sugars should be purchased from reputable suppliers, stored carefully, and preferably sterilized by heat treatment. Water can be an ingredient in foods, such as soft drinks, or used as a processing aid, for example in washing or cleaning machinery or raw materials. Contaminated cooling water or washing water used in rinsing bottles are frequent sources of infection (Scharf 1960; Pitt and Richardson 1973; Sand and Kolfschoten 1971; Schmidt 1995). Air can be a source of yeast infection, but less frequently than

Table 11.3 Extremophilic spoilage yeast species. Most are normally rare yeast species and found in foods only when selected for by an extreme environment. Almost all are highly fermentative. Excess gas production is indicated by *an asterisk*. When occurring in environments not containing the specific selective agents, these yeasts become "second division" species. Inclusion of *Debaryomyces hansenii* is debateable, as it is less extreme in proportion to other species, is relatively common and is not highly fermentative

Yeast species	Extreme environmental resistance
(*Debaryomyces hansenii*)	Salt
	Low temperature
*Dekkera anomala** and *Dekkera bruxellensis**	Carbonated beverages
	Ethanol
	Sorbic acid
*Saccharomyces cerevisiae** and *Saccharomyces bayanus**	Carbonated beverages
	Ethanol
	Sulphite
*Saccharomycodes ludwigii**	Carbonated beverages
	Ethanol
	Sulphite
	Acetic acid
*Schizosaccharomyces pombe**	Benzoic acid
	Acetic acid
*Z. bailii**	Sugar
	Ethanol
	Sorbic acid and benzoic acid
	Acetic acid
*Z. bisporus**	Sugar
	Sorbic acid and benzoic acid
	Acetic acid
*Z. lentus**	Low temperature
	Sorbic acid and benzoic acid
	Acetic acid
*Z. rouxii**	Sugar
	Salt

might be expected (Ingram 1949), yeast cells being larger and denser than bacteria. Amongst airborne yeasts, basidiomycetes have been reported to predominate (Ingram 1958), the airborne flora being largely composed of black and red yeasts (*Aureobasidium pullulans*, *Rhodotorula* and *Sporidiobolomyces* spp.) together with *Cryptococcus albidus*, *Cryptococcus laurentii* and *Cryptococcus diffluens*. These species are essentially those described by Davenport (1996) as group 3, hygiene-indicator species, not spoilage yeasts. Insects can be regarded as potent carriers of spoilage yeasts, especially fruit flies (*Drosophila* spp.) and bees and wasps. Fruit fly infestation in fruit processing factories is notorious (Ingram 1958) and fruit flies have been shown to carry noted spoilage yeasts such as *Debaryomyces hansenii*, *Candida parapsilosis*, *P. membranifaciens*, *Saccharomyces cerevisiae*, *I. orientalis* and

Saccharomycodes ludwigii (Recca and Mrak 1952; Cooper 1960; Lachance et al. 1995). Bees and wasps may also carry osmophilic spoilage yeasts (Ingram 1958; Tilbury 1976) including *Z. rouxii*. Recently, it has been noted that most members of the *Starmerella/Candida* clade have been implicated in spoilage of high-sugar foods (Steels et al. 2002a; Stratford and James 2003). These include *Candida apicola*, *Candida etchelsii*, *Starmerella bombicola*, *Candida lactis condensii*, *Candida stellata*, *Candida davenportii* and *Candida magnoliae*. These yeasts are osmotolerant and most species in this group have been found associated with wasps, bees, bumblebees and leafcutter bees. It has been suggested that bees and wasps attracted to sugary foods form the principle source of infection of these relatively uncommon spoilage yeasts.

Contact with unhygienic, dirty, contaminated equipment is probably the commonest cause of spoilage yeast infection in food-processing factories (Ingram 1958; Tilbury 1976). It has been estimated that 95% of soft drinks spoilage was due to poor factory hygiene (van Esch 1987). The list of possible contaminated equipment includes returned bottles and packaging, filling and capping machines, conveyors and their motors, soap lubricating systems, meters and proportioning pumps and valve seals (Scharf 1960; Sand 1971a, b; Sand and Kolfschoten 1971; Pitt and Richardson 1973; Schmidt 1995; Davenport 1997). This is in addition to the yeasts living within the general factory environment, being particularly concentrated in areas where sugary products are spilled, or washed away and diluted, into the soak-aways and drains (Davenport 1996). What are the species that inhibit the general factory environment, the commonest source of spoilage yeast infection?

11.8.3 Species Frequency in Factory Environments

How commonly do different species of spoilage yeasts occur? In a far-sighted attempt to answer this question, Deak and Beuchat (1996) assembled a table of "calculated frequencies (%) of yeasts in foods". Ninety-nine yeast species were considered to be found in foods. The frequency of occurrence was calculated from the literature reports of (1) the number of types of food in which a given species was found, (2) the number of times the species was detected and (3) the number of strains of the species isolated from foods. These frequencies are therefore of reports of species in foods in the literature, and it is not surprising that spoilage yeasts are well represented, particularly the major gas-forming species. The order of frequency in all foods (Deak and Beuchat 1996) is as follows: *Saccharomyces cerevisiae* 7.04%, *Debaryomyces hansenii* 6.72%, *P. anomala* 4.56%, *P. membranifaciens* 4.32%, *R. mucilaginosa* 3.78%, *T. delbrueckii* 3.64%, *Kluyveromyces marxianus* 3.36%, *I. orientalis* 3.20% and *Z. bailii* 3.05%. However, this will include a number of spoiled foods and is understandably biased towards spoilage species. This may not reflect the frequency of occurrence of yeast species, in a food production environment prior to infection.

A series of investigations carried out some 30 years ago by Sand and colleagues may come closer to answering this question. Surveys to determine the yeast flora were carried out in soft drinks factories in Europe, the Middle East and Scandinavia, and on factory machinery (Sand and van Grinsven 1976a, b; Sand et al. 1976a). Yeasts found typically included *Candida stellata*, *Candida sake*, *Candida guilliermondii* and *Z. fermentati*. Unexpectedly, recognized spoilage yeasts were rarely or not detected (Sand et al. 1976a).

Recent surveys of the yeast flora of soft drinks factories support the findings of Sand and colleagues. Typical factory floras of a European and an Asian soft drinks factory are shown on Table 11.4. While several of the second division or group 2 spoilage yeasts are present (Tudor and Board 1993; Davenport 1996), the absence of the *Zygosaccharomyces* and *Saccharomyces sensu stricto* spp. spoilage yeasts was remarkable.

11.8.4 Yeast Frequency by Numbers of Isolates

A species list like that shown in Table 11.4 is misleading in terms of frequency of occurrence. The table lists 14 species and implicitly the reader assumes all to be present in equal proportions. Reality is very different, if the numbers of isolates of each species is taken into account. Red and black yeasts usually predominate in numbers, approximately 50% of isolates; *Rhodotorula*, *Sporidiobolus* and *Sporobolomyces* comprising the red genera and *Aureobasidium* being the black genus. Also common in numbers are *Cryptococcus* spp. and certain *Candida* spp. such as *Candida pseudointermedia*. Interestingly, this factory flora frequency very closely resembles the aerial flora frequency described by Ingram (1958). If the factory flora is plotted against the resistance to an antimicrobial agent, such as sorbic acid, it becomes obvious that the great majority of the factory flora is easily inhibited by very small quantities of preservative (Fig. 11.7). Isolates of resistant species are found in very low numbers, and at the European legal limit for sorbic acid, 300 ppm (just under 3 mM), no isolates were found able to grow. This means that in this factory, no spoilage would be expected in sorbic acid preserved soft drinks, if any of the factory flora of yeast species were to gain access to the product. Similar results and profiles

Table 11.4 The yeast flora of a European soft drinks factory and an Asian soft drinks factory. All species were identified by D1/D2 26S ribosomal DNA sequencing. The species are dominated by red, *Rhodotorula* spp., and black, *Aureobasidium* sp., yeasts. Extremeophilic spoilage species, such as *Z. bailii*, are notable by their absence

European soft drinks factory	Asian soft drinks factory
Aureobasidium pullulans	*A. pullulans*
Bulleromyces albus	*Candida boidinii*
Candida boidinii	*Candida diddensiae*
Candida oleophila	*Candida oleophila*
Candida parapsilosis	*Candida pseudolambica*
Candida pseudointermedia	*Candida pseudointermedia*
Cryptococcus albidus	*Candida silvae*
Debaryomyces hansenii	*Candida sojae*
H. meyerii	*Cryptococcus diffluens*
P. anomala	*P. anomala*
R. mucilaginosa	*P. jadinii*
R. nothofagi	*R. graminis*
Saccharomyces exiguus	*R. dairenensis*
Yarrowia lipolytica	*R. nothofagi*

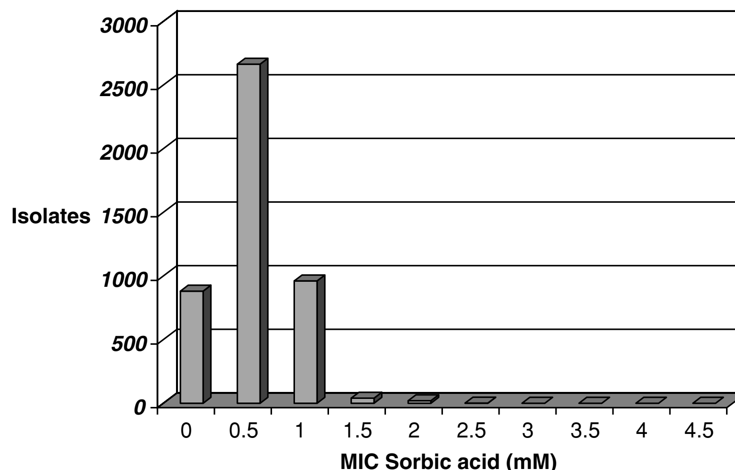

Fig. 11.7. Profile of the resistance of yeast isolates from a European soft drinks factory (Table 11.4) to sorbic acid in a soft drink, pH 3.4. The legal limit for sorbic acid in a soft drink is 300 ppm, 2.7 mM, in Europe (Anon 1989)

of resistance can be obtained with other preservatives systems/factors, for example heat and acetic acid.

The conclusions that can be drawn from this are:

1. The infamous extremophile spoilage yeasts, *Z. bailii*, *Z. rouxii*, *Saccharomyces sensu stricto*, *Saccharomycodes ludwigii*, *Dekkera bruxellensis* (Table 11.3), are rare in factories, either absent or present in very, very low numbers.
2. The second division (Table 11.5), group 2 yeasts (Tudor and Board 1993; Davenport 1996) are commonly present in factories, albeit at moderate/low frequency of isolation.
3. These second division, group 2 spoilage/hygiene species can be controlled by the food preservation system (Fig. 11.7). Group 2 yeasts will not normally cause spoilage unless there is a mistake in manufacturing (Davenport 1996), such as omission of preservation or poor hygiene.
4. Poor factory hygiene has been suggested to account for 95% of yeast infections (van Esch 1987). Improvements in factory hygiene can dramatically reduce spoilage (Beech and Davenport 1970; Sand 1971a; Rankine and Pilone 1974; Windisch and Neumann-Duscha 1974).
5. Poor factory hygiene typically increases the microbial loading of the food, i.e. increases the yeast inoculum, to such an extent that the preservation system/factors are overwhelmed, rather than altering the factory yeast flora to a great extent.

It therefore follows that the great majority of cases of yeast spoilage of foods are caused by the second division, group 2 species. Such food spoilage tends to be

Table 11.5 Commonly encountered second division, group 2 spoilage/hygiene yeast species. These are normally frequently isolated yeasts and can be regarded as opportunistic species, causing spoilage following any mistake in manufacturing or storage. Gas production is indicated by *one asterisk*, excess gas by *two asterisks*. It is likely that the majority of yeast spoilage is caused by these lesser-known species

*Candida albicans**	*H. uvarum**
*Candida boidinii**	*I. occidentalis***
*Candida glabrata**	*I. orientalis*** (*Candida krusei*)
*Candida intermedia**	*P. anomala***
*Candida lambica**	*P. galeiformis*
*Candida parapsilosis**	*P. guilliermondii**
*Candida pseudointermedia**	*P. membranifaciens*
*Candida pseudolambica**	*Saccharomyces exiguus*** = *Kazachstania exigua*
*Candida sake**	*Torulaspora delbrueckii***
*Candida sojae**	*Y. lipolytica*
*Candida tropicalis**	*Z. fermentati*** = *Lachancea fermentati*
Candida zeylandoides	*Z. florentinus*** = *Zygotorulaspora florentinis*
*Clavispora lusitaniae**	*Z. microellipsoides*** = *Torulaspora microellipsoides*

unspectacular and consequently tends to be underreported. The group 1 extremophile species cause spoilage much more rarely, but these are much more likely to be reported, owing to the spectacular nature of their high gas spoilage. To use an analogy, this resembles an iceberg (Fig. 11.8) where the visible 5% of the iceberg is the gas-forming extremophilic species (Table 11.3). However, 95% of the iceberg is beneath the surface, a great underreported mass of yeast spoilage caused by the second division group 2 spoilage yeasts. These common yeasts are opportunists and will cause spoilage following mistakes or poor hygiene, either in the factory or in the domestic environment.

11.9 Future Trends in Yeast Spoilage

11.9.1 Spoilage Yeast Identification and Nomenclature

Future trends in yeast spoilage of foods can potentially originate with the food, or with the yeasts. Any changes in the yeasts themselves are unlikely over a reasonable timescale. However, there will undoubtedly be changes in the nomenclature of spoilage yeast species, through changes in yeast phylogeny and taxonomy, and through more accurate identification of spoilage yeast species using molecular techniques. Fortunately identification of the first division, group 1 yeasts as the cause of the most-obvious spoilage is likely to remain almost unchanged. Yeasts such as *Zygosaccharomces bailii*, *Schizosaccharomyces pombe* or *Saccharomycodes ludwigii* are rarely misidentified owing to their distinctive morphology and physiology. There are, however, likely to be a number of unexpected names appearing in the second division, group 2 spoilage/hygiene yeasts, following better identification. Small, round, budding-yeasts, with smooth white colonies are often very difficult to correctly identify.

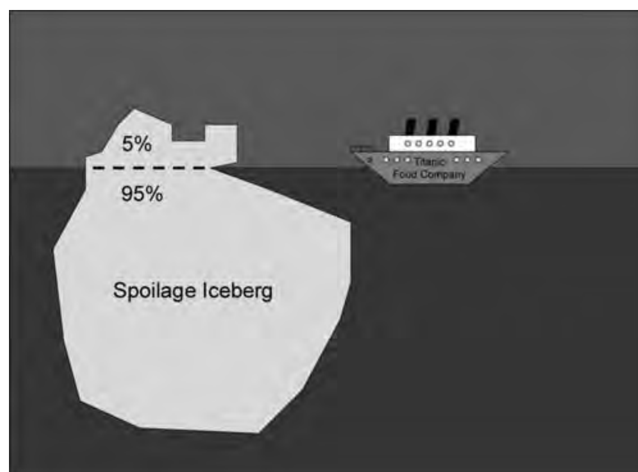

Fig. 11.8. Metaphorically, yeast spoilage resembles an iceberg. The 5% easily visible spoilage is caused by the extremophilic, highly fermentative species (Table 11.3). Ninety-five percent of spoilage is under the surface; caused by poor hygiene allowing the opportunistic "second division" spoilage yeasts (Table 11.5) to proliferate. Spoilage by the second division yeasts is much less obvious, is greatly underreported, but is still sufficient to sink a food company

11.9.2. Packaged and Processed Foods

Any future trends in yeast spoilage of foods and beverages are therefore likely to depend almost entirely on changes in food, food processing and food preservation. The current trend in foods and beverages is for more foods to be purchased ready prepared and packaged. This inevitably means a higher degree of food processing by factories. Over the past 20 years owing to customer demand, a high proportion of processed foods have been prepared containing more sugar and salt than in non-processed foods. It is therefore probable that any increase in food processing will result in more potential targets for yeast spoilage. Recent moves, particularly in the USA, to limit human obesity by lowering sugar concentrations are not likely to affect yeast spoilage. A soft drink, for example, containing 5% sugar is equally likely to suffer yeast spoilage as one containing 10% sugar.

11.9.3 New Preservation Techniques

New mechanisms of food preservation are often discussed in the scientific literature, but are not likely to be widely applied over the next few years. Any new methods require stringent safety testing, must also be acceptable to the public, and must be compatible with current legislation where applicable. Possible new food preservation techniques include use of ultrahigh pressure, irradiation, high-intensity light, pulsed electric fields and use of antimicrobial essential oils. Any new technique is likely to select for extremophilic yeast species able to survive the treatment; these may not be

the species currently recognized as spoilage species. For example, limited studies on irradiation suggest that *Z. bailii* is irradiation-sensitive, but *R. glutinis* is resistant (Youssef et al. 2002).

11.9.4 Less Preserved, More "Natural" Foods

Following the discovery of traces of pesticide and herbicide residues in foods, there is now a strong adverse public reaction to any perceived adulteration to food or additives in food. This has also extended in certain cases to an opposition to physical treatments of food such as pasteurization. Foods are required to be more "natural", with all of the goodness left in and not overprocessed or containing chemical additives, including preservatives. This desire by the public can be counterproductive, as in the USA in natural apple juices. In a celebrated outbreak of verotoxic *E. coli* 0157:H7 food poisoning in California, bacteria in untreated, unpreserved, unpasteurized apple juices survived up to 30 days, and caused 49 cases of illness and one death (McLellan and Splittstoesser 1996).

While food additives may not be completely eliminated from foods, there has been a general trend over the past 20 years for the concentrations of additives in foods to be lowered, often accompanied by changes in legislation. The overall effect of lowering preservative concentrations in foods is that a higher proportion of foods will be subject to yeast spoilage. The extremophilic yeasts will remain a threat, but more of the second division spoilage species will be able to survive in lowered preservative concentrations. It is therefore probable that any increase in yeast spoilage brought about by removal of preservatives will be primarily due to an increase in spoilage by the second division species.

11.9.5 Increased Role of Factory Hygiene

Any shortfall in the preservation systems used to prevent yeast spoilage in foods must result in an unacceptable increase in spoilage, unless the gap is filled by an increase in factory hygiene. There is always an uneasy balance between preservation and factory hygiene. A food containing excessive preservative, for example 1,000 ppm benzoic acid, is almost immune to spoilage and could be produced in very unhygienic conditions. At the other end of the scale, a food with 0-ppm preservatives will require excessive hygiene to prevent *any* microbial infection; an expensive process known as aseptic filling, carried out within clean rooms containing filtered air.

Really good hygiene in factories requires competent and well-educated personnel. Human error or poor training ultimately causes many, perhaps the majority of hygiene breakdowns in factories. As food production becomes more technically complex, it is likely that the human factor will become the weakest link in the successful production of foods and the greatest cause of yeast spoilage in foods and beverages.

QED – the human factor: While working in a British beverage factory some years ago, the writer found that one of the workers in the factory repeatedly did not apply the necessary disinfectant to clean the pipe work. When asked why this was, the worker replied "I knew it was not important, because if it was, you would have come down and done it yourself".

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Chapter 12

The Public Health and Probiotic Significance of Yeasts in Foods and Beverages

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12.1 Introduction

The microbiological safety of foods is generally discussed in relation to the occurrence and significance of pathogenic bacteria, infectious viruses, mycotoxigenic fungi, parasitic protozoans and toxigenic algae. Mention of yeasts in the context of food safety is conspicuously absent (Lund et al. 2000; Doyle et al. 2001; Hocking 2003). Compared with other microbial groups, yeasts are not seen as aggressive pathogens, but they are capable of causing human disease in opportunistic circumstances (Hurley et al. 1987; Rippon 1988; Segal and Baum 1994; Georgiev 2003; Hazen and Howell 2003). *Candida albicans* (Hurley 1980; Calderone 2002) and *Cryptococcus neoformans* (Campbell and Mackenzie 1980; Casadevall and Perfect 1998) are well known in this regard, and are responsible for causing a range of mucocutaneous, cutaneous, respiratory, central nervous and systemic infections. However, an increasing number of other yeast species are now associated with these disorders and have been added to the list of opportunistic pathogens (Hazen 1995; Georgiev 2003). Although consumption of food contaminated with yeasts may not have a direct role in causing these infections, there is increasing concern that foods could be an underestimated environmental source of these yeast pathogens.

The development of allergic and other adverse responses in humans as a consequence of food consumption is well documented, and food allergy has now become a significant branch of food safety (Metcalfe et al. 2003). The role of yeasts in eliciting these types of responses is relatively minor compared with that of other causative agents, but there is increasing and justifiable interest in this topic.

Public health considerations in relation to microorganisms in foods are not always negative, and the concept of probiotic microorganisms has developed into a major area of scientific and commercial interest (Klaenhammer 2001). For many years, viable and nonviable yeasts have been used as supplements in stock feed to enhance the growth of domesticated animals and poultry (Lyons et al. 1993; Lyons

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2002; Dawson 2002). Recently, the yeast *Saccharomyces boulardii* has emerged as a probiotic species for consumption by humans (van der Aa Kuhle et al. 2005), paving the way for a broader consideration of yeasts as probiotic organisms in foods.

Although yeasts are well known for producing fermented foods and beverages, as sources of food ingredients and as spoilage yeasts, their public health significance in foods has largely been overlooked. This chapter addresses this gap in knowledge and brings together a range of topics that broadly cover the relationship between yeasts, foods and public health.

12.2 Yeasts and Foodborne Gastroenteritis

As part of normal, daily food consumption, humans are unknowingly and inadvertently ingesting large populations of viable yeast cells without adverse impact on their health. Table 12.1 lists a range of food and beverage commodities that are likely to harbour significant populations of viable yeasts at the time of consumption. These products contain a diversity of ascomycetous and basidiomycetous yeast species, often at populations as high as 10^6–10^8 cfu/g or 10^6–10^8 cfu/ml. Despite this exposure, outbreaks or cases of foodborne gastrointestinal infections or intoxications attributable to yeasts are rarely encountered. Epidemiological statistics on foodborne microbial disease from various countries over many years are notable for their absence of any data on yeasts (Bean and Griffin 1990; Mead et al. 1999; Lee et al. 2001; Sewell and Farber 2001). Similarly, searches of this topic in computer data bases yield no information. Nevertheless, there are occasional reports of yeast-associated gastroenteritis that are worthy of discussion.

Table 12.1 Diversity of food and beverage commodities with significant populations of viable yeast cells at the time of consumption

Commodity group	Prevalent yeast species	Reference
Fresh fruits	*Aureobasidium, Rhodotorula, Cryptococcus, Candida, Metschnikowia, Hanseniaspora*	Deak and Beuchat (1996), Fleet (2003)
Fruit juices, salads	*Saccharomyces, Zygosaccharomyces, Candida, Hanseniaspora*	Deak and Beuchat (1996), Fleet (2003)
Cheeses, other fermented dairy products	*Debaryomyces, Yarrowia, Candida, Kluyveromyces, Saccharomyces, Galactomyces* (*Geotrichum*)	Fleet (1990), Jakobsen and Narvhus (1996), Frohlich-Wyder (2003)
Delicatessen and fermented meat products	*Debaryomyces, Candida, Yarrowia, Cryptococcus*	Dillon and Board (1991), Samelis and Sofos (2003)
Alcoholic beverages	*Saccharomyces, Hanseniaspora, Pichia, Candida, Brettanomyces, Metschnikowia*	Fleet (1998)
Traditional fermented foods	*Saccharomyces, Candida, Pichia*	Steinkraus (1996), Beuchat (2001)

Jensen and Smith (1976) noted malaise, fever and nausea in a patient who regularly consumed tablets of brewer's yeast as a health-food supplement. These symptoms disappeared when the patient stopped consuming tablets of the yeast. In a review of foodborne disease statistics for Canada over the period 1973–1977, Todd (1983) referred to 39 cases that were attributed to consumption of foods (punctured canned foods, bottled drinks, baked products and infant food) contaminated with yeasts and mould. He noted the need for further research to determine the significance of these observations. *Hanseniaspora uvarum*, (*Kloeckera apiculata*), possibly associated with seafood, was suspected of causing a case of gastroenteritis in Spain (Garcia-Martos et al. 1999).

Under some circumstances, *Candida* species can colonise areas of the intestinal tract, leading to diarrhea and other gastroenteritis symptoms, along with excretion of high yeast populations (more than 10^6 cfu/g) in the faeces (Gupta and Ehrinpreis 1988). Over the years, there have been numerous reports of such cases (reviewed in Talwar et al. 1990; Danna et al. 1991; Levine and Dykoski 1995), where *C. albicans* was the main species involved, but many other *Candida* species were also implicated (e.g. *C. tropicalis*, *C. kefyr*, *C. krusei*, *C. parapsilosis*, *C. lusitaniae*, *C. guilliermondii*). Generally, these cases are associated with individuals who are receiving antibacterial therapy, are immunocompromised, or who have some other underlying disorder. Consumption of yeast-contaminated food was not the initiating factor. Nevertheless, such findings establish the fact that, given appropriate circumstances, yeasts can colonise the intestinal tract, and contribute to gastroenteritis and other infections (Cole et al. 1996; Bernhardt and Knoke 1997).

It may be concluded from the overall epidemiological evidence that, as causative agents of foodborne gastrointestinal infections and intoxications, yeasts present very little risk to consumers. This, of course, correlates with their widespread acceptance as safe agents in the production of fermented foods and beverages. However, some caution and vigilance is required to consider changing demographics, and the increasing numbers of individuals with compromised immune systems.

12.3 Yeasts as Opportunistic Pathogens

As mentioned already, various species of yeasts are considered as opportunistic pathogens. *C. albicans* and *Cr. neoformans* head this list, but many other yeasts are now considered in this category (Hazen 1995; Georgiev 2003). Infection of the blood (fungaemia) is the main pathological effect caused by yeasts. From this source, however, yeast cells are disseminated throughout the human body and can infect almost any organ (e.g. heart, lungs, kidney, brain), often with fatal consequences. They can colonise the skin and membraneous areas, and infections of the oral cavity, vagina, anal region and respiratory system are not uncommon (Hurley et al. 1987; Rippon 1988; Ahearn 1998; Hazen and Howell 2003; Georgiev 2003). Antifungal agents such as amphotericin, fluconazole, itraconazole and ketoconazole are used to treat yeast infections, but the development of resistance to these antibiotics is an on-going issue.

Table 12.2 lists the *Candida* and *Cryptococcus* species that have been reported to cause yeast infections in humans. Only their anamorphic or nonteleomorphic names

Table 12.2 Species of *Candida* and *Cryptococcus* causing opportunistic infections in humans

Candida albicans	*Candida famata*
Candida parapsilosis	*Candida pulcherrima*
Candida tropicalis	*Candida zeylanoides*
Candida krusei	*Candida rugosa*
Candida glabrata	*Candida utilis*
Candida lusitaniae	*Candida lipolytica*
Candida guilliermondii	*Candida dubliniensis*
Candida stellatoidea	*Cryptococcus neoformans*
Candida norvegensis	*Cryptococcus albidus, Cryptococcus laurentii*

Hazen (1995), Hazen and Howell (2003), Georgiev (2003)

are listed, as this is how they are most frequently described in the medical literature. Infections caused by *Candida* species are, by far, the most frequently reported cases, and there is an extensive literature on this topic (reviewed by Hazen 1995; Krcmery and Barnes 2002; Hobson 2003). Although infections with *C. albicans* have been most prevalent and significant in the past, infections by other *Candida* species are increasing in frequency and importance. The species of main concern, here, are *C. parapsilosis*, *C. krusei*, *C. tropicalis* and *C. glabrata* (Pfaller 1996; Krcmery and Barnes 2002). Epidemiological statistics correlate this trend with their increased resistance to antibiotics used to treat infections with *C. albicans*, but other factors are also operative, and include the virulence properties of the yeast and particulars of the host.

Cryptococcus infections generally begin by inhalation of the yeast into the lungs, after which it enters the blood system and is spread to other parts of the body (Hurley et al. 1987). *Cr. neoformans* is the main species involved, but on rare occasions infections by other species such as *Cr. albidus* and *Cr. laurentii* have been reported (Hajjeh et al. 1995; Georgiev 2003).

Table 12.3 lists yeast species other than those of *Candida* and *Cryptococcus* that have been reported to cause infections (primarily fungaemia) in humans. Most notable are species of *Rhodotorula*, *Pichia anomala* (formerly *Hansenula anomala*), *Issatchenkia orientalis* (anamorph *C. krusei*) and *Kluyveromyces marxianus* (formerly *K. fragilis*) (Kremery et al. 1999). The industrialised yeast, *S. cerevisiae*, widely used in the production of foods and beverages, has attracted significant attention as an opportunistic pathogen. It has been linked to various cases of fungaemia, vaginitis and organ infections over the past 50 years (Eschete and West 1980; Aucott et al. 1990; McCullough et al. 1998a; Murphy and Kavanagh 1999; Xu et al. 1999; Cherifi et al. 2004; Llanos et al. 2004). Murphy and Kavanagh (1999) have reviewed the pathogenic significance of *S. cerevisiae* and its implications in the biotechnological applications of this yeast. There is convincing evidence that this yeast is an opportunistic pathogen and that industrialised strains of baker's yeast have been linked to cases of vaginitis (Nyirjesy et al. 1995; McCullough et al. 1998a; Llanos et al. 2004), and brewer's yeast to other infections (Jensen and Smith 1976). Various molecular methods have been used to differentiate

Table 12.3 Yeast other than *Candida* and *Cryptococcus* species causing opportunistic infections in humans

Species	References
Saccharomyces cerevisiae	Eschete and West (1980), Aucott et al. (1990), Bassetti et al. (1998), McCullough et al. (1998a), Murphy and Kavanagh (1999), Wheeler et al. (2003), Llanos et al. (2004)
Saccharomyces cerevisiae var. *boulardii*	McCullough et al. (1998b), Piarroux et al. (1999), Lherm et al. (2002), Cassone et al. (2003)
Rhodotorula spp.	Papadogeorgakis et al. (1999), Petrocheilou-Paschou et al. (2001), Braun and Kaufmann (1999), Diekema et al. (2005)
Pichia anomala	Murphy et al. (1986), Haron et al. (1988), Klein et al. (1988), Yamada et al. (1995), Garcia-Martos et al. (1996), Cermeno-Vivas et al. (1999), Georgiev (2003)
Pichia farinosa	Garcia-Martos et al. (1996)
Pichia membranifaciens	Garcia-Martos et al. (1996)
Issatchenkia orientalis	Merz et al. (1986), Goldman et al. (1993), Abbas et al. (2000), Georgiev (2003)
Kluyveromyces marxianus	Lutwick et al. (1980), Nielsen et al. (1990), Garcia-Martos et al. (1996)
Hanseniaspora uvarum	Garcia-Martos et al. (1999)
Yarrowia lipolytica	Shin et al. (2000), Georgiev (2003)

See also Rippon (1988), Hazen and Howell (2003), Georgiev (2003)

clinical, nonclinical and industrial strains of *S. cerevisiae*, but more research is needed to define the genotypic and phenotypic properties of pathogenic and non-pathogenic strains, as well as the conditions of the host that are predisposing to colonisation and infection by the yeast. Both immunocompromised and immuno-competent hosts have been infected by *S. cerevisiae* (McCullough et al. 1998a; Xu et al. 1999; Llanos et al. 2004). Pathogenic strains of *S. cerevisiae* exhibit the ability to grow at 42°C, produce proteinase and are capable of pseudohyphal growth (Murphy and Kavanagh 1999). However, the composition and the structure of the cell wall are also properties that may confer virulence on strains of *S. cerevisiae* (Wheeler et al. 2003). *S. boulardii* is a yeast with biotherapeutic and probiotic functions and these activities are discussed in a later section. Taxonomically, it is considered to be a variety of *S. cerevisiae*, although it can be differentiated from other strains of *S. cerevisiae* by various molecular criteria (van der Aa Kuhle and Jespersen 2003). Unfortunately, its use as a biotherapeutic or probiotic agent has caused numerous cases of fungaemia in recent years (Piarroux et al. 1999; Lherm et al. 2002; reviewed by Cassone et al. 2003), prompting some authors to suggest that its application for these purposes should be prohibited.

The factors that contribute to infections with opportunistic yeasts are well recognised and are listed in Table 12.4. Usually, healthy, immunocompetent individuals are not at risk of such infections. Generally, individuals with weakened health and immune function are at greatest risk, and include cancer and AIDS

Table 12.4 Factors contributing to human infections with opportunistic yeast pathogens

Weak health; hospitalisation
Cancer, AIDS patients
Weak immune system; treatment with immunosuppressive drugs; chemotherapy
Treatment with broad spectrum bacterial antibiotics
Insertion of catheters
Recent surgery (especially gastrointestinal tract)
Total parenteral nutrition
Hazen (1995), Annaissie et al. (1998), Hobson (2003)

patients, hospitalised patients, and those with catheter insertions (Hart et al. 1969; Hazen 1995; Anaissie et al. 1998; Hobson 2003). Yeasts, especially *Candida* species, are normal inhabitants of the human gastrointestinal tract, and there is sound experimental evidence to demonstrate that they translocate from this source to the blood system (Krause et al. 1969; Cole et al. 1996). This mode of transmission is facilitated by conditions which increase the populations of yeasts in the gastrointestinal tract (e.g. diet, treatment with bacterial antibiotics) and which damage the intestinal mucosa (e.g. immunosuppressive and chemotherapeutic agents, diarrhea episodes). The presence of an indwelling catheter serves as a focus for yeast contamination and growth as a biofilm, that is more resistant to elimination by the host's defence mechanisms, and antifungal agents (Douglas 2003; Kojic and Daroviche 2004). Generally, any factor that increases the exposure of susceptible individuals to yeasts will increase their risk of acquiring an opportunistic infection. Three studies have suggested that foods with substantial yeast loads (e.g. processed meats, soft cheeses) pose a risk to susceptible hospital patients. These patients should not be offered such foods for consumption and, furthermore, these foods introduce yeasts into the general hospital environment, from where they could contaminate catheters and patients (Staib et al. 1980; Radosavljevic et al. 1999; Bouakline et al. 2000). Staib et al. (1980) noted the ability of pathogenic yeasts (*C. parapsilosis*, *C. tropicalis* and *Cr. neoformans*) to grow in some meat products.

Many of the yeast species listed in Tables 12.2 and 12.3 are commonly found in foods at the time of consumption (Fleet 1992) [see Table 12.1 and also the extensive tables of yeasts and foods given in Deak (1991), Tudor and Board (1993) and Deak and Beuchat (1996)]. To establish a stronger linkage between the role of foods in contributing to opportunistic yeast infections, more research is needed to understand (1) the survival and growth of foodborne yeasts throughout the gastrointestinal system, (2) the potential for such yeasts to translocate from the gastrointestinal tract to the blood stream, and (3) the general occurrence and ecology of these yeasts in hospital and health-care environments.

12.4 Allergic and Other Adverse Responses to Yeasts

The ability of foods and food contaminants to elicit allergic and other adverse reactions in humans is attracting increasing scientific and consumer interest, although

there is little reference to foodborne yeasts in this regard (Emerton 1992; Metcalfe et al. 2003; Sampson 2004). Nevertheless, there is a significant body of "lay" and "alternative" literature that connects yeasts to a broad range of allergic and hypersensitive reactions in humans. These include a variety of gastrointestinal, respiratory, skin, migraine and even psychiatric disorders (Truss 1981; Crook 1986; Eaton 2004). Chronic fatigue syndrome, dysfunctional gut syndrome, irritable bowel syndrome and gut dysbiosis are prominent among these disorders. It is thought that overgrowth of yeasts in the gastrointestinal tract leads to the development of these conditions. *C. albicans* is reported to be the main species of concern, but other species are likely to be involved, and this highlights the need for a better understanding of the yeast ecology of the human gut (Bernhardt and Knoke 1997). Metabolites (e.g. acetaldehyde) produced by yeast growth pass into the circulatory system and are believed to trigger the various adverse responses; however, the underlying mechanisms are probably more complex as there is increasing evidence demonstrating the immunogenic or immunomodulating effects of yeast cell-wall components such as the 1,3-β-glucans and mannans (Lindberg et al. 1992; Kim et al. 2002; Instanos et al. 2004). The linkage between yeasts and these human disorders is largely based on dietary observations. If foods suspected to contain yeasts are removed from the diet, symptoms of the disorder generally disappear, but return when these foods are reintroduced into the diet (Grant 1979; Wuthrich and Hofer 1986; Eaton and Howard 1998). Such foods include yeast extract, leavened bread, alcoholic beverages and mould-ripened cheeses (Eaton and Howard 1998).

Biogenic amines (e.g. histamine, tyramine, phenylethylamine, putrescine, cadaverine) cause a diversity of adverse responses in humans, including headaches, hypotension, migraines and digestive disturbances (Shelaby 1996; Silla Santos 1996). Yeasts produce an array of biogenic amines through the decarboxylation of amino acids and contribute to the amine levels found in alcoholic beverages (Izquierdo-Pulido et al. 1995; Torrea-Goni and Ancin-Azpilicueta 2001; Torrea and Ancin 2002; Caruso et al. 2002) and other fermented products such as cheeses (Wyder et al. 1999). The concentrations produced vary with the yeast species (Caruso et al. 2002; Wyder et al. 1999). More research on amine production by yeasts is required, but the evidence to date suggests they do not produce sufficient amounts to be of concern to public health.

Sulphur dioxide has a certain degree of toxicity to some humans, causing respiratory, hypotension, flushing and tingling responses. Its production by strains of *S. cerevisiae* is well documented, where most strains produce less than 10 mg/l of SO_2; however, some strains can produce up to 100 mg/l, which could have public health implications (Romano and Suzzi 1993; Rauhut 1993). The production of SO_2 by other species of yeasts is not well known and requires investigation.

Ethyl carbamate (urethane) is a potential carcinogen, and its production in fermented foods and beverages needs to be considered (Ough 1976). It is formed by the reaction of urea with ethanol under acidic conditions. Yeasts produce urea as a consequence of nitrogen (e.g. arginine) metabolism, with some strains generating more urea than others. Ethyl carbamate formation during wine production is of particular concern where management strategies are used to minimise the presence and production of urea (Henschke and Jiranek 1993).

12.5 Yeasts as Probiotics

Probiotics are viable microorganisms that are beneficial to the host when consumed in appropriate quantities. Benefits include reduction in the incidences of diarrhea, constipation and bowel cancer, stimulation of the immune system, reduction in serum cholesterol levels, and enhanced nutrient uptake (Klaenhammer 2001; Holzapfel and Schillinger 2002; Marteau and Boutron-Ruault 2002). Particular species and strains of lactic acid bacteria (e.g. *Lactobacillus acidophilus*, *Bifidobacterium* spp.) are well known in this context and have received widespread application in the production of yogurts. Generally, greater than 10^6–10^7 viable cells of the probiotic organism need to be consumed on a regular basis for the health benefits to be realised. The probiotic microorganisms survive the ingestion process, and then colonise areas of the intestinal tract to assert their beneficial influence and impact. There is a significant and increasing body of scientific evidence that demonstrates their beneficial functions, but the underlying physiological and molecular mechanisms remain uncertain and require on-going research for their clarification (Mombelli and Gismondo 2000; Sullivan and Nord 2002).

The concept of using yeasts as human probiotics is at an early stage of development. However, there is significant experience in using viable yeasts, principally baker's, brewer's and distiller's yeasts (*S. cerevisiae*), as supplements to feeds for cattle, pigs and poultry, where improvements in growth and health of animals and birds are observed (Lyons et al. 1993; Dawson 2002). There is a substantial literature in this field (Aros-Garcia et al. 2000) and an expanding interest in using yeasts as probiotics in the aquaculture industry (Gatesoupe 1999; Tovar et al. 2002).

S. boulardii has been listed in recent literature as a potential human probiotic (Klaenhammer 2001). It was described in 1984 as an isolate from tropical fruit, and subsequently reported as an effective biotherapeutic agent for the clinical treatment of a range of diarrheal disorders (Surawicz et al. 1989; McFarland and Bernasconi 1993). The yeast is available commercially as lyophilised cultures that are resuspended in sterile saline and administered orally to patients who usually have been hospitalised as a consequence of severe diarrhea. The yeast colonises the intestinal tract, but is eliminated once administration is stopped, or the patient is given fungal antibiotics. The yeast has been reported to be effective in treating antibiotic-associated diarrhea, traveller's diarrhea, Crohn's disease and other inflammatory bowel disorders, acute gastroenteritis in adults and children, chronic diarrhea in HIV infected patients and diarrhea caused by *Clostridium difficile*, *Vibrio cholerae* and various *Enterobacteriaceae* (Czerucka and Rampal 2002; Sullivan and Nord 2002). Although treatment with the yeast has been considered to be safe, an increasing number of outbreaks of *S. boulardii* fungemia are being reported, causing some authors to question its safety status (Table 12.3) (Piarroux et al. 1999; Cassone et al. 2003). Also, there has been significant controversy over the correct nomenclature and taxonomic status for this yeast (McFarland 1996; McCullough et al. 1998b; Mitterdorfer et al. 2002). On the basis of phenotypic criteria, it is difficult to differentiate *S. boulardii* from *S. cerevisiae*. However, a range of molecular methods (sequencing, pulsed-field gel electrophoresis, restriction fragment length polymorphism, randomly amplified polymorphic DNA) clearly distinguish *S. boulardii*

strains from other strains of *S. cerevisiae*, but they fall within the overall cluster or clade for *S. cerevisiae* (Mitterdorfer et al. 2002; van der Aa Kuhle and Jespersen 2003; Posteraro et al. 2005). It is now generally accepted that the original species description of *S. boulardii* is taxonomically invalid, and that the yeast is correctly assigned as *S. cerevisiae* var. *boulardii*.

The mechanisms by which *S. cerevisiae* var. *boulardii* functions as a biotherapeutic or probiotic agent and prevents a range of diarrhea disorders are not fully understood, but multiple activities are probably operating (Czerucka and Rampal 2002). In the case of *Cl. difficile* induced diarrhea, it appears that *S. cerevisiae* var. *boulardii* produces a serene protease which degrades specific diarrhea-causing toxins produced by this bacterium, as well as the receptor sites for these toxins on the colonic mucosa. The yeast may stimulate particular enzymatic activities of the intestinal mucosa, as well as stimulate the host's intestinal mucosal immune response. Also, the cell wall of the yeast could adsorb and bind toxic products of diarrhea-causing bacteria. Among other strains of *S. cerevisiae*, van der Aa Kuhle et al. (2005) screened probiotic *S. cerevisiae* var. *boulardii* for tolerance of low pH (2.5) and bile salts, adhesion to epithelial cells, and effects on proinflammatory cytokine levels. While all strains were acid- and bile-tolerant, only some showed adhesion to epithelical cells and one (only one tested) decreased the expression of cytokine IL-1α. Attachment to intestinal mucosa, therefore, might not be a requirement for probiotic function.

For *S. cerevisiae* var. *boulardii* to be useful as a probiotic in foods or beverages, it needs to satisfy important technological criteria. Apart from conveying a health benefit to the consumer, the yeast should not have detrimental effects on the shelf-life and sensory properties of the product, and it should remain viable at functional probiotic populations (usually greater than 10^6 cfu/g) until the food/beverage is consumed. To date, there has been little investigation of these requirements. Lourens-Hattingh and Viljoen (2001) demonstrated that populations (10^7–10^8 cfu/g) of *S. boulardii* did not decrease after inoculation into plain yogurts and UHT milk, and remained relatively stable during storage for 28 days at 5°C. However, the yeast exhibited significant growth on the sugars in fruit-based yogurts, producing gas and ethanol that spoiled the product. Heenan et al. (2004) examined the survival and sensory impact of *S. boulardii* inoculated into a frozen, soymilk-based dessert. There was a tenfold decrease in viability of the yeast during the first 10 weeks of storage and, moreover, the yeast gave an unacceptable off-flavour to the product. On the basis of these criteria, more research would be needed to develop *S. boulardii* as an acceptable probiotic in this product. Betoret et al. (2003) have examined the feasibility of incorporating a probiotic mixture of *S. cerevisiae* and *L. casei* into dried apples. Sindhu and Khetarpaul (2001, 2003) described the nutritional enhancement of an indigenous fermented food produced within a mixture of *L. casei* and *S. boulardii*, and its ability to decrease the serum cholesterol level in mice. Psomas et al. (2003) have reported the ability of *S. boulardii*, *S. cerevisiae* and other potential, probiotic yeasts to assimilate cholesterol.

There is a developing interest in using yeast species other than *S. cerevisiae* var. *boulardii* or *S. cerevisiae* as probiotic organisms. Such species include *Debaryomyces*

hansenii, *K. marxianus*, *Yarrowia lipolytica* and *I. orientalis* that are frequently associated with yogurts and cheeses and have been isolated from human faeces (Lourens-Hattingh and Viljoen 2002; Psomas et al. 2001). Other possibilities include *P. farinosa*, *P. anomala* and *Galactomyces geotrichum*, also isolated from human faeces (Mo et al. 2004). The fermented milk products kefir and koumis are frequently noted for their health-promoting, probiotic properties and, in addition to lactic acid bacteria, contain significant populations of yeasts, including *K. marxianus*, *C. kefyr*, *S. cerevisiae* and other *Saccharomyces* species, and *Zygosaccharomyces* species (Oberman and Libudzisz 1998; Beshkova et al. 2002; Frohlich-Wyder 2003; Witthuhn et al. 2005). *D. hansenii* has been studied as a potential probiotic in fish aquaculture where its surface properties and polyamine production appear to be relevant criteria (Gatesoupe 1999; Tovar et al. 2002).

12.6 Other Health and Nutritional Benefits

For many years, now, yeasts and yeast products (principally from *S. cerevisiae*) have been used as ingredients and additives to enhance the sensory and nutritional qualities of foods. Discussion of this topic is beyond the scope of this chapter, but the reader is referred to Dziezak (1987), Halasz and Lasztity (1991), Reed and Nagodawithana (1992), Dawson (2002) and Abbas (Chap. 10) for further information. Recent interests include the use of yeasts to enrich foods and diet with vitamins (e.g. folic acid), antioxidants (e.g. glutathione) and metal ions such as selenium and chromium (Chap. 10; Dawson 2002). The β-(1→3)- and β-(1→6)-glucans of the yeast cell wall exhibit some very attractive functional properties (Nguyen et al. 1998). They can stimulate the immune system (Williams et al. 1992; Jamas et al. 1996; Sutherland 1998), lower serum cholesterol (Robbins and Seeley 1977; Bell et al. 1999) and exhibit antitumour activity (Bohn and Be Miller 1995). In addition, yeast cell-wall polysaccharides also adsorb mycotoxins (Dawson 2002; Yiannikouris et al. 2004; Bejaoui et al. 2004).

12.7 Conclusion

Along with bacteria, viruses and filamentous fungi, yeasts are part of the microflora of many foods and beverages. However, they are rarely (if ever) associated with outbreaks or cases of foodborne illness. In this context, they have an excellent public health track record. Nevertheless, they are opportunistic pathogens, and the role of foods and beverages as a source of the infecting yeast should not be underestimated. Numerous species other than *C. albicans* and *Cr. neoformans* are now considered in the category of opportunistic pathogens, and many of these species frequently occur as contaminants of foods and beverages. More information is required about the ecology of yeasts in the human gastrointestinal tract and how this is impacted by the yeast ecology of foods and beverages. The probiotic and beneficial functions of yeasts in the human diet require further development. While *S. cerevisiae* var. *boulardii* has received most attention in this regard, other species should not be overlooked as having the potential to contribute a diversity of health benefits.

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Chapter 13

The Development of Superior Yeast Strains for the Food and Beverage Industries: Challenges, Opportunities and Potential Benefits

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13.1 Introduction: Who is Moulding Whom?

When one thinks about domesticated organisms that have been exploited and shaped by humans over the millennia one probably thinks of such things as crop plants, dogs, cats, and livestock. Interestingly many evolutionary biologists and anthropologists who study the relationships between such organisms and humans describe this in the context of co-evolution. They argue that we have been shaped by domesticated species as much as they have been shaped by us. It is argued, for example, that they have 'manipulated' us away from a nomadic existence into the sedentary lifestyle required for growing crops and raising livestock, thereby ensuring their own survival and reproductive success. How would industrial yeasts fare if considered in this light? We have exploited these fungi for millennia in the making of bread, wine and beer, and over this time they have been moulded (forgive the pun) by artificial selection to perform for us in a range of different settings (Fig. 13.1). So central are yeasts to human cultures that we are probably as dependent on them as we are on many of our agricultural domesticated species. From a co-evolutionary perspective one might argue that industrial yeasts such as *Saccharomyces cerevisiae* have used this dependency to exploit us over the millennia; they get us to facilitate their reproduction and dispersal in very large numbers. Because of us, *S. cerevisiae* enjoys phenomenal reproductive success with, for example, an estimated 600,000 t of baker's yeast being produced every year (Pretorius et al. 2003).

This review looks at the next stage in the ongoing relationship between industrial yeasts and humans. We will look at how humans are continuing to make improvements in the performance of such yeasts and how new technologies, particularly recombinant DNA techniques, are being utilised to this end. Or, perhaps we might

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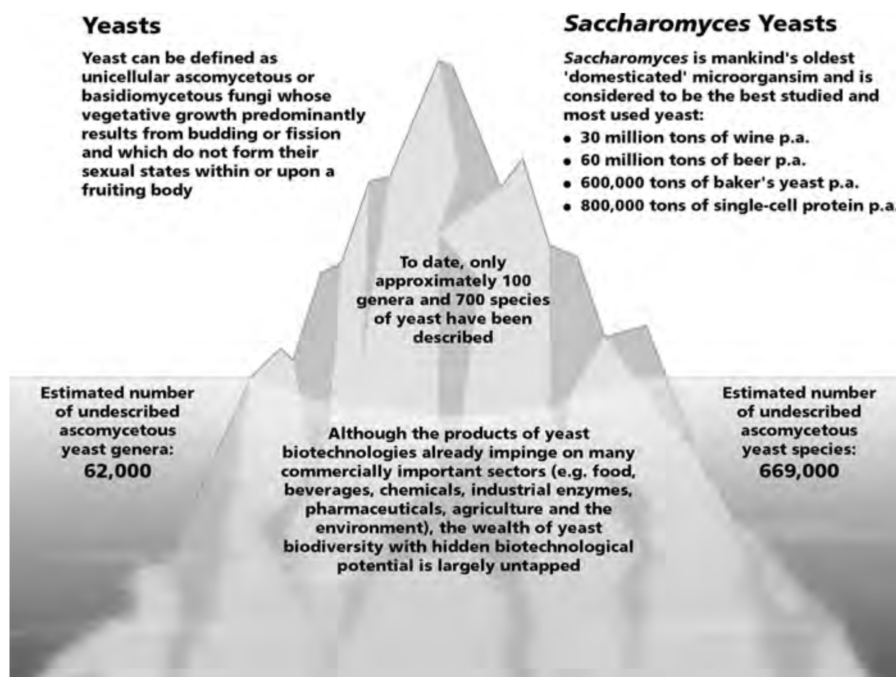

Fig. 13.1. Yeasts comprise a huge group of unicellular ascomycete and basidiomycete fungi. *Saccharomyces* spp., the yeasts that we are most familiar with, represent a tiny tip of this very large iceberg of genera and species, most of which are yet to be described. Who knows what untapped resources lie hidden in the depths of this biodiversity?

just as legitimately say that this review looks at what humans are being 'manipulated' to do by yeasts to find ever more successful ways of using them, thereby increasing their reproductive success.

Our relationship with yeasts probably started at least 7,000 years ago. References to winemaking date back to 5,000 BC, when yeasts were unwittingly used in spontaneous fermentations in Egypt and Phoenicia, and historians believe wine production probably occurred much earlier than this (Robinson 1994). Archeological evidence of a 'brewery' dating back to about 1,500 BC was uncovered beneath the Sun Temple of Queen Nefertiti, suggesting that beer was produced on an industrial scale in ancient Egypt. Selection of yeasts with desirable properties was presumably under way from these very early times but it was not until 1881 that Emil Hansen isolated the first pure yeast culture, a prerequisite for the systematic selection and improvement of strains. Not long after this, Hermann Müller-Thurgau introduced the idea of inoculating fermentations with pure yeast starter cultures and this was rapidly adopted by many wineries of the day. Yeast starter cultures are, of course, now used widely in large-scale wine production, where rapid and reliable fermentations are essential to obtain wines of consistent quality (Henschke 1997).

As far as domesticated organisms go, *S. cerevisiae* is exceptional in what it has to offer us (Fig. 13.2). Not only is it very efficient at fermentation, it also has a long

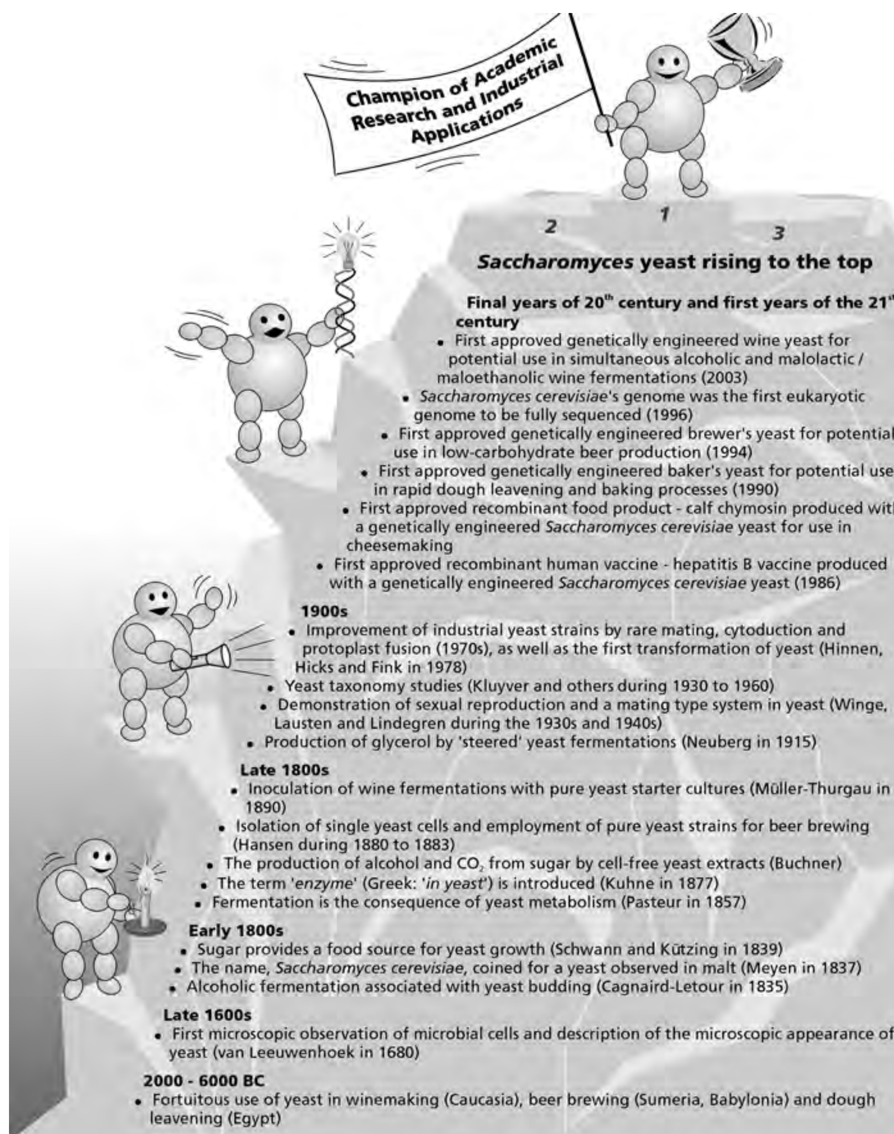

Fig. 13.2. *Saccharomyces* spp. have a very long history of domestication, dating back at least as far as ancient times; however, the past 150 years has seen the greatest growth of knowledge of these yeasts and their systematic application to a range of industrial processes and scientific research programmes. This long history of safe and fruitful association with humans has given *Saccharomyces* spp. a privileged place among domesticated organisms and explains why *S. cerevisiae* has 'generally recognised as safe' (*GRAS*) status and why it was the first genetically modified organism to be approved for applications in a range of industries

history of use in food and beverage production with a proven safety record for human consumption, and this has ensured it of 'generally recognised as safe' (GRAS) status by the US Food and Drug Administration (FDA). It was therefore inevitable that *S. cerevisiae* would be one of the first organisms to be genetically modified (GM) to produce food additives. Indeed, the first GM-based food additive to be approved for use in human food, calf chymosin (which is used to make cheese), was produced using transgenic *S. cerevisiae* cells (Walker 1998). Furthermore, GM *S. cerevisiae* was the first GM organism (GMO), as distinct from a GM product, to be cleared for use in human food production (Gopal and Hammond 1992; Hammond 1995; Walker 1998).

The application of yeasts has now been extended beyond the food world to the bio-ethanol and pharmaceutical industries. In some countries (e.g. Brazil) there is considerable dependence on yeasts for bio-ethanol production, and in a world of depleting fossil fuels and accumulating greenhouse gases it is likely that we will see considerable growth in the production of this renewable fuel. This will, of course, lead to ongoing development of new strains of yeasts that are able to produce greater amounts of ethanol and from cheaper sources of carbon, such as lignocellulose waste (Lynd et al. 2002). The *S. cerevisiae* that we currently have at our disposal is unable to use such carbohydrates, but we can be quite confident that it is already 'working on us' to engineer its genome so that it will be able to access such resources, thus increasing its range even further than is already the case.

Perhaps not surprisingly, given its GRAS status, *S. cerevisiae* was the host of choice for production of the first recombinant human vaccine, against hepatitis B (McAleer et al. 1984). Thus, *S. cerevisiae* continues to make itself more and more indispensable to us as our dependence on it for fuel and medicines grows.

A major issue faced by the fermentation and baking industries is the cost of purchasing or preparing yeast biomass. While beer makers usually repitch (i.e. recycle) their yeast several times before resorting to a fresh starter culture, and some winemakers rely on spontaneous fermentations, most wine and bread producers utilise fresh yeast for each fermentation. Interestingly, some winemakers claim that the unique contributions of different yeast species living on grapes or in the winery confer a complexity upon wine not seen in inoculated, controlled fermentations (Fugelsang 1997), but the risks associated with using such an approach can be considerable. This is why, in beer and bread production and for most wine fermentations, starter cultures of single, known yeast strains are used; they minimise the risk of failed fermentations and spoilage, and ensure a predictable product for the consumer. Thus, despite associated costs, there will be an ongoing need to continue producing large quantities of 'single culture' yeasts. Anything that yeast scientists can deliver with regard to reducing yeast production costs would therefore be welcomed by industry and consumers, who would benefit from savings, passed down the line.

Today's consumers have an increasing interest in luxury, individualised foods and beverages. Thus, there is an ongoing drive to make novel products, but in fermentation industries this is limited when one is restricted to using single strains of yeasts. Thus, scientists associated with the beer and wine industries have an interest in making yeasts capable of delivering more 'interesting' and varied sensory properties to

beverages, without having to resort to mixed ferments. Genetic modification offers numerous possibilities in this regard, enabling the development of products with novel sensory properties and improved quality (for recent reviews, see Pretorius and Van der Westhuizen 1991; Pretorius 2000, 2003, 2004, 2005; Verstrepen et al. 2001a; Akada 2002; Pretorius and Bauer 2002; Pretorius et al. 2003, 2005; de Barros Lopes et al. 2005; Pretorius and Høj 2005). Of course, research and development to produce superior yeast strains for industrial use is very costly, making it accessible only to large multinational companies or highly co-ordinated industry bodies.

Interestingly, when trying to improve yeast-based industrial processes, attention has generally focused on various highly technological solutions ranging from the refinement of bioreactors to the optimisation of various production parameters. It is only recently that there has been a shift to modify yeasts to improve the processes they are applied to, and this is, in no small part, due to the development of modern microbiology and molecular biology (Fig. 13.3). Once the public are more accepting of using GMOs we will be able to construct yeasts in ever more defined and refined ways to meet the demands of industry. The potential of this is already apparent from experimental work that will be described later in this review.

One of the challenges facing scientists working on industrial yeasts is the fact that the strains used in bakeries, breweries and wineries are usually polyploid or aneuploid. This makes crossing and sporulating, two techniques commonly used in 'classic' yeast breeding programmes, difficult (Akada 2002; Estruch and Prieto 2003). In 1978, however, genetic engineering offered an alternative approach when, independently, Hinnen et al. (1978) and Beggs (1978) described a method to genetically transform *S. cerevisiae*, thereby negating the need for sexual reproduction to recombine genes from different genetic backgrounds. But even with this in place industrial strains are still less straightforward to work with than laboratory strains because it is usually undesirable to have auxotrophic markers in strains that are to be used in an industrial setting; auxotrophy can impact on the performance of the yeast and the quality of the product that the yeast is employed to make. This has meant that new dominant genetic markers and new strategies for genetic engineering have had to be developed for selection of transformants in industrial yeasts (Webster and Dickson 1983; Akada 2002; Estruch and Prieto 2003; Verstrepen and Thevelein 2004).

Although this chapter is about industrial yeasts there is only one group of yeasts that we have truly domesticated and co-evolved with, at least in a cultural (again, forgive the pun) sense. That group is the *Saccharomyces sensu stricto* complex, which includes the most thoroughly researched and scientifically described of all domesticated organisms, *S. cerevisiae*. This chapter focuses on industrial strains of *S. cerevisiae*, describing examples of how it has been engineered to improve its performance in the food and alcoholic beverage industries (Figs. 13.4–13.6), future possibilities offered by exciting gene technologies, and some of the hurdles we have to get over to exploit it more fully. Consideration is also given to some of the legal and political issues associated with the application of GM yeasts to the food and beverage industries. Philosophically, however, one might argue that this chapter is really about how this yeast has exploited humans to make it more and more indispensable to us. In this respect we are a means to an end for its reproductive success.

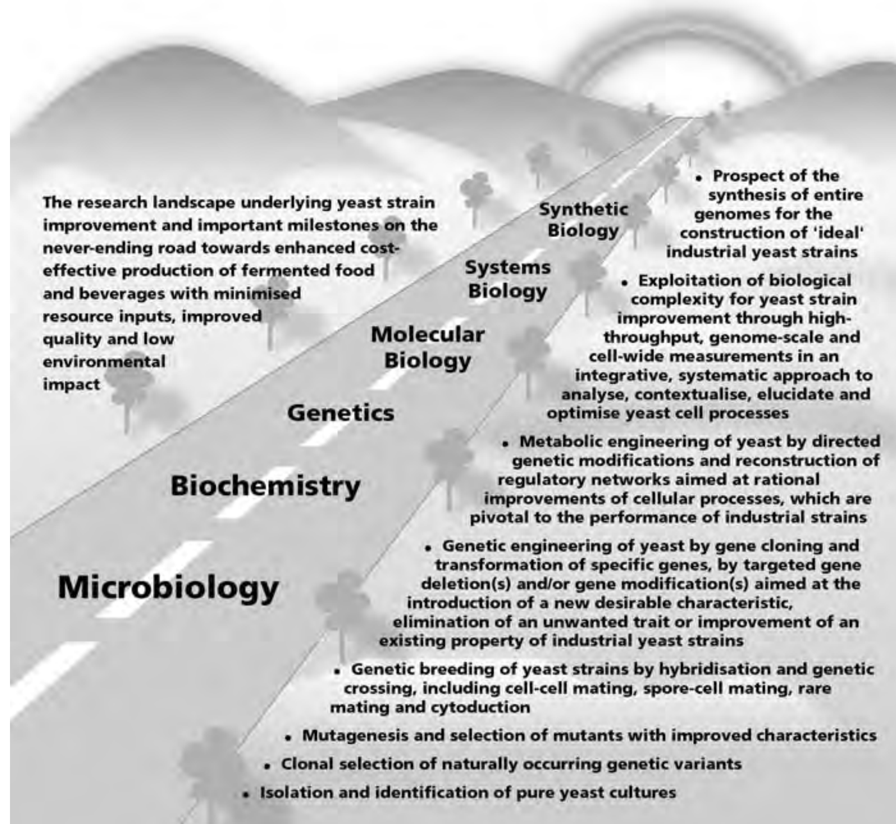

Fig. 13.3. The road of discovery and application in yeast science started in microbiology laboratories. Moving through the twentieth century, the application of biochemistry and genetics to research on yeasts increased our knowledge and understanding of cell physiology enormously, and the advent of molecular biology revolutionised the field. We are now moving into systems biology, and we can only guess what this era will deliver. One thing we can be sure about, however, is that it holds enormous potential for fermentation industries in that it will enable us to design yeasts that are more robust and deliver an increasing array of tailor-made products to meet consumer demand

13.2 Genetically Engineering Yeasts for Improved Performance and Product Quality

This section presents an overview of some of the types of recombinant DNA-based modifications that have been introduced into *S. cerevisiae* to improve fermentation performance or quality of end products (Fig. 13.7). In the current political climate it is unlikely that these yeasts will be used to produce food or beverages, but they give some indication of what will be possible in the future when GMOs are viewed with less suspicion by the general community.

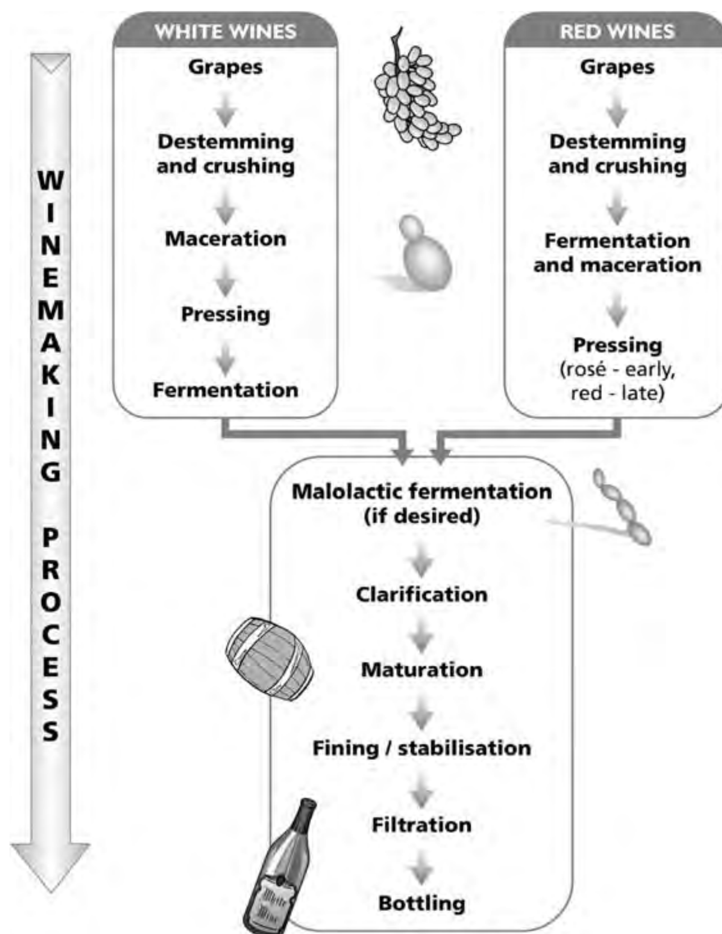

Fig. 13.4. Winemaking is probably the oldest application of yeast fermentation. Illustration of the main steps in this process

13.2.1 Improving Fermentation Performance

Different strains of *S. cerevisiae* vary considerably in their efficiency and reliability in fermentations. This tells us that there is considerable genetic variation that we can tap into when we select or manipulate this yeast for improved performance, and when this variation proves to be too limiting we can use genetic engineering techniques to borrow genes from elsewhere. The following section will consider what are now known as self-cloned (i.e. GM but not transgenic) and transgenic variants of *S. cerevisiae*, that have been created to this end.

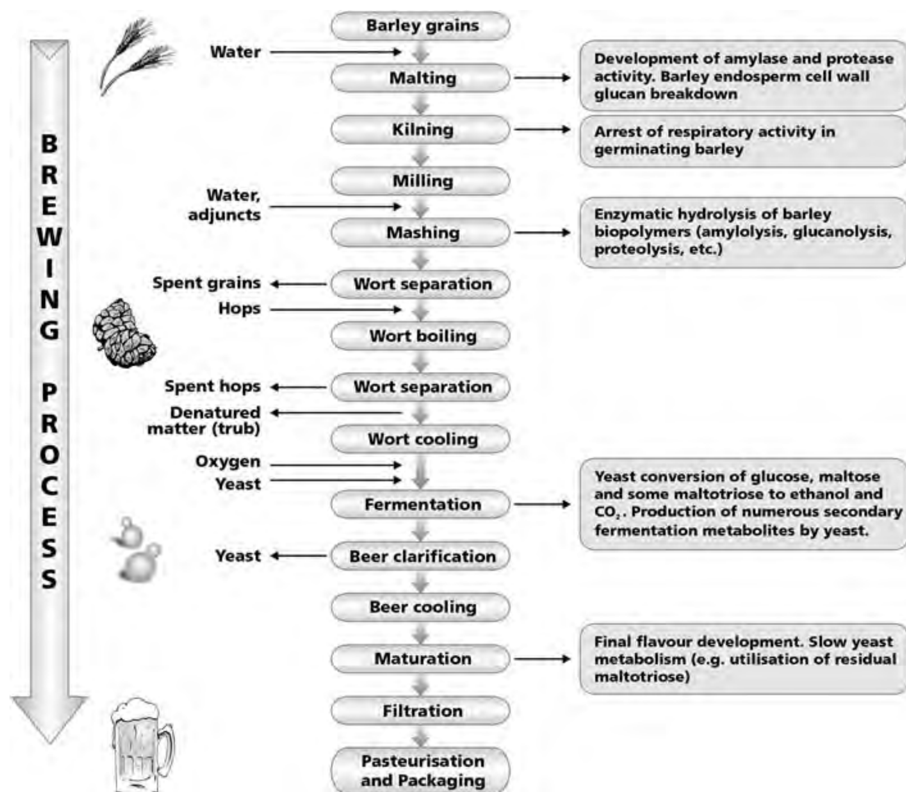

Fig. 13.5. Brewing has been with us for thousands of years and, while there are many variations in the steps involved, the key stages that are central to most brewing processes are illustrated

13.2.1.1 Making Improvements by Manipulating Fermentation Rate, Glycolytic Flux and Sugar Uptake

Fermentation-based production processes (Figs. 13.4–13.6) are time-consuming; beer fermentation takes 4–7 days and wine fermentation often takes more than 20 days. The time needed to complete a fermentation cycle is a major determinant of productivity and cost efficiency. A faster fermentation rate results in lower running costs, greater flexibility, and the total fermenter volume needed to obtain a targeted production volume is dramatically reduced.

Temperature control in the fermentation vessel is the traditional way to manage fermentation rate. The optimal growth temperature of *S. cerevisiae* is 28–30°C and, in general, temperatures close to this result in relatively rapid fermentations. Cooler temperatures slow overall yeast metabolism (Sablayrolles and Barre 1993; Speers et al. 2003) and higher temperatures compromise product quality. For example, many important volatile aroma compounds are lost at higher than optimal temperatures

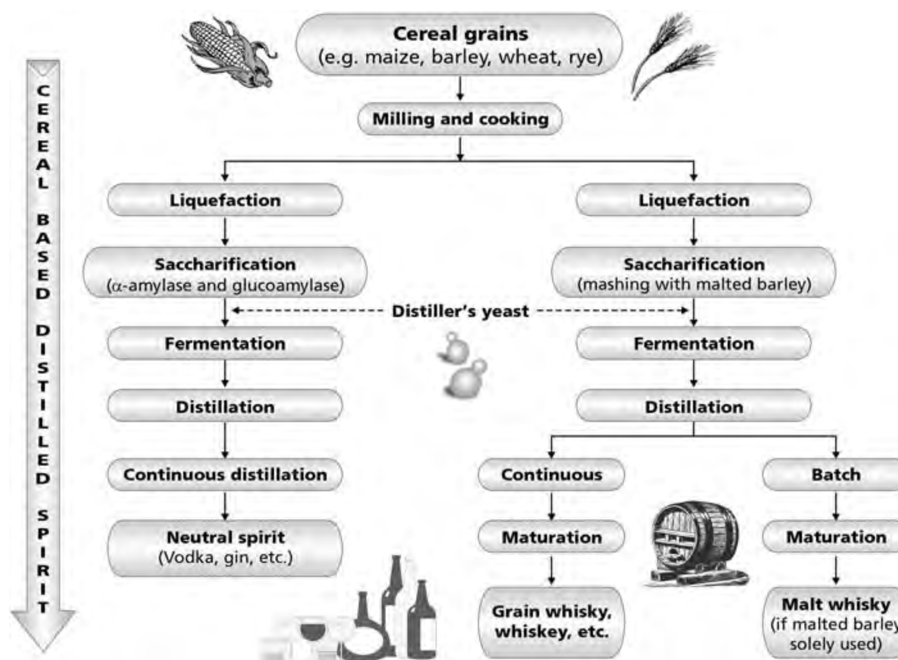

Fig. 13.6. Making alcoholic spirits utilises distiller's yeasts, which are strains of *S. cerevisiae* that produce, amongst other things, high levels of ethanol and have a high level of tolerance to this alcohol. Illustration of the main stages in a fermentation to generate spirits

because of increased evaporation and by the entrainment action of carbon dioxide (Verstrepen et al. 2003a). In addition, production of flavour-active secondary metabolites such as fusel alcohols and esters is disproportionately increased as temperatures increase, causing an unbalanced aroma profile (Sablayrolles and Ball 1995; Verstrepen et al. 2003a). Use of increased fermentation temperatures is therefore limited, particularly in lager beer and white wine production, where temperatures are generally kept below 15°C to preserve the typical aromas associated with these beverages (Nykänen and Suomalainen 1983; Nykänen 1986; Robinson 1994; Dominé 2000; Verstrepen et al. 2003a).

Attempts have been made to change fermentation rates using GM yeast strains engineered to produce higher levels of glycolytic enzymes. In one remarkable study genes encoding key enzymes of the glycolytic pathway were overexpressed in order to increase glycolytic flux (Schaaff et al. 1989). The overexpressed genes encoded hexokinase (HXK), glucokinase (GLK), phosphoglucose isomerase (PGI), phosphofructokinase (PFK), aldolase (FBA), triosephosphate isomerase (TPI), glyceraldehyde-3-phosphate dehydrogenase (TDH), phosphoglycerate kinase (PGK), phosphoglycerate mutase (PGM), enolase (ENO), pyruvate kinase (PYK), pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH), each being overexpressed

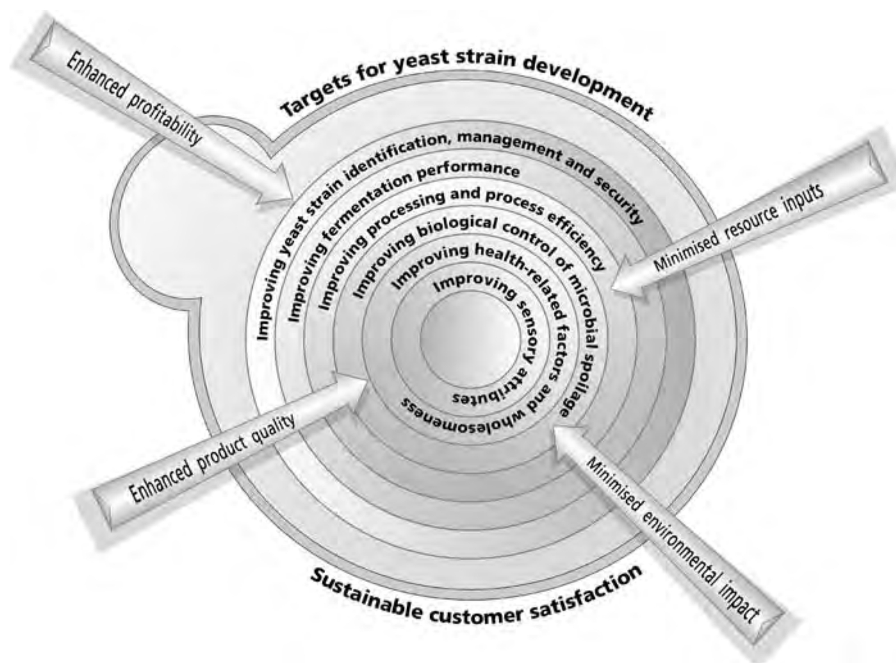

Fig. 13.7. There are many improvements that can be made to yeast performance and product quality in industrial applications. Genetic engineering is a very powerful means to achieving such ends, by enabling the development of new strains of yeasts with novel phenotypes that can lead to, amongst other things, improvements in sensory properties of products and efficiency of processing

in a different strain. However, overexpression of these enzymes had no significant effect on the rate of ethanol formation. Simultaneous overexpression of a group of seven enzymes in the lower glycolytic pathway resulted in a very limited increase of fermentative capacity (Smits et al. 2000). Together, these results suggest glycolytic flux is not regulated at the level of glycolytic gene expression. Perhaps an increase in glycolytic flux may require overactivation of all glycolytic enzymes simultaneously. However, it is likely that the import of sugars into the cell and/or sugar hydrolysis are the main rate-limiting steps for fermentation (Boulton et al. 1996). Over recent years much research has focused on carbohydrate transport and the genes involved.

In most commercial fermentations the medium contains a mixture of carbohydrates. The main sugars in bread dough are sucrose, glucose, fructose and maltose; grape must contains mainly glucose and fructose; beer and whiskey wort contains glucose, fructose, sucrose, maltose and maltotriose; and the fermentation medium for ethanol production usually contains a mixture of any of these sugars in variable concentrations, depending on the origin of the molasses (Bamforth 2003; Yoon et al. 2003). Because carbohydrates are too hydrophilic to cross the cell membrane by free diffusion, specialised carriers are required for their transport into the cell. Yeast cells

have more than 15 general hexose transporters (Stambuk and de Araujo 2001) as well as a set of more specialised transporters, including the five maltose transporters encoded by the *MALx1* genes (where *x* is the number of the *MAL* gene family) (Vanoni et al. 1989; Han et al. 1995), and Fsy1 (Goncalves et al. 2000; de Sousa et al. 2004), a specialised fructose transporter identified in *S. pastorianus* and *S. bayanus*.

Carbohydrate import is a rate-limiting step in fermentation, at least in part, because glucose and sucrose repress the expression of transporters necessary for the import of other sugars such as maltose, maltotriose and galactose (for reviews, see Winderickx et al. 2003; Verstrepen et al. 2004a). Glucose also slows down the uptake of fructose because both sugars are imported by the same HXT carriers, which have a greater affinity for glucose than fructose. Apart from this competitive inhibition of fructose uptake, recent research demonstrates that glucose may also repress the expression of specific fructose transporters such as Fsy1 (Goncalves et al. 2000; Berthels et al. 2004; de Sousa et al. 2004). The minimal concentration at which glucose and sucrose induce signals differs for different signalling pathways and targets but, in general, concentrations higher than 20–40 mM are sufficient to elicit a strong response (Meijer-Michelle et al. 1998; Meneses et al. 2002).

In beer wort, grape must, molasses and bread dough, the initial glucose and/or sucrose concentration greatly exceeds the aforementioned threshold for catabolite repression; therefore, the transport of other sugars is delayed until glucose and sucrose levels drop. In some cases, yeast cells fail to attain a proper de-repressed state, leading to sluggish or 'hanging' fermentations, in which not all of the available sugars are fermented (Oda and Ouchi 1989; Meneses et al. 2002; Meneses and Jiranek 2002; Verstrepen et al. 2004a). Also, towards the end of fermentation, transport and consumption of sugars often slows down. This has been attributed to the various stresses yeast cells experience at the end of industrial fermentations, such as high ethanol and carbon dioxide levels and low levels of carbon and nitrogen compounds (Ivorra et al. 1999; Bauer and Pretorius 2000; Brosnan et al. 2000; Puig and Pérez-Ortin 2000; Blateyron and Sablayrolles 2001; Carrasco et al. 2001). However, the precise mechanisms responsible for this late-fermentation drop in sugar consumption are not yet understood.

On the basis of current knowledge of the regulatory mechanisms that control sugar transport, several attempts have been made to use genetic modification to increase fermentation rates. For example, it has been shown that constitutive expression of the *MAL61* gene, encoding a maltose transporter protein, results in a clearly greater fermentation rate of high-gravity beer worts (Kodama et al. 1995). Similarly, overexpression of maltose permease and maltase resulted in a yeast with improved dough-leavening capacities (Osinga et al. 1988). Increasing sugar uptake through the general hexose transporters, however, is more complicated. This is because some of these have specific roles during fermentation; in fact overexpression of a single *HXT* gene cannot fully restore the fermentation capacity of an *HXT* null strain (Luyten et al. 2002). In order to obtain increased general hexose uptake, it may be necessary to co-overexpress several members of the *HXT* gene family. Other strategies, such as constitutive expression of the heterologus fructose transporter-encoding gene *FSY1*, which may improve the fermentation performance of wine yeasts, remain to be explored.

13.2.1.2 Making Improvements in Oligosaccharide (Dextrin) Utilisation

S. cerevisiae ferments only some mono-, di- and trihexoses, while more complex or branched oligosaccharides are not metabolised. Molasses, a commonly used medium for the production of bio-ethanol, biopharmaceuticals and baker's yeast biomass, contains up to 8% raffinose. This trisaccharide (fructose–glucose–galactose) is hydrolysed by baker's yeast invertase to fructose and the non-fermentable disaccharide melibiose. Expression of the α-galactosidase-encoding *MEL1* gene from *S. bayanus* in baker's yeast resulted in an 8% increase in biomass yield, yet other desirable characteristics were retained (Gasentramirez et al. 1995).

Beer wort contains about 30% non-fermentable higher dextrins. While some residual sugars in fermented beverages contribute to the 'wholesomeness' and the 'mouthfeel' (Ragot et al. 1989), non-fermentable sugars represent a considerable economic loss for brewers. A second disadvantage associated with these carbohydrates is that the popular light beers end up carrying a considerable number of kilojoules. Frequently, glucoamylases are added to break down the higher dextrins in wort into less complex sugars that can be fermented by brewer's yeast. The dextrins are hydrolysed and subsequently fermented, resulting in a relatively high ethanol product. After dilution to an appropriate alcohol level, the resulting beer contains fewer sugars and therefore less energy. By enabling yeast to ferment these dextrins, light beers could be produced without the addition of expensive purified enzymes. In fact, the fermentation of a greater proportion of wort sugars would make the production of 'light' and 'low-carbohydrate' beers cheaper than that of standard beers (Hammond 1995). A commercial lager yeast strain was transformed with a multicopy plasmid in which the *S. diastaticus* glucoamylase-encoding *STA2* gene was placed under the control of the constitutive yeast *PGK1* promoter. Transformants were superattenuating, producing about 1% (v/v) more ethanol than the wild-type parental strain (Perry and Meaden 1988), but they were not very stable. This problem was easily addressed, however, by integrating the *PGKP::STA2* construct into the *S. cerevisiae* genome (Vakeria and Hincliffe 1989). Similar results were obtained with expression of the *S. diastaticus STA1* gene (Sakai et al. 1989) in brewing yeast.

13.2.1.3 Achieving Fermentation of Branched Oligosaccharides and Polysaccharides

Fermentation of media containing a mixture of various complex sugars that are low in cost is important for many ethanol-producing companies, but particularly the bio-ethanol industry; accessing cheaper forms of substrate would make bio-ethanol a more economically viable alternative to fossil fuel (Wheals et al. 1999; Zaldivar et al. 2001). Cellulose and starch are the most abundant and widespread polysaccharides on the planet but *S. cerevisiae* does not have the cellulase or amylase enzymes necessary to access these polymers. The potential of these carbohydrates to be used as cheap sources of carbon in fermentation processes has therefore fuelled research aimed at creating transgenic yeasts carrying the requisite enzymes. Ethanol production using other types of microorganisms that encode their own cellulases and/or amylases is probably impossible because of constraints such as poor ethanol tolerance, poor growth rates and lack of GRAS status (Eksteen et al. 2003).

Starch (and dextrins) are composed of linear α-1,4-linked chains of glucose with various degrees of α-1,6 branching. At least two different classes of enzymes are needed to efficiently digest these molecules into fermentable maltose and glucose: α-amylase (endoamylase) and glucoamylase (exoamylase). Specific debranching enzymes that digest the α-1,6 linkages, such as pullulanase and isoamylase, enable complete digestion of these polymers. Thus to make transgenic amylolytic yeasts requires engineering genes for at least some of the aforementioned enzymes into *Saccharomyces* spp.

Amylolytic yeasts are, of course, also of interest to the whiskey and beer industries because of their potential to ferment all available sugars without the need for extensive malting. In the beer world, digestion of branched carbohydrate molecules has been achieved using enzymes derived from organisms such as *Aspergillus niger* (Gopal and Hammond 1992) and *Aspergillus amawori* (Cole et al. 1988), both of which have enzymes with α-1,6 and α-1,4 hydrolytic activities. An expression cassette of the *A. niger* glucoamylase gene has been inserted into the *S. cerevisiae HO* gene and the resultant strain was tested at a semi-industrial scale in fermentations of up to 10,000 l. The trials were successful, producing beer that was both superattenuated and of good quality (Gopal and Hammond 1992; Hammond 1995). The GM yeast strain was the first (and so far only) GM brewer's yeast to be cleared for the production of human food. Large-scale commercialisation of the beer was not attempted, however, because of the low consumer acceptance of GMOs in food production (see later) (Hammond 1995; Roller and Harlander 1998).

Increased dextrin degradation has similarly been obtained by overexpression of the amyloglucosidase of *Schwanniomyces occidentalis* in a brewing yeast (Lancashire et al. 1989). Like the *A. niger* and *A. amawori* amyloglucosidase activities, the *S. occidentalis* enzyme combines α-1,6 and α-1,4 hydrolytic activities. However, this enzyme has the additional advantage of being heat-labile, and therefore can be inactivated during beer pasteurisation. This reduces the risk of beer sweetening due to further breakdown of unfermented starch during storage (Lancashire et al. 1989).

Research on whiskey yeasts has shown that it is possible to create industrial strains of *S. cerevisiae* that can ferment starch. Amylase genes *LKA1* and *LKA2*, from the yeast *Lipomyces kononenkoae*, were transformed into four different whiskey strains and a wine yeast, and the transformants (unlike the original parent strains) were able to utilise starch for ethanol production (La Grange-Nel et al. 2004). This again highlights the potential of genetic engineering for creating novel yeast phenotypes that are of value to industry.

13.2.2 Breakdown of Haze-Causing Polysaccharides, Phenols and Proteins

Haze is a precipitate that appears in some beers and wines as they mature. The resultant cloudy product can cause filtration problems during processing and is usually regarded as undesirable by consumers. Polysaccharides derived from raw materials and including β-glucans, pectins and xylans are partially responsible for such problems. β-Glucans are found in barley cell walls and are cleaved by a specific *endo*-β-glucanase; however, this cleavage is often only partial because enzymatic activity

is lost owing to the elevated temperatures of kilning, thus leaving residual insoluble carbohydrate. Grapes contain pectins, cellulose (a source of glucans) and hemicellulose (mainly xylans).

Another cause of haze is the precipitation of proteins. In wine this type of haze is caused by grape-derived, pathogenesis-related proteins such as thaumatin-like proteins and chitinases, which aggregate and then precipitate over time, and particularly on heating.

Haze-causing molecules are normally removed by sedimentation, filtration and centrifugation, or by the addition of enzymes (usually bacterial) that break them down. In the case of wine, fining agents such as casein, isinglass, albumin, gelatin, bentonite or polyamide materials are commonly used to bind or filter phenols and colloidal particles that can contribute to haze formation (Boulton et al. 1996). However, filtration, centrifugation, enzymatic treatment and fining are costly and laborious processes. Moreover, as much as 20% of the product can be lost during these clarification and stabilisation steps (Canal-Llauberes 1993). Thus, it would be of great benefit to the alcoholic beverage industries if yeast strains that have an inherent ability to hydrolyse haze-causing molecules could be developed, and there have been several exciting advances in this regard (van Rensburg and Pretorius 2000).

Several yeast strains that can hydrolyse one or more of the known haze-causing agents have been produced. For brewer's yeast, most attention has focused on the development of β-glucan degrading strains. For example a *Bacillus subtilis* β-glucanase gene was fused to the *S. cerevisiae* α-factor secretion signal ($MF\alpha 1_S$) and expressed in *S. cerevisiae* (Lancashire and Wilde 1987), leading to reductions in β-glucan content and haze reduction in the final product. More efficient glucan hydrolyis in beer fermentation was obtained by expressing *Trichoderma reesei* β-glucanase, which has a lower optimal pH (4–5 compared with 6.7 for the *B. subtilis* enzyme) (Pentillä et al. 1987a; LaGrange et al. 1996). Industrial brewer's yeast carrying the *T. reesei EG1* gene driven by the *S. cerevisiae ADH1* promoter and devoid of any bacterial sequences has been tested in pilot beer fermentations. The strain hydrolysed virtually all β-glucans, resulting in a significant reduction of beer viscosity, while all other brewing characteristics of the parent strain remained unchanged (Pentillä et al. 1987b; Suihko et al. 1991). Similarly, several glucan-hydrolysing wine strains were constructed that expressed the *Butyrivibrio fibrisolvens* endo-β-1,4 glucanase gene *END1*, the *B. subtilis* endo-β-1,3-1,4-glucanase gene *BEG1*, the *Ruminococcus flavefaciens* cellodextrinase gene *CEL1*, the *Phanerochaete chrysosporium* cellobiohydrolase gene *CBH1* or the *Saccharomycopsis fibuligera* cellobiase gene *BGL1* (van Rensburg et al. 1994, 1995, 1996, 1997, 1998, 2005). However, these strains have not yet been tested in large-scale fermentation trials.

A pectolytic wine yeast was developed by co-expressing the *Erwinia chrysanthemi* pectate lyase gene *pelE* and the *Erwinia carotovora* polygalacturonase gene *peh1* (Laing and Pretorius 1992, 1993a, b), both of which were fused to *S. cerevisiae* $MF\alpha 1_S$ secretory signals. Xylan-fermenting yeasts were constructed by expressing the endo-β-xylanase genes from *A. kawachii* (*XYN1*) and *A. niger* (*XYN4* and *XYN5*), *T. reesei* (*XYN2*) as well as the *B. pumilis xylosidase XLO1*, the *A. niger* α-L-*arabinofuranosidase ABF2* and the *A. nidulans* genes *xlnA* and *xlnB* (Pérez-Gonzalez et al. 1993, 1996; Crous et al. 1995, 1996; LaGrange et al. 1996, 1997;

Luttig et al. 1997). While these constructs show great promise in laboratory-scale work, further tests are needed to evaluate the exciting possibilities they offer in large-scale fermentation.

The possibility of creating wine yeasts capable of hydrolysing haze-active proteins has also been investigated. Overexpression of *S. cerevisiae* vacuolar protease A, encoded by the *PEP4* gene, was chosen because of its activity at the low pH of wine. The *PEP4* gene was cloned and expressed in wine yeast using several promoter, leader and terminator sequences. When *PEP4* was driven by the *ADH1* promoter and preceded by the *MFα1* pheromone secretion signal, biologically active protease A was secreted into the medium. However, this was not sufficient to replace bentonite fining because many of the haze-causing proteins are particularly resistant to proteolysis (Pretorius 2000).

A novel method to prevent haze in white wine is the use of haze protective factors (hpfs); specific mannoproteins from *S. cerevisiae* that visually reduce protein haze (Waters et al. 1993, 1994). While the exact mode of action of these proteoglycans is unknown it is possible that overexpression of genes for hpfs in wine yeast during fermentation will lead to a reduction in the levels of haze-causing proteins in wine.

13.2.3 Improving Flocculation and Sherry Flor Formation

At the end of a primary beer, wine or whiskey fermentation, yeast cells have to be separated from the product. The natural tendency of yeast cells to flocculate can make this biomass separation relatively easy. Flocculation is a reversible, asexual and calcium-dependent process in which yeast cells adhere to form flocs consisting of thousands of cells (Verstrepen et al. 2003b). Upon formation, the flocs rapidly separate from the bulk medium by sedimentation or, having entrapped gas bubbles, by rising to the surface. Flocculation is important to the fermentation industry because it provides an environmentally friendly, cost-free and effective way to separate yeast cells from the fermentation product. However, the timing of flocculation is critical: cells should not flocculate before fermentation is complete because this can lead to slow or stuck fermentations, while late or poor flocculation increases the need for extra filtration and centrifugation. The ideal industrial yeast should exhibit strong flocculation towards the end of fermentation. Many of the yeast strains presently used in industrial fermentations have a less than optimal flocculation profile (for a review, see Verstrepen et al. 2003b). Furthermore, the flocculation behaviour of a specific yeast strain is often variable and difficult to predict (Jibiki et al. 2001; Sato et al. 2001; Verstrepen et al. 2003b, 2004b). Improvement and control of flocculation is therefore a constant concern for the alcoholic beverage industries.

Flocculation of *S. cerevisiae* involves lectin-like adhesins, called flocculins, that stick out of the cell wall (Stratford and Carter 1993). Following activation by calcium ions (Stratford 1989; Watari et al. 1994; Teunissen and Steensma 1995; Bony et al. 1997b; Kobayashi et al. 1998), the N-termini of flocculins selectively bind mannose residues of adjacent cells, thereby creating a group or floc of cells. More than 30 genes are known to be involved in this process (Teunissen and Steensma 1995; Teunissen et al. 1995b; Kobayashi et al. 1999; Verstrepen et al. 2003b) but only a few of them encode flocculins; the others are regulatory genes, which are often not

specific for flocculation. The best-known flocculation gene is *FLO1*, which is relatively large, encoding a cell wall flocculin of 1,537 amino acids (Stratford and Carter 1993; Teunissen et al. 1993a, b; Watari et al. 1994; Bony et al. 1997b; Kobayashi et al. 1998). Other dominant genes encoding flocculins include *FLO5*, *FLO9* and *FLO10* (Teunissen and Steensma 1995; Teunissen et al. 1995a; Guo et al. 2000; Batlle et al. 2003). Lager-*FLO1* (Lg-*FLO1*) is a *FLO1*-like gene found in lager yeast (often referred to as *S. pastorianus* or *S. carlsbergensis*). This gene is believed to encode a slightly different flocculin that binds glucose and maltose as well as mannose. Hence, flocculation caused by the Lg-*FLO1* gene product is competitively inhibited by free glucose and mannose in the medium (Kobayashi et al. 1998; Sato et al. 2002).

The *FLO11* gene (also referred to as *MUC1*) encodes a special adhesin that enables cells to adhere to various surfaces, including agar and plastic (Lambrechts et al. 1996; Lo and Dranginis 1996, 1998; Guo et al. 2000; Reynolds and Fink 2001; Verstrepen et al. 2004b). In addition, expression of *FLO11* is required for filamentous growth (Lambrechts et al. 1996; Guo et al. 2000). Filamentous growth occurs when mother and daughter cells do not detach, forming long chains of slightly elongated cells. These chains can penetrate the semi-solid growth medium of agar plates. In liquid medium, filamentous growth is believed to be responsible for flor formation (a layer of floating yeast chains found during the oxidative fermentation stages in sherry production) (Ishigami et al. 2004).

Several attempts have been made to introduce flocculence into non-flocculating *S. cerevisiae* strains. The first strategies were based on the electrofusion or mating of a non-flocculent industrial strain with a flocculating strain (Urano et al. 1993a, b). Later approaches involved the use of genetic engineering to overexpress dominant flocculation genes such as *FLO1* (Barney et al. 1980; Watari et al. 1993, 1994). Self-cloning of *FLO1* into a non-flocculent industrial strain was first reported in 1998 (Ishida-Fujii et al. 1998); self-cloning strains contain no non-*Saccharomyces* DNA sequences and therefore are more likely to be exempt from the strict legislation regulating the use of GMOs in food production (Sect. 13.3.2.1).

Since expression of *FLO* genes results in flocculation, the controlled expression of these genes may lead to controllable flocculation; the main problem with most of the strains produced so far is that they constitutively flocculate, making them unsuitable for industrial use. Recently, a strain was constructed in which *FLO1* was brought under the control of an *HSP30* promoter (Verstrepen et al. 2001b) (Fig. 13.8). This promoter is activated only towards the end of fermentation, when ethanol levels increase and carbohydrates are depleted (Piper et al. 1997; Riou et al. 1997; Donalies and Stahl 2001; Hahn and Thiele 2004). This self-cloning strain showed an appropriate fermentation and flocculation behaviour in small-scale fermentation trials (Verstrepen et al. 2001b); however, further tests are needed to evaluate the performance of this construct in large-scale commercial fermentations. The controlled expression of Lg-*FLO1*, which will potentially prevent early flocculation because Lg-*FLO1* binding is inhibited by residual unfermented glucose and mannose, is also awaiting exploration.

Flor formation is one of the defining features of sherry production. A 'flor' is a dense mat of yeast that grows on the surface of maturing sherry after ethanolic

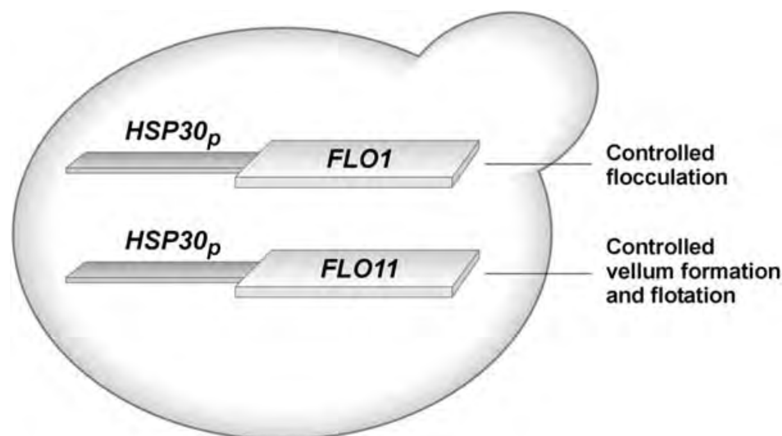

Fig. 13.8. Flocculation of yeast cells involves adhesin molecules called flocculins, one of which is encoded by *FLO1*. Transforming the yeast with this gene behind an *HSP30* promoter ensures that it will be expressed at the end of fermentation, when flocculation is required by the brewer or winemaker. Another flocculin, *FLO11*, is involved in filamentous growth and is important for flor formation during sherry production. If this gene were to be cloned behind the *HSP30* promoter presumably this would also lead to enhanced flor formation at the end of fermentation, when the flor is required

fermentation; failure of the mat to form or its breakdown leads to spoilage. Thus, improving flor formation and stability is of interest to sherry producers. In this context, overexpression of *FLO11* (Fig. 13.8) should help to create strains better able to form stable flors, and with improved adherence to carrier materials sometimes used in fermentations. A first step towards this was recently taken with the investigation of the role of *FLO11* in an industrial sherry yeast (Ishigami et al. 2004).

13.2.4 Engineering Antimicrobial Properties into Industrial Yeast

In contrast to controlled laboratory experiments, commercial-scale industrial fermentations are usually contaminated with other microbes. Industrial equipment is not easy to sterilise or even clean, many raw materials are contaminated with microorganisms and traditional fermenters may have airflow from the industrial plant. Thus there is a risk that 'wild' yeasts and/or bacteria may contaminate the fermentation. This is usually not a problem, but care must be taken to ensure that unwanted microorganisms do not become a predominant species in the fermenter or the packaged product. This can be achieved by arming industrial yeasts with mechanisms that allow them to fight and contain the growth of other microorganisms.

Some feral yeast strains secrete toxins known as zymocins, which kill non-resistant yeasts; in fact contamination with zymocin-producing wild yeasts has been identified as one of the causes of sluggish and stuck fermentations (van Vuuren and Wingfield 1986). Five types of killer toxins have been identified in *S. cerevisiae*: K_1, K_2, K_3, K_{28} and K_{3GR1}, with K_3 and K_{3GR1} probably being variants of K_2. Production and

resistance to killer toxins are conferred by a cytosolic double-stranded RNA fragment; thus, introduction of this RNA into an industrial yeast will confer resistance to zymocin produced by contaminating yeasts and also allows the host cell to fight off non-resistant contaminants (Hammond and Eckersley 1984; Sasaki et al. 1984; Boone et al. 1990). However, even when different killer factors are combined in one yeast strain, there is only limited protection. Application of zymocins produced by non-*Saccharomyces* yeast species, such as *Pichia* and *Hanseniaspora*, will perhaps be more effective and is now being given attention.

The antibacterial capacity of yeasts expressing bacteriocin-encoding genes, such as the *pedA* pediocin gene of *Pediococcus acidilactici* and the *lcaB* leucocin gene of *Leuconostoc carnosum*, has been investigated (Schoeman et al. 1999; du Toit and Pretorius 2000). In laboratory tests, transformants carrying these genes inhibited the growth of sensitive bacterial strains, indicating that it is possible to create bactericidal yeasts. Also under exploration is the expression of hydrolytic enzymes that disrupt the microbial cell wall. Lysozyme, for example, is officially approved as an additive to control malolactic fermentation and to enhance wine stability. Lysozyme is an *N*-acetylhexosaminidase capable of lysing the β-1,4 glucosidic linkages of the peptidoglycan layer in some Gram-positive bacteria. The lysozyme-encoding gene from chicken egg white was successfully expressed in *S. cerevisiae*, but the activity was hampered by hyperglycosylation, and the protective effect of this recombinant strain in industrial fermentations has yet to be demonstrated (Nakamura et al. 1993).

Environmental protection is an important issue associated with the development and use of GM zymocidal and bactericidal yeasts. Most industrial fermentations do not guarantee containment of the yeasts used and the liberation of GM yeasts with an obvious selective advantage may be risky, even if their chance of survival and associated disturbance of other ecosystems is very small. Further research is needed to assess and limit these risks before these yeasts can be used in commercial applications (Sect. 13.3.2.2).

13.2.5 Engineering Improved Stress Tolerance in Industrial Yeasts

Industrial yeasts encounter numerous stresses, ranging from mechanical shear stress during handling to complex biochemical stresses during fermentation. Yeasts unable to cope with such stresses exhibit reduced fermentation performance and compromise product quality (Attfield 1997; Bisson 1999; Ivorra et al. 1999; Bauer and Pretorius 2000; Verstrepen et al. 2004a). Attempts have therefore been made to improve stress tolerance in these organisms.

A high level of tolerance to freeze-thawing in yeasts is desirable for the increasingly popular use of frozen dough for bread production; reduced yeast vitality after freezing and thawing makes it necessary to use a larger amount of yeast (Park et al. 1997; Teunissen et al. 2002). UV mutants of an industrial baker's yeast with increased tolerance to this stress have been described, but the mutations that are responsible for the favourable phenotype are not known. In another approach, the yeast aquaporin genes *AQY1* and *AQY2*, that encode proteins involved in transport of water and solutes across cellular membranes, were overexpressed in an industrial yeast strain. The strain showed a remarkable increased resistance to freezing and

thawing, presumably because aquaporins allowed water efflux out of the cells during freezing of the extracellular matrix, thereby preventing cell damage by intracellular ice formation (Tanghe et al. 2002).

Tolerance to other stressors, such as ethanol, is more complex. Attempts have been made to increase the resilience of yeasts by overexpressing genes known to be associated with stress tolerance, but these experiments have met with varying, usually limited, levels of success (Chen and Piper 1995; Kajiwara et al. 2000). This is probably due to the enormous complexity of stress tolerance. For example, it is has been estimated that more than 250 genes are involved in ethanol tolerance (Boulton et al. 1996), and manipulation of one or just a few of these genes may not be sufficient to cause a significant improvement. One area that might be explored is simultaneous overproduction of multiple factors thought to be involved in stress protection, such as trehalose (Majara et al. 1996; Wiemken 1990; Boulton 2000) and some of the heat shock proteins (Sanchez et al. 1992; Varela et al. 1995; Piper et al. 1997). Another approach might be manipulation of the expression of *PMA1* and *PMA2*, which encode cellular ATPases and are known to be important in maintaining a proper intracellular pH and membrane potential under stressful conditions, although again the impacts of the products of these genes appear to be different for different stresses (Monteiro et al. 1994; Attfield 1997; Fernandes et al. 1998). Sterol biosynthesis is also a possible target because sterols are known to be important to maintain membrane integrity during stress (Swan and Watson 1998; Beney and Gervais 2001). But perhaps the most promising strategy is to manipulate genes involved in the various regulatory cascades that control stress resistance, e.g. the Ras/cyclic AMP (cAMP)/protein kinase A (PKA) pathway, the fermentable growth medium (FGM) induced pathway and the target of rapamycin (TOR) pathway. This strategy enables alterations in the expression levels of a large group of genes, and mutations in the Ras/cAMP/PKA pathway have already been shown to increase the general stress resistance (Van Dijck et al. 2000; Teunissen et al. 2002; Versele et al. 2004; Verstrepen et al. 2004a). A recent genome-wide monitoring of the transcriptome during wine fermentation also showed that the TOR pathway plays an important role in the regulation of gene expression during fermentation (Rossignol et al. 2003). Further tests are needed to evaluate whether or not this strategy allows the creation of stress-tolerant industrial strains without affecting the important industrial properties of the respective yeast.

Because of the complexity of stress tolerance, the large number of genes and metabolic processes that are likely to be involved, and our poor understanding of what is required, perhaps approaches other than those targeting small numbers of genes or specific pathways might be more successful for raising stress-tolerant mutants. Adaptive evolution, driven by natural/artificial selection, can lead to the generation of yeasts that are more resilient than the parent from which they were derived (Stephanopoulos 1994; Ferea et al. 1999; Lassner and McElroy 2002; Petri and Schmidt-Dannert 2004; Cakar et al. 2005; Giudici et al. 2005). The approach used involves exposing a parental strain of yeast to ongoing selection in the form of stresses the yeast will encounter during fermentation. After many (typically hundreds of) generations, mutants are raised with increased stress tolerance. The beauty of this method is that no prior knowledge of the genetics or molecular biology of

the system is required, and the strains generated are non-GM and therefore no special approval is needed for their use in food and beverage production. However, stress-tolerant strains generated in this way in the laboratory have not yet been applied in a production-scale industrial setting.

13.2.6 Genetically Engineering Yeasts to Impart Improved Sensory Qualities on Fermentation Products

Yeasts play a significant role in determining the sensory properties and wholesomeness of fermentation products (Swiegers and Pretorius 2005). This section gives an overview of some of the ways *S. cerevisiae* has been genetically engineered to improve these aspects of its performance.

13.2.6.1 Reducing Diacetyl Levels in Alcoholic Beverages

During primary fermentation, yeast cells produce a vast array of secondary metabolites, and despite their low concentrations, some of these contribute enormously to aromas and flavours in fermented beverages. One of the most important flavour-active metabolites is diacetyl, a by-product of yeast valine synthesis. While moderate concentrations of diacetyl impart the typical buttery character of some white wines and Scotch ale beer, diacetyl is unwanted in most other beverages. In fact the prime reason for beer lagering (a 7–20-day maturing period after the primary fermentation) is to reduce diacetyl levels to a concentration below the flavour threshold of about 0.15 mg L^{-1}. During lagering, yeast cells take up the diacetyl produced during primary fermentation and convert it into acetoin and 2,3-pentanediol (Bamforth and Kanauchi 2004).

A variety of strategies have been employed to minimise diacetyl levels in beer, one of which is the introduction of a heterologous gene encoding α-acetolactate decarboxylase (ALDC). This enzyme catalyses the conversion of the diacetyl precursor, acetolactate, to acetoin, which has a far higher organoleptic threshold value than diacetyl (Meilgaard 1975a). ALDC is found in several bacteria, including *Lactococcus lactis* and *Acetobacter* species, which are currently used for food production (Goelling and Stahl 1988). The ALDC gene derived from *Acetobacter aceti* ssp. *xylinum* has been cloned behind the yeast constitutive *PGK1* promoter and introduced into the *S. cerevisiae* genome. The resultant recombinant strain has been used in pilot-scale brewing trials and consistently produced beers of high quality that were comparable to beers produced by the wild-type parent except for drastically reduced diacetyl levels (Yamano et al. 1994a, b, c, 1995). Similarly, the *Enterobacter aerogenes* ALDC, cloned behind the *S. cerevisiae ADH1* promoter and introduced in brewer's yeast, resulted in significantly reduced levels of diacetyl in small-scale fermentation trials (Sone et al. 1988; Fujii et al. 1990).

Another strategy to reduce diacetyl levels is to lower the activity of the *ILV2*-encoded α-acetolactate synthase, which catalyses the synthesis of α-acetolactate. This has been accomplished by screening yeast populations for resistance to the herbicide sulfometuron methyl (SMM), which inhibits the action of α-acetolactate synthase (Casey et al. 1988). Mutants with SMM resistance produce less α-acetolactate and

therefore less diacetyl. However, the reduced activity of this gene product, which is crucial for valine biosynthesis, may affect overall yeast performance. Perhaps the most elegant way to reduce diacetyl formation is to increase flux through the valine biosynthesis pathway. In this way, accumulation of intermediary products such as α-acetolactate is avoided, while valine biosynthesis is not compromised. A high flux was accomplished by overexpressing the *ILV3* and *ILV5* genes and this led to a big reduction in diacetyl production, especially when only *ILV5* was overexpressed (Goossens et al. 1987; Villa et al. 1995).

13.2.6.2 Engineering Yeasts for Improved Flavour Profiles of Alcoholic Beverages

Like diacetyl, volatile esters are only trace compounds in fermented beverages, but they are extremely important for the flavour profile of these drinks (Drawert and Tressl 1972; Engan 1972, 1974; Meilgaard 1975a, b, 1991, Suomalainen 1981; Nykänen and Suomalainen 1983; Nykänen 1986; Kruger 1998a, b; Debourg 2000; Lambrechts and Pretorius 2000; Cristiani and Monnet 2001; Pisarnitskii 2001). The most important flavour-active esters in beer are the three acetate esters: ethyl acetate (solvent-like aroma), isoamyl acetate (fruity, banana aroma) and phenyl ethyl acetate (flowery, roses, honey aroma); and the medium-chain fatty acid ethyl esters ethyl caproate and ethyl caprylate (sour apple). Since most esters are present in concentrations around their flavour threshold values, minor changes in concentration may have dramatic effects on beer flavour (Hammond 1993). This can be problematic in high-gravity brewing because the use of high specific gravity worts results in a severe overproduction of acetate esters. The concentration of acetate esters after dilution of beers produced through ultra-high-gravity fermentations (20°P) can be up to 75% higher than in beers produced with standard 12°P wort (Anderson and Kirsop 1974; Hammond 1993; Meilgaard 2001). In contrast to this, fermentations performed in tall, cylindroconical 'Apollo' fermenters result in decreased formation of esters, so that the beers produced lack desirable fruity tones (Meilgaard 2001; Verstrepen et al. 2003a). Other novel fermentation systems, such as continuous-flow fermenters or fermenters with immobilised yeast also give rise to unbalanced ester profiles (Moonjai et al. 2002; Verstrepen et al. 2003a; Shen et al. 2004).

Much research has focused on the enzymes responsible for the formation of volatile esters. So far, two related alcohol acetyltransferases have been described: Atf1 and Atf2 (Fujii et al. 1996b; Nagasawa et al. 1998). Whereas the physiological role of these ester synthases remains unknown, it is generally believed that volatile esters may be side products of other, physiologically relevant reactions, such as certain steps in the cellular lipid metabolism (Verstrepen et al. 2003e).

The deletion and overexpression of the alcohol acetyltransferase genes (*ATF1* and *ATF2*) have been reported for both brewing and wine strains (Fujii et al. 1994, 1996a, b). From this work it was found that deletion of *ATF1* reduces isoamyl acetate production by 80% and ethyl acetate production by 30%. *ATF2* deletion has similar but smaller effects on ester production (Nagasawa et al. 1998; Verstrepen et al. 2003d). Accordingly, overexpression of these genes in sake yeast led to a tenfold increase in ethyl acetate production and a 30-fold increase in isoamyl acetate

formation. Similarly, it was shown that overexpression of *ATF1* in a commercial wine yeast resulted in wine and distillates with substantially greater ester concentrations (Lilly et al. 2000). In similar experiments on beer fermentations, *ATF1* and *ATF2* were overexpressed in a commercial brewer's strain (Verstrepen et al. 2003c, d). The pilot-scale beers produced with an *ATF1*-overexpressing strain contained 5 times more acetate esters than the beers produced with the wild strain. Overexpression of *ATF2* led to smaller increases in isoamyl acetate formation and no significant change in ethyl acetate levels (Verstrepen et al. 2003c). More detailed analysis using gas chromatography coupled with mass spectrometry revealed that *ATF1* and *ATF2* are capable of esterifying a broad range of different alcohol substrates (Verstrepen et al. 2003d). While most of the esters produced have not been studied intensively, it is possible that these lesser known esters also contribute to the aroma of various fermented beverages.

The work just described indicates that it is possible to use genetic modification to create new yeast strains with desirable characteristics for ester production. In addition, the highly elevated ester levels obtained using these strains clearly indicate that ester synthesis during brewery fermentations is not strictly limited by substrate availability. Indeed, it can be concluded that it is not the substrate concentration, but rather the expression level of the *ATF* genes that is one of the main controlling factors affecting ester synthesis during wort fermentations, as first suggested by Yoshioka and Hashimoto (1981, 1984) and later elaborated by Malcorps et al. (1991).

Genetic engineering can also be used to generate yeast strains with enhanced capacity to liberate volatile flavour compounds from non-volatile precursor molecules that are present in wort or must. A classic example of such precursors is the monoterpenyl glucosides, which release terpenols (e.g. geraniol and nerol) when the glucose residue is cleaved off by glucosidases. However, the glucosidases present in yeasts and grapes are repressed by glucose and are unstable at the pH of wort and must. Expression of other, more active and/or more stable glucosidases, such as the recently described β-glucosidase from *Aspergillus oryzae* (Riou et al. 1998) or glucosidases found in *Candida*, *Hanseniaspora* and *Pichia* species, may increase the varietal aroma (Canal-Llauberes 1993). Another possibility is the use of GM yeasts with alcohol oxidase activity, which can convert fusel alcohols into aldehydes with specific aroma properties (Vanderhaegen et al. 2003).

Another potential application of genetic engineering to construct wine yeasts with increased capacity to release flavour-active molecules from grape juice comes from research showing that carbon–sulfur lyase enzymes release the volatile thiol 4-mercapto-4-methylpentan-2-one (4MMP) from its non-volatile precursors (Tominaga et al. 1995). In subsequent work using deletion strains of *S. cerevisiae* with carbon–sulfur lyase genes knocked out, four genes encoding enzymes capable of releasing 4-MMP in wine fermentations were identified (Howell et al. 2005). In related work it was also shown that Atf1p releases another flavour-active volatile thiol, 3-mercaptohexan-1-ol, from its precursor (Swiegers et al. 2005). With this knowledge it should now be possible to construct yeasts that express carbon–sulfur lyase genes and *ATF1* in a highly regulated manner, thus controlling the amount of flavour-active volatile thiols present in wine. Such yeasts would be of enormous interest to winemakers, brewers and whiskey producers.

13.2.7 Engineering Yeasts with Altered Levels of Ethanol and Glycerol Production

Glycerol, an abundant by-product of fermentations performed by *S. cerevisiae*, is implicated in the mouthfeel, viscosity, perceived sweetness and 'roundness' of wine and beer (Klopper et al. 1986; Scanes et al. 1998). Several attempts have therefore been made to increase glycerol production. Another reason for re-routing glycolysis to glycerol is the expected accompanying decrease in alcohol production, which would be beneficial in the production of low-alcohol beers and wines. Such an approach would be an alternative to current methods of alcohol reduction, which include dialysis, dilution and evaporation, all of which are detrimental to sensory quality. Conversely, decreasing glycerol formation and increasing ethanol production may be useful for producing high-alcohol beverages, such as whiskey, brandy and gin, as well as for the production of bio-ethanol. However, since glycerol serves as an osmoregulator and also has a role in maintaining redox balance (Hohmann 1997; Taherzadeh et al. 2002; White et al. 2003), tampering with glycerol production will probably have its limitations.

Glycerol is an important by-product of glycolysis. About 4–10% of all fermented carbon sources are converted into glycerol, resulting in levels that are about 7–10% those of ethanol (Scanes et al. 1998). Glycerol is produced by the conversion of the glycolytic intermediate dihydroxyacetone phosphate into glycerol 3-phosphate by glycerol 3-phosphate dehydrogenase, of which there are two isozymes, *Gpd1* and *Gpd2*. This step is followed by glycerol 3-phosphatase-driven dephosphorylation. Once produced, glycerol can exit the cell by passive diffusion or facilitated transport via Fps1 protein channels. The rate-limiting steps in glycerol production are the conversion of dihydroxyacetone phosphate into glycerol 3-phosphate and the export of glycerol into the medium (Remize et al. 2001).

Overexpression of *GPD1*, *GPD2* or *FPS1* (Fig. 13.9) can result in as much as a 2% (v/v) reduction in ethanol and a 1.5–4-fold increase in glycerol production, depending on the genetic background of the yeast strains used (Michnick et al. 1997; Remize et al. 2001; de Barros Lopes et al. 2000; Eglinton et al. 2002). However, as a consequence of glycerol overproduction and the resultant changes in redox balance, the formation of other secondary metabolites changed significantly, with increased formation of pyruvate, acetate, butanediol and succinate (Remize et al. 1999; de Barros Lopes et al. 2000; Eglinton et al. 2002). The most negative of these consequences, the increased formation of acetate, can be circumvented, however, by deletion of *ALD6*, which encodes cytosolic $NADP^+$-dependent acetaldehyde dehydrogenase (Eglinton et al. 2002) (Fig. 13.9).

These approaches for diverting carbons away from ethanol production are self-cloning strategies (as long as construction of the *ALD6* deletion does not leave any foreign DNA behind), and this will probably be advantageous for consumer acceptance. However, there is a limit to how much carbon can be diverted to glycerol production without having detrimental effects on cell physiology, which would inevitably lead to problems in industrial applications. An additional strategy which holds great promise for reducing ethanol levels in fermentation products involves constructing transgenic yeasts that carry the glucose oxidase gene, *GOX1*, from

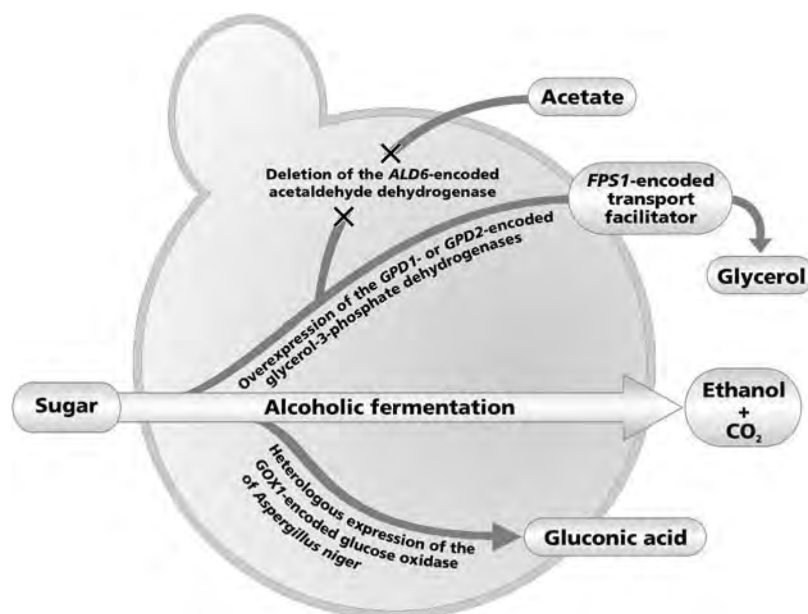

Fig. 13.9. Diverting metabolic processes in yeasts can be used to change the chemical composition of alcoholic beverages. Illustration of how carbons can be diverted from ethanol production to the synthesis of glycerol or gluconic acid. This is achieved by overexpressing native yeast glycerol synthesis genes, *GPD1* or *GPD2*, or introducing a heterologous glucose oxidase (*GOX1*) gene. Overexpressing *FPS1*, the yeast gene for transport of glycerol across the plasma membrane, also helps divert carbons away from ethanol production by facilitating removal of glycerol from the cell. One of the problems of increasing glycerol synthesis is that acetate levels are also increased. This can be ameliorated, however, by deleting the *S. cerevisiae* aldehyde dehydrogenase gene *ALD6*

A. niger. This enzyme converts glucose to gluconic acid, thus diverting carbons away from ethanol production, and it has been demonstrated to reduce ethanol levels by up to 2% in a microvinification trial using a *GOX1*-transformed *S. cerevisiae* strain (Malherbe et al. 2003). While this strain was transgenic, the *GOX1* gene has GRAS status and should therefore not be of great concern to the general community once there is greater acceptance of GMOs in foods and beverages.

13.2.8 Engineering Yeasts to Control Acid Levels in Wine

Acid levels are sometimes too high or too low in wines immediately after fermentation of grape juice. Deacidification processes, performed largely by lactic acid bacteria, are therefore necessary to make some wines more palatable, whereas in others, acids have to be added. In the following sections we will consider how yeasts might be engineered to deal with these problems.

13.2.8.1 Constructing Yeasts that Perform Malolactic Fermentation

Wine producers rely on a secondary fermentation by lactic acid bacteria such as *Lactobacillus* spp., *Pediococcus* spp., and, most importantly, *Oenococcus oeni* (formerly *Leuconostoc*) to convert the very sour and tart malate, found in wine at the end of the primary fermentation, into lactate. This malolactic fermentation is needed for the deacidification and stabilisation of many wines. However, it is difficult to control and delayed or stuck fermentations lead to economic losses and logistic problems. To circumvent the need for secondary malolactic fermentation, *S. cerevisiae* strains that convert malate into lactic acid and carbon dioxide have been constructed (Fig. 13.10). This was achieved by expressing the malolactic enzymes of *Lactococcus lactis* (Ansanay et al. 1993, 1996; Denayrolles et al. 1994, 1995), *Lactobacillus delbrueckii* (Williams et al. 1984) and *Oenococcus oeni* (Labarre et al. 1996) in yeasts. Since *S. cerevisiae* does not have an efficient system for malate uptake, the malate permease of *Schizosaccharomyces pombe* has also been coexpressed with the bacterial malolactic enzyme (Bony et al. 1997a; Volschenk et al. 1997) (Fig. 13.10). This led to the conversion of up to 7 g of malate per litre in 4 days, without significantly affecting the ongoing primary fermentation (Bony et al. 1997a). However, a further

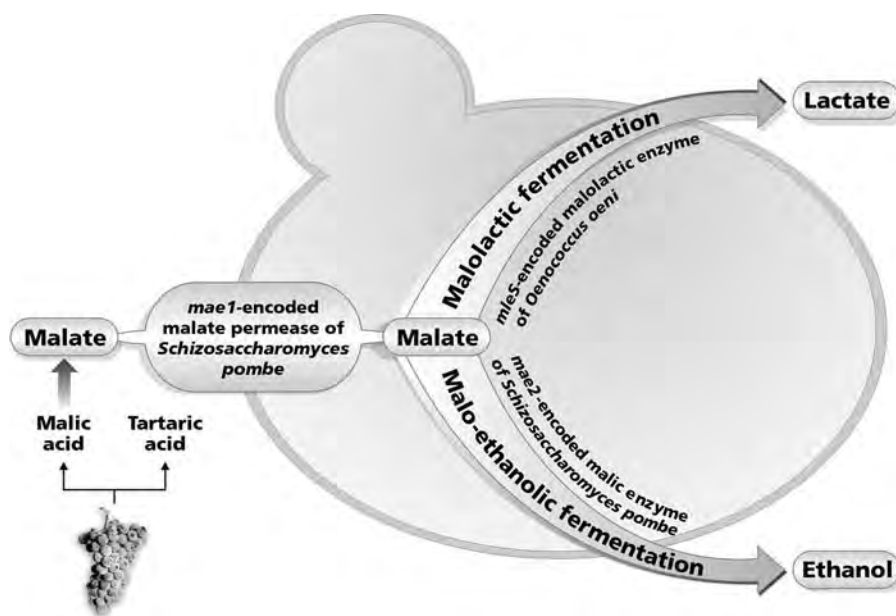

Fig. 13.10. Grape juice often has high levels of malate and this makes wines very acidic and sour to taste. Wine yeasts are unable to ferment this organic acid and winemakers therefore have to rely on bacteria that can convert malate to the less acidic lactate. A strain of *S. cerevisiae* has been constructed that carries a gene for a malate transport protein and genes for the enzymes necessary for malolactate fermentation, thus negating the need for bacterial inputs. The genes that drive this process in the genetically modified *S. cerevisiae* are from a very distant relative of wine yeasts, namely *Schizosaccharomyces pombe* and from the lactic acid bactarium *Oenococcus oeni*

large-scale evaluation is needed to determine the effectiveness of this yeast under industrial conditions and to test the sensory properties of the resultant wine.

13.2.8.2 Constructing Yeasts that Produce Increased Levels of Lactic Acid

A good balance of sweetness and acidity is crucial for wine. In hot climates, grape must often contains low levels of acids and, to correct this, other acids (e.g. tartaric acid) can be added. However, artificial acidification is an added cost to the winemaker and is not always allowed. Attempts have therefore been made to create yeast strains that produce increased levels of lactate from sugar metabolism. This has been achieved by expression of the *Lactobacillus casei* lactate dehydrogenase encoding gene *LDH* (Dequin and Barre 1994; Dequin et al. 1999). The transformants performed a dual fermentation: the normal ethanol-producing, as well as lactic acid-producing fermentations. The strains produced around 5 g L^{-1} lactate, which corresponds to a decrease in pH of around 0.25 units (Dequin et al. 1999). Since part of the alcoholic fermentation is diverted to lactate fermentation, alcohol levels in the wines are about 0.25% (v/v) lower. This is generally not a problem however, because many of the wines produced in hot climates contain high levels of ethanol. Technically, it should be possible to develop a set of yeast strains that produce different amounts of lactic acid, depending on the acid level of the must. In addition, these acid-producing yeasts may be useful in the production of sourdough bread (Dequin 2001).

13.2.8.3 Constructing Yeasts that Produce Low Levels of Volatile Acidity

Acetic acid contributes to volatile acidity in wines, leading to harsh off-flavours and should be kept to below 0.8 g L^{-1}. *S. cerevisiae* forms acetic acid during alcoholic fermentation. While the mechanisms involved in regulating its production remain unknown, it has been shown that the levels of this acid can be controlled by changing the activity of the acetaldehyde dehydrogenase gene *ALD6* (Remize et al. 2000) (Fig. 13.9). Inactivation of both copies of this gene in a diploid wine yeast led to a significant decrease in acetate production during wine fermentation.

In work on sake yeast, a spontaneous mutant with low NADH dehydrogenase activity was selected (Kurita et al. 2003). This mutant showed a 15-fold increase in acetate formation, resulting in a more favourable balance between acetate and lactic acid in the sake wines produced. However, the mutant strains produced altered levels of other secondary metabolites, such as glycerol, which was not desirable. An approach based on the genetic engineering of a target solely involved in acetate formation may help to avoid these undesirable effects.

13.2.9 Engineering Yeasts to Produce Decreased Levels of Hydrogen Sulfide

Hydrogen sulfide is a by-product of sulfur metabolism in yeasts, and with its characteristic 'rotten eggs' aroma, it is highly undesirable in fermented beverages. It is formed by the reduction of sulfate during methionine synthesis. Most of the hydrogen sulfide

secreted during primary fermentation is removed from wine by yeast cells during a maturation phase. However, the need for this time-consuming process could be eliminated or the time required greatly reduced by using yeast strains that have been genetically altered to release less of this compound. This can be achieved by increasing the rate of reactions that consume hydrogen sulfide, including the final steps in methionine, cysteine and homocysteine production. Increased expression of the cystathione β-synthase-encoding gene, *NHS5*, in brewer's yeast has been shown to decrease hydrogen sulfide accumulation without affecting other fermentation parameters (Tezuka et al. 1992). Similarly, overexpression of *MET25*, which encodes *O*-acetyl homoserine sulfhydrolase/*O*-acetyl serine sulfhydrolase, resulted in a tenfold decrease in hydrogen sulfide formation in pilot-scale beer fermentation (Omura et al. 1995).

Another method to decrease hydrogen sulfide production is to reduce activity of the methionine, cysteine and homocysteine synthesis pathway, thus reducing the formation of hydrogen sulfide. Elimination of *MET10*, for example, leads to a significant reduction in hydrogen sulfide formation, and an increase in the formation of sulfite, a desirable antioxidant (Hansen and Kielland-Brandt 1996b). However, as the maximum concentration of sulfite in wine is legally limited, care needs to be taken not to exceed this level.

13.2.10 Engineering Yeasts to Make Alcoholic Beverages with Increased Storage (Antioxidative) Potential

As mentioned previously, sulfite is an important natural antioxidant in fermented beverages but it also acts as a flavour-stabiliser because it binds to flavour-active aldehydes, forming stable products with less intense flavours and higher flavour thresholds. Limited amounts of sulfite are sometimes added to white wines to stabilise and protect them from oxidation. Genetic modification can be used to produce yeasts with modified sulfite production, eliminating the need for addition of this preservative. Overexpression of the *MET3* and *MET14* genes, which are involved in the biosynthesis of sulfur-containing amino acids, leads to increased sulfite production in a brewer's yeast (Korch et al. 1991). Alternatively, reduction of the copy number of *MET2*, encoding serine acetyltransferase, also increases sulfite production (Hansen and Kielland-Brandt 1996a). Another strategy that has been tested is the inactivation of *MET10*, which not only led to increased sulfite levels, but also reduced the production of hydrogen sulfite (Hansen and Kielland-Brandt 1996b).

In contrast, some industrial yeast strains produce concentrations of sulfite that are too high, leading to off-flavours and posing possible health concerns for consumers (Rauhut 1993). Presumably decreased sulfite levels could be achieved by using the inverse of the strategies just described.

13.2.11 Engineering Yeasts for Decreased Formation of Ethyl Carbamate

Ethyl carbamate is a suspected carcinogen found in most fermented beverages (Ough 1976) and is thought to be produced from urea and ethanol during storage. Levels of ethyl carbamate increase with elevated storage temperatures and with higher ethanol levels, and can therefore be particularly problematic in fortified wines

and distilled beverages. In *S. cerevisiae*, urea is formed by the *CAR1*-encoded arginase, which is responsible for the conversion of L-arginine into L-ornithine, ammonia and carbon dioxide. Urea is an intermediate in this conversion, and is partially secreted into the medium. Commercial enzyme preparations of acidic urease can be used to hydrolyse this (Ough and Trioli 1988), but having access to yeast strains that produce less urea or strains that produce urease would be highly desirable.

Disruption of the *CAR1* gene in a sake yeast proved to be successful in reducing urea and ethyl carbamate production (Kitamoto et al. 1991), but this strain could not metabolise arginine, resulting in a growth defect that would limit its potential for industrial applications. Expression of the *Lactobacillus fermentum* urease operon was trialled in yeast and, while expression levels of the urease subunits was sufficient it was not able to break down urease, presumably because essential auxiliary proteins were missing (Visser et al. 1997). It therefore seems accessory genes of *Lactobacillus fermentum* would have to be co-expressed with the urease operon in order to create a functional, secreted urease complex.

13.3 GM Industrial Yeasts of the Future

From Sect. 13.2 it is clear that there is enormous potential for the application of genetic engineering to construct yeasts with improved performance, and with the capacity to deliver fermentation products that have improved qualities. The following section looks at what we are moving into with respect to the application of DNA technologies for improving industrial yeasts, and challenges that lie ahead with respect to legislation and public perception of GMOs in food and beverage production.

13.3.1 The Latest Generation of GM Yeasts

The development of several dominant selectable markers (Goldstein and McCusker 1999), as well as gene knockout cassettes that can be removed following gene disruption (Güldener et al. 1996), has enabled researchers to sequentially introduce multiple genetic alterations into a single yeast genome. Perhaps the best example of this has been the development of yeast strains capable of efficient starch and cellulose fermentation, which requires the simultaneous expression of several heterologous genes (for reviews, see Moraes et al. 1995; Petersen et al. 1998; van Rensburg et al. 1998; Zaldivar et al. 2001; Altinas et al. 2002; Lynd et al. 2002; Eksteen et al. 2003). Another remarkable example is the development of a yeast strain capable of producing the steroid hydrocortisone from a simple carbon source (Szczebara et al. 2003). An artificial and fully self-sufficient biosynthetic pathway involving as many as 13 engineered genes was assembled and expressed in a single yeast strain, and endogenous sterol biosynthesis was re-routed to produce compatible sterols to serve as substrates for the heterologous pathway. Biosynthesis involved the expression of eight mammalian genes and the disruption of unwanted side reactions associated with the *ATF2*, *GCY1*, and *YPR1* gene products.

The trend to target complex phenotypes regulated by several genes does not always require genetic alterations in multiple loci. Instead of engineering a multitude of individual structural genes, researchers can instead manipulate genes encoding

proteins involved in cell signalling pathways or regulation of transcription. Indeed, the improved knowledge of biological pathways and systems as a whole (systems biology) makes it possible to target upstream regulators instead of the multitude of downstream effectors. The advantage of such approaches is that multiple targets involved in common processes can be affected by targeting one or a few genes involved in regulatory networks. This technique also has the potential to lead to more balanced changes in a network of protein activities involved in shaping the same aspect of a phenotype, even if all details of this network are not yet known. An example of this approach is found in the work of Roca et al. (2004). In an already GM, xylose-fermenting *S. cerevisiae* strain, these scientists deleted the *MIG1* and *MIG2* genes, which encode repressors of genes involved in the metabolism of carbon sources such as galactose and maltose. Wild-type *S. cerevisiae* is unable to utilise xylose, but this strain had been engineered to carry and express *Pichia stipitis* XYL1 and XYL2 genes, which encode enzymes for xylose catabolism. The original XYL1/XYL2 strain showed unsatisfactory fermentation of xylose but, after deletion of MIG1 and MIG2, and thereby removal of repression of the metabolism of many sugars, the fermentation rate was increased by 25%.

Modification of cell signalling pathways or transcription regulation will, however, only work for pathways or transcription factors that regulate single or a limited number of cell processes. Downstream branches of regulatory pathways often target several physiological processes, and modification of such a pathway would have pleiotropic, probably undesirable, effects. A good example can be seen in the metabolic engineering of the GAL regulatory pathway (a downstream branch of the main glucose repression pathway). Østergaard et al (2000) eliminated three known negative regulators of this pathway (Gal6, Gal80 and Mig1), and a 41% increase in galactose consumption was obtained. However this increased galactose flux did not lead to the predicted increase in biomass formation, but instead caused excessive fermentative metabolism and increased ethanol production (Østergaard et al. 2000).

A third approach by which complex phenotypes can be improved combines old-fashioned breeding and selection with modern techniques such as genome shuffling, stimulation of mutation and/or random insertion or deletion of genetic elements, e.g. the random insertion of strong, constitutive promoter elements into the yeast genome, which can be accomplished using the Ty yeast transposons (Lassner and McElroy 2002; Patnaik et al. 2002; Petri and Schmidt-Dannert 2004; Giudici et al. 2005). These methods have the advantage that no knowledge about underlying cellular biology is needed to improve traits. A large pool of yeast cells is transformed, and the few transformants that show beneficial alterations are selected from the pool. The desired phenotypic trait can be enhanced by repeated rounds of mutation and selection. A great disadvantage of these 'random' methods, however, is the need for an efficient selection method, which may not be trivial. A second drawback is the 'black-box' character of the method; indeed, nothing is known about the alterations that make the mutants better than their progenitors.

'Functional foods', i.e. foods offering a potential health benefit, is an area of increasing interest in the food and beverage industries, and industrial yeast-driven processes are potential sources of several such products, including resveratrol. Resveratrol is a stilbene produced in grapes during infection, radiation or wounding.

This compound is mainly formed in the grape skins, and acts as a protectant. It attracted scientific attention when it appeared to provide an explanation for the 'French Paradox', i.e. the low incidence of heart disease among French people who combine a relatively high-fat diet with the regular consumption of wine; it is thought to have cardioprotectant and anticancer properties (Gao et al. 2002). In order to create a yeast capable of resveratrol synthesis, the co-enzyme A (CoA) ligase encoding gene (*4CL216*) from a hybrid poplar and the grapevine resveratrol synthase gene (*vst1*) were co-expressed in a laboratory yeast (Becker et al. 2003). Expression of *4CL216* enabled the yeast cells to synthesise *p*-coumaroyl-CoA. This compound and 3-malonyl-CoA produced by the yeast were then able to be used by resveratrol synthase to produce resveratrol. While the concentration of resveratrol produced by this recombinant yeast was low compared with levels found in standard red and even white wines, the study proved it was possible to genetically engineer yeasts to produce this important health-promoting substance.

Another example of engineering yeasts to obtain a healthier product for consumers is the construction of a sucrose-fermenting baker's yeast that is incapable of fermenting fructooligosaccharides. Fructooligosaccharides are regarded as functional foods because they stimulate the growth of favourable intestinal bifidobacteria. This carbohydrate would therefore be a useful additive to foods such as bread, but such an approach would be pointless because it is hydrolysed by invertase, secreted by baker's yeast during leavening. However, yeast strains lacking the *SUC* genes, which encode invertase, and overexpressing *MAL* genes, which encode proteins involved in maltose utilisation, still ferment sucrose, but are unable to hydrolyse fructooligosaccharides, so this functional food could be added and retained in bread if recombinant yeast is used (Oda and Ouchi 1991).

13.3.2 Future Challenges: Legislation, Marketing and Consumer Perception

The first GM yeast strains approved for use in food production were a baker's yeast that constitutively expressed maltose permease and maltase genes, resulting in an increased leavening capacity, and a brewer's strain that expressed a glucoamylase gene allowing the partial fermentation of dextrins (Hammond 1995; Walker 1998). However, it is interesting to note that although GM yeasts have been used to create enzymes and additives used in food production, and that *S. cerevisiae* had been granted GRAS status, no GM yeast strain is currently used for food production. This can be attributed to two factors: the complex regulations for the use of GMOs in food production and, more importantly, today's negative consumer perception of genetic modification.

13.3.2.1 Legislation and Its Impacts on the Use of GM Yeasts

Before the early 1990s, there were virtually no regulations on the use of GMOs in food production. Since that time things have changed dramatically. Research scientists and biotechnology companies began to seriously explore the possibilities offered by the newly developed genetic tools and this quickly prompted countries to

develop new guidelines and laws regulating the use of GMOs in food production. But these laws vary greatly from country to country.

In the USA, the FDA issued its 'statement of policy regarding foods derived from novel plant varieties', which also comprises plants that have undergone genetic modification. This statement provides the basis for the FDA's assessment of all GMOs used in food production. The FDA's basic policy with respect to bioengineered foods is that they are not inherently different from other foods: each product is examined on a case-by-case basis for safety and quality (Foramanek 2001). Tests for conventional food and food produced with, or containing, GMOs are similar and include the investigation of the nutritional value and allergenic properties. Moreover, the FDA does not require producers to indicate if their products contain or have been produced using GMOs or GM products. However, in response to pressure from consumer organisations, the FDA has issued guidelines for voluntary labelling that indicates whether foods have or have not been developed using bioengineering. Apart from the FDA, which controls food safety, GMOs such as plants that are released into the environment also need to comply with standards set by the US Department of Agriculture (USDA) and the Environmental Protection Agency (EPA). This ensures the protection of the environment from GMOs that could potentially harm the ecosystem.

In Europe, things are more restrictive. As in the USA, all novel foods are subjected to stringent tests to give assurance of their safety. This 'Novel Food Regulation' or '258/97/EC' is comparable to the FDA's policy in the USA. It is important to note that these regulations apply to all novel foods, not only foods produced with, or containing, GMOs. In addition, the European Union has issued several guidelines, directives and regulations that specifically regulate the use of GMOs in food production. The directive 90/219/EC and its amendment, 98/81/EC, provide the basis for the contained use of GM microorganisms in food production, while 90/220/EC and 2001/18/EC regulate the deliberate release of GMOs into the environment. These regulations basically define precautions, procedures and tests required to permit the use of a GMO for industrial production. Regulation 1829/2003 also stipulates that all food containing, or derived from, GMOs should be tested in order to guarantee its safety for the consumer. Other directives ensure that the GMOs are not harmful for the ecosystem. Finally, 1139/98/EC, 49/2000/EC, 1829/2003/EC, 1830/2003/EC and 641/2004/EC oblige the producer to indicate on the label if a product contains or is derived from a GMO. This is a very important directive, as many companies are afraid that a GM-label may scare consumers, which partially explains why at present hardly any GM-derived food products can be found on the shelves of European supermarkets. Directive 1830/2003/EC also states that producers working with GMOs should assure the traceability of their products, meaning they should keep track of who buys and sells them.

In general, regulations in other parts of the world lie between those of the USA and the European Union. Canada's policies resemble those in the USA. Australia, New Zealand, Japan and China require labelling of GM foods, but the regulations on the use of GMOs are less restrictive than the European guidelines. However, laws concerning the use of GMOs are informed by new scientific findings, public opinion and lobby groups, and therefore are always open to review. It is difficult to predict how the GMO legislation will evolve.

Despite the differences in regulations between different countries there are general guidelines for approval of GMO-derived products. Approval usually requires a few obvious guarantees. For example, the inserted or altered DNA sequence should be fully defined, the GMO should not have any selective advantage that would favour its survival and/or proliferation in nature, and the GMO should not pose any risks to consumers or the environment. A common way for producers to satisfy these demands is to prove that the GMO and/or the derived product is 'substantially equivalent' to analogous conventional organisms or products (Cockburn 2002). When substantial equivalence is shown, few other tests are necessary to comply with safety regulations. If the GMO or the product is not substantially equivalent, further examination is required, including rigorous chemical analyses and animal tests. In the specific case of a yeast used in a contained industrial environment and modified to better suit the producer's and consumer's needs, the risks are very low. In many cases, obtaining a licence for these GM yeasts may be merely a matter of applying and providing the necessary data.

Special, more flexible regulations have been developed for GMOs that contain only 'self-DNA', i.e. DNA sequences that also occur in the respective wild strain. In Japan and Europe 'self-cloning' strains are exempt from the strict regulations governing the use of GMOs, including the important labelling requirement. Many of the possible applications of genetic engineering for the improvement of industrial yeasts are realisable without the introduction of heterologous DNA, especially when some of the recent technologies are used (Ishida-Fujii et al. 1998; Verstrepen et al. 2001b; Akada 2002; Aritomi et al. 2004).

13.3.2.2 Public Perception

Despite complex and strict legislation, the real limiting factor for the introduction of GMO-derived foods and beverages lies with the consumer. A significant proportion of today's consumers are extremely suspicious of the application of gene technology in food production. Many consumers believe GM-derived food may cause long-term health problems, such as cancer. From a scientific point of view, these reservations are not justified (Pretorius 2000; Miles and Frewer 2001; Finucane 2002). There is no reason to suspect that consumption of a GM-derived food could affect the consumer any differently from consumption of a conventional food. Other fears, such as the concern that GMOs could disturb the ecosystem or affect biodiversity, are more realistic (Miles and Frewer 2001; Freckleton et al. 2003). It is precisely for this reason that rigorous GMO legislation was set up. In many cases, there is no reason to expect a problem in this area because the GMOs and their products are subjected to elaborate, extensive testing. In the specific case of industrial yeasts, it has to be highlighted that most of these organisms have been selected for use in an artificial environment, often with copious amounts of nutrients and optimal growth temperatures. Many of these industrial strains therefore cannot compete with feral strains, limiting the threat they pose on the ecosystem. Moreover, most of the traits that are introduced to make these strains better performers for their industrial tasks do not offer any competitive advantage in the 'real world'. In addition, it should be highlighted that relying on traditional

approaches rather than utilising GM yeasts has its consequences; in many cases traditional methods, such as the use of additives and enzymes, can lead to increased costs, additional waste products and higher energy consumption during production process.

What factors need to be addressed so that GMOs can fully realise their great promise? It is clear consumers need to be better informed about the genetic techniques used to create GMOs and the scientific foundation on which gene technology relies. This would arm the general public against the often misleading, biased and in some cases blatantly incorrect information provided by some consumer organisations and pressure groups. GMO legislation should be based on rigorous scientific data and aim solely to protect consumers and the environment, not the interests of pressure groups. Producers, on the other hand, must not take risks, must comply with regulations and should always provide complete and honest information on their products. GM foods and beverages should be designed to deliver benefits for consumers and/or the environment by offering improved products for similar prices, by reducing the price of equivalent products, and/or introducing products with reduced negative impact on the environment. In summary, consumers need to be provided with good information, allowing them to assess correctly the risks and benefits of the use of GMOs (Pretorius and Bauer 2002; Rowe 2004), and GM products must deliver benefits compared with traditional foods and beverages.

13.4 General Conclusion

It is clear that the industrial application of GM yeasts has many potential benefits with exciting possibilities in the bio-ethanol and pharmaceutical industries, as well as providing some potentially huge leaps forward for the traditional food and beverage industries. Biotechnologists in these latter industries would do well to investigate the potential for modifications that can lead to health benefits while ensuring environmental risks are nullified, and without transgressing current laws and regulations.

Community concern over the use of GM products is apparently a great deal less intense for applications in the pharmaceutical and bio-ethanol industries than it is for food and beverage production; ironically we already use GM yeast to express pharmaceutical agents such as vaccines that are injected into us. Consumer information and education about the use of GM yeasts in pharmaceutical and bio-ethanol industries may therefore provide a stepping stone for the introduction of GM yeasts in the food and beverage worlds. The use of self-cloning yeasts may further reduce concerns of consumers, and it may be that such constructs will be the first to find their way into foods and beverage production.

In conclusion then, we can now access and modify the yeast genome in amazingly precise and innovative ways and we are able to tailor this microbe for an increasing array of applications, making it more and more indispensable to us. Thus, our good friend the yeast continues to offer new possibilities for us to increase its usefulness and consequently our dependence on it. Which brings us back to where we started this chapter: who, exactly, is moulding whom?

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