Andriy A. Sibirny *Editor*

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Preface

The reader is referred to the new Springer book *Biotechnology of Yeasts and Filamentous Fungi*. The proposed topic is quite important as biotechnology of yeasts and filamentous fungi is apparently the largest branch of biotechnology in terms of annual sales. It is enough to tell that yeast is responsible for the production of nearly 100 billion liters of fuel ethanol per year and much more of alcoholic beverages. Fungal antibiotics have the largest market among all anti-infectives. A huge market relies on the traditional use of *S. cerevisiae* for baking. Yeasts and fungi are responsible for production of glycerol, lipids, organic acids, vitamins, and other low-molecular-weight compounds as well as enzymes. There are several popular yeast platforms for expression of heterologous proteins of pharmaceutical, food, agricultural, and industrial importance, which have a large and steadily growing market. Finally, yeast and fungal enzymes and intact cells are widely used for analytical purposes as components of enzymatic kits and biosensors. This book contains 14 chapters which cover the latest achievements in the traditional and modern use of yeasts and filamentous fungi. The authors are leaders in their research fields. They represent 12 countries from the continents of Europe, Asia, North and South Americas, and Oceania. The reviews describe the history and current state of the particular field of yeast and fungal biotechnology; moreover, perspectives of further development of research and implementation into industry are also discussed. It became clear that new achievements in yeast and fungal biotechnology are possible and they largely depend on the search for new, still unknown species of yeasts and filamentous fungi with some novel features, as well as on the development of the new tools of molecular research.

The first two chapters deal with yeast alcoholic fermentation and describe construction of efficient producers of first- and second-generation ethanol as well as high-temperature alcoholic fermentation. The next two chapters describe modern approaches to the traditional use of yeast for baking, including stress-tolerant yeast and yeast dehydration. Yeast metabolic engineering was developed a 100 years ago by Carl Neuberg and was directed for glucose conversion to glycerol instead of ethanol production. Consequently, one of the chapters of this book describes modern approaches of metabolic engineering of *S. cerevisiae* for anaerobic glycerol production. Yeasts and filamentous fungi are known as the industrial producers of low-molecular-weight metabolites, so the book contains comprehensive reviews

describing using these organisms for production of lipids and carotenoids. It is not surprising that the book also offers chapters on the production of organic acids and riboflavin by native and recombinant strains of yeasts and filamentous fungi. An interesting review is dedicated to psychrophilic yeasts as the source of cold-active enzymes. Methylotrophic yeasts are considered in another chapter as convenient expression platforms and promising industrial producers of recombinant proteins. Finally, three chapters are dedicated to yeast and fungal cells and enzymes or other proteins isolated from these organisms as components of the analytical tools for assaying various chemical substances.

I hope that this book will be useful to biotechnologists, microbiologists, geneticists and biochemists.

Lviv, Ukraine Andriy A. Sibirny

Contents

Genetic Improvement of Conventional and Nonconventional Yeasts for the Production of First- and Second-Generation Ethanol

1

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Abstract

Production of fuel ethanol is one of the largest fields of industrial biotechnology. Currently, most of fuel ethanol is produced from conventional feedstock (first-generation ethanol); however, lignocellulose-derived (second-generation) ethanol has great future. Current review describes achievements in construction of the advanced strains of *Saccharomyces cerevisiae* with increased yield and productivity of ethanol synthesis from glucose. Among producers of second-generation ethanol, review presents data on three species, conventional yeast *S. cerevisiae*, and two nonconventional yeast species: one of the most efficient natural xylose-fermenting yeast *Scheffersomyces stipitis* and the thermotolerant yeast *Ogataea polymorpha*. Improvements of the available strains using methods of rational metabolic engineering and classical selection are discussed.

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Contents

1.1 Introduction

Global energy and environmental problems have stimulated an increase in efforts for biofuels production from renewable resources as a drop in replacements for the currently used fossil fuels such as gasoline produced from oil (Lennartsson et al. 2014). Due to the current political instability in oil-producing nations, and the concerns about global warming (attributed to harmful greenhouse gas emissions), there has been a call for greater reliance on domestic energy sources for the development of economically viable and environmentally friendly biofuels.

Fuel ethanol is a promising renewable liquid transportation fuel that is expected to become a dominant renewable biofuel in the transport sector within the next 20 years (Hahn-Hägerdal et al. 2006). This can be documented by the worldwide exponential in ethanol production during the last decade, reaching 97 billion liters in 2015 (http://www.afdc.energy.gov/data/10331). Ethanol can be blended with gasoline or used as a neat alcohol in dedicated engines, taking advantage of the higher octane number and higher heat of vaporization. Furthermore, it is considered as an excellent fuel for advanced flexi-fuel hybrid vehicles.

The current review summarizes the recent progress in the construction of more robust efficient yeast producers of ethanol from conventional and nonconventional feedstocks or what is referred to as first- and second-generation ethanol (1G, 2G). *Saccharomyces cerevisiae* is considered as the major industrial producer of 1G ethanol. Construction of the producers of 2G ethanol will be described for three organisms: *S. cerevisiae*, one of the best natural xylose fermenters, *Scheffersomyces (Pichia) stipitis*, and the most thermotolerant yeast known that also ferments xylose, *Ogataea (Hansenula) polymorpha*. Each of these organisms has some advantages and drawbacks. *S. cerevisiae* is the primary industrial 1G ethanol producer and is the most ethanol-tolerant natural yeast known. However, this yeast cannot metabolize xylose at all. *S. stipitis* can effectively ferment both glucose and xylose; however, it has much lower ethanol tolerance than *S. cerevisiae* and requires oxygen for growth. *O. polymorpha* can grow and ferment glucose and xylose at temperatures of up to 50 °C and for this reason can be promising for use in the process of simultaneous saccharification and fermentation (SSF). This yeast also shows relatively high ethanol tolerance, but the wild-type strains of *O. polymorpha* produce very low amounts of ethanol from xylose. The drawbacks of all three yeasts that are discussed can be successfully overcome by

combining metabolic and evolutionary engineering in some cases with classical mutation and selection approaches.

1.2 *Saccharomyces cerevisiae*

Today, industrial-scale production of fuel ethanol uses primarily conventional food-based feedstocks such as glucose (derived from starch) and sucrose and is known as the first-generation (1G) ethanol.

The yeast *Saccharomyces cerevisiae* is the organism of choice for the industrial production of ethanol. The production of ethanol by fermentation represents the largest industrial biotechnological application of yeast. *S. cerevisiae* is characterized by several desirable industrial properties which include fast growth, efficient glucose anaerobic metabolism, high ethanol productivity, high yield, and high tolerance to several environmental stress factors, such as high ethanol concentration, low pH, and low oxygen level. In addition to this, yeast is insensitive to bacteriophage infection, which is particularly relevant in large industrial processes that use bacteria as the production microorganism. During alcoholic fermentation, *S. cerevisiae* produces ethanol with a yield close to the theoretical maximum (0.51 g ethanol per g of consumed glucose) (Gombert and van Maris 2015). At industrial scale, ethanol is produced with a yield that is higher than 90% of the theoretical maximum yield possible from glucose (Della-Bianca et al. 2013). Taking into account the large worldwide ethanol production, an increase of ethanol yield of even 1% can provide an additional estimated profit that approaches hundreds of millions of dollars annually.

The yeast *S. cerevisiae* catabolizes glucose via the Embden-Meyerhof-Parnas (EMP) pathway yielding anaerobically of 2 moles ATP per mole of consumed glucose. The efficiency of this pathway for anabolic processes is low with a maximal biomass yield of around 7% and an ethanol yield in the range of 90–93% from the glucose consumed (Ingledew 1999). In contrast to *S. cerevisiae*, the bacterium, *Zymomonas mobilis*, ferments glucose through the Entner-Doudoroff (ED) pathway. This pathway provides only 1 mole of ATP per mole of glucose and consequently directs only 3% of glucose used to biomass production, while the remaining 97% is converted to ethanol at almost the possible theoretical value (Sprenger 1996). When ATP is used for growth, cell biomass is formed at the expense of glucose that is not converted to ethanol. In other words, cell biomass is a by-product that is produced during alcoholic fermentation. Therefore, the lowering of ATP yield during alcoholic fermentation increases ethanol yield with reduced substrate conversion to cell mass. To achieve this goal several strategies can be applied such as (1) substitution of EMP pathway in yeast by ED pathway, (2) activation of energy-consuming plasma membrane sugars symporters, (3) generation of futile cycles, and (4) elevation activity of ATP-degrading enzymes (De Kok et al. 2012). An alternative possibility to increasing ethanol yield during alcoholic fermentation is based on decreasing production of glycerol, which is another major by-product of ethanol production in yeast (Gombert and van Maris 2015).

Notwithstanding the former patent application of Lancashire et al. (1998) which describes the functional integration of the ED pathway to bypass glycolysis in *S. cerevisiae* with positive impact on ethanol yield, more recent work demonstrates the inability to express in this yeast one of the two unique enzymes to the ED pathway, namely, 6-phosphogluconate dehydratase (PGDH) (Benisch and Boles 2014). Several attempts to improve the availability of iron-sulfur clusters in the yeast cells and to attract the iron-sulfur cluster assembly machinery to PGDH protein did not result in improved enzyme activities (Benisch and Boles 2014).

The impact of the disaccharide-proton symport on biomass and ethanol yields was studied in *S. cerevisiae* by comparing anaerobic growth on maltose, which is transported by a maltose-proton symporter and intracellularly hydrolyzed by maltase, to that of glucose (Weusthuis et al. 1993). It was demonstrated that biomass and ethanol yields in anaerobic maltose-limited cultures were 25% lower and 8% higher, respectively, than that of glucose-limited cultures. Relocation of sucrose hydrolysis from the extracellular space to the cytosol with additional evolutionary engineering resulted in a strain that had elevated sucrose uptake kinetics with a 30% decrease in the biomass yield and an 11% increase in the ethanol yield relative to the reference strain. Such evolved strain was characterized by increased transcript level of *AGT1* gene encoded sucrose-proton symporter (Basso et al. 2011). Therefore, in principle the improved kinetics of sucrose transport with concomitant ATP reduction can be achieved by targeted overexpression of *AGT1* or the heterologous genes encoding sucrose-proton symporters from yeasts that naturally hydrolyze sucrose intracellularly (Kaliterna et al. 1995). Replacement of *S. cerevisiae* facilitated diffusion systems by heterologous proton symporters for the other industrially relevant sugars (viz., glucose and fructose) presents an opportunity to explore the energetic impact on the biomass accumulation and ethanol yield from the introduction of such heterologous transporters in *S. cerevisiae* (De Kok et al. 2012).

A futile cycle is a set of biochemical reactions of at least two metabolic pathways that run simultaneously in opposite directions which result in energy dissipation.

Phosphofructokinase is a glycolytic enzyme that phosphorylates fructose 6-phosphate to fructose-1,6-bisphosphate in an ATP-dependent manner. Fructose-1,6-bisphosphatase (FBPase) is one of the gluconeogenesis enzymes. This enzyme hydrolyzes fructose-1,6-bisphosphate to fructose-6-phosphate in an ATP-dependent reaction. The simultaneous action of both enzymes—phosphofructokinase and fructose-1,6-bisphosphatase—leads to a futile cycle generation between fructose-1,6-bisphosphate and fructose-6-phosphate with ATP dissipation. Phosphofructokinase is primarily regulated at the level of activity and the differences in the amount of the enzyme in glycolytic and gluconeogenic conditions do not exceed twofold (Bafuelos and Gancedo 1978; Clifton and Fraenkel 1981). In contrast, the FBPase activity is tightly regulated by catabolic repression, inactivation through ubiquitination, and inhibition by AMP and fructose-2,6-bisphosphate (Navas et al. 1993; Navas and Gancedo 1996). Therefore, intracellular FBPase activity level is maintained at basal level in cells grown in media containing fermentable carbon sources. To overcome the tight regulation of the yeast FBPase,

the bacterial FBPase from *E. coli*, that is, insensitive to fructose-2,6-bisphosphate inhibition, was constitutively expressed in yeast (Navas and Gancedo 1996; Semkiv et al. 2016). The resulting recombinant strain was characterized by a threefold increase of FBPase activity, a 30% reduced intracellular ATP level, and up to 9% increase of ethanol production relative to that of parental strain (Semkiv et al. 2016).

An alternate futile cycle was generated via the simultaneous activation of two enzymes, pyruvate carboxylase and the gluconeogenic enzyme, phosphoenolpyruvate carboxykinase (Semkiv et al. 2016). Pyruvate carboxylase catalyzes the conversion of pyruvate into oxaloacetate in an ATP-dependent reaction, and phosphoenolpyruvate carboxykinase uses ATP energy to convert oxaloacetate into phosphoenolpyruvate. ATP is also synthesized when phosphoenolpyruvate is converted into pyruvate by pyruvate kinase. Therefore, the resulting total loss in ATP is one molecule for each one turn of the cycle. The specific activity of pyruvate carboxylase was increased three- to fivefold via substitution of target gene promoter with a strong constitutive one.

The activity of phosphoenolpyruvate carboxykinase is carefully regulated at the posttranslational level (Wilson and Bhattacharjee 1986). In order to avoid such regulation, a heterologous gene coding for the corresponding enzyme from *E. coli* was overexpressed. As a result, the specific activity of phosphoenolpyruvate carboxykinase was elevated six- to sevenfold. Ethanol production by the constructed recombinant strains revealed a twofold increase over the parental strain by the end of the first day of fermentation (Semkiv et al. 2016).

Potential futile cycle based on the interconversion between glucose and trehalose, which theoretically could lead to ATP dissipation in cells of *S. cerevisiae*, was generated by the activation of trehalose-6-phosphate synthase and the neutral trehalase responsible for the synthesis and degradation of trehalose (Semkiv et al. 2016). Despite the increase in the activities of both enzymes, biomass accumulation remained unchanged.

Lowering of intracellular ATP content in yeast cell with concomitant increase in ethanol yield can also be achieved by the activation of some of the cytosolic ATPases. A decrease in cellular ATP pool and activation of alcoholic fermentation was achieved by the overexpression of the soluble part (F_1) of H^+-ATPase or a portion of F_1 exhibiting ATPase from different origin in *S. cerevisiae* (Jensen et al. 2006). Similar results were obtained after the overexpression of *PHO5* coding for acid phosphatase which is a non-specific enzyme that also hydrolyzes ATP (Rogers and Szostak 1993). Overexpression of the vacuolar alkaline phosphatase Pho8 leads to an increase in yield and ethanol production from glucose in both a laboratory and an industrial strains of *S. cerevisiae* (Fig. 1.1), whereas the expression of the truncated cytosol localized form is detrimental to cell growth (Semkiv et al. 2014).

The galactose-induced expression of ATP hydrolysis region of the ribosome-associated molecular chaperon encoded by gene *SSB1* of *S. cerevisiae* and the ATP diphosphohydrolases also known as apyrases from *E. coli* resulted in an increase in ethanol yield of 39 and 29%, respectively, during fermentation of the corresponding

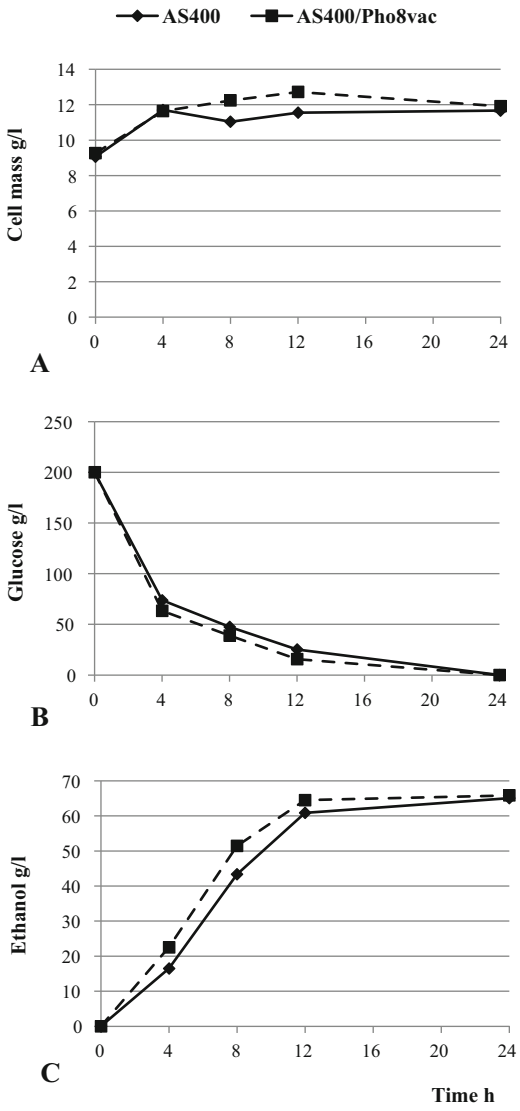

Fig. 1.1 Growth, glucose consumption, and ethanol production during alcoholic fermentation of recombinant strains overexpressing vacuolar form of alkaline phosphatase. Strains were cultured under semianaerobic conditions in YNB medium with 200 g/L glucose. Cultures were incubated at 30 °C, with limited aeration using a gyratory shaker at a setting of 120 rpm. AS400–WT, recipient strain. AS400/Pho8vac—strain containing vector pUC57-delta1_2-ADHpr-PHO8-CYCt-kanMX. (**a**) Biomass accumulation (g of biomass/L of medium); (**b**) glucose consumption (g of glucose/L of medium); (**c**) ethanol production (g of ethanol/L of medium)

recombinant *S. cerevisiae* strains in galactose-containing media (Semkiv et al. 2016).

Glycerol is the second primary by-product after cell biomass during the ethanol production. Glycerol is formed at the expense of sugar that is not converted to ethanol (Gombert and van Maris 2015). In yeast, the reduction of glycerol formation can most probably result in an increase in ethanol yields. Therefore, significant research efforts were directed to reduce glycerol formation during fermentation. This can be accomplished by deletion of one or both genes *GPD1* and *GPD2*,

coding for glycerol-3-phosphate dehydrogenase (Ansell et al. 1997). Deletion of both genes affected anaerobic growth. Deletion of *GPD2* resulted in an increase in ethanol yield with concomitant decrease of glycerol production; however, such a mutation also reduced growth and ethanol productivity (Valadi et al. 1998). Glycerol formation is caused by the oxidation of the excess NADH, which is produced from glycolysis under anaerobic conditions. In order to decrease cytosolic NADH formation, the gene *GDH1*, encoding NADPH-dependent glutamate dehydrogenase, was deleted, while *GLN1* and *GLT1* coding for glutamine synthetase and NAD-dependent glutamate synthase were overexpressed. During ammonium assimilation that is linked with NADH and ATP consumption, a recombinant strain showed a decrease in the glycerol yield to 38%, while ethanol yield was increased by 10% (Nissen et al. 2000). Another approach for reducing the intracellular pool of NADH and ATP production consisted of replacing the glyceraldehyde-3-phosphate dehydrogenase gene by that of the non-phosphorylating heterologous NADP-dependent analog from *Bacillus cereus* or *Streptococcus mutans* (Guo et al. 2011; Zhang et al. 2011). The combination of this approach with the overexpression of NAD-dependent fumarate reductase or acetaldehyde dehydrogenase in both cases increased ethanol yield to 95% of the theoretical maximum (Zhang et al. 2011).

Other work has also demonstrated that the expression of the NAD-dependent acetaldehyde dehydrogenase from an *E. coli* in *gpd1Δ gpd2Δ* strain supports the growth in the medium with glucose supplemented with acetate under anaerobic conditions (Guadalupe Medina et al. 2010). The constructed strains can be used for alcoholic fermentation of acetate-containing substrates, as they are able to convert acetate into ethanol. The main drawback of these *S. cerevisiae* strains is that the reduced glycerol production resulted also in reduced osmotolerance and overall viability (Hohmann 2002). This finding imposes certain restrictions on the application of these strains in industrial fermentation processes that normally run at high concentration of fermented substrate. Significant other research efforts have focused on improving the stress resistance of yeast strains with reduced glycerol production. Osmotolerance, thermotolerance, and tolerance to high concentrations of ethanol of several target strains were elevated by applying methods of metabolic engineering and genomic shuffling (Wang et al. 2012; Hou 2010). The combination of the deletion of the *GPD1* gene with the overexpression of the *nadF* gene from *B. cereus* that encodes for an NADP-dependent glyceraldehyde-3-phosphate dehydrogenase with the derepression of homologous genes of trehalose synthesis *TPS1* and *TPS2* (encoding trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase) resulted in increased ethanol production and reduced glycerol formation but did not exhibit a negative influence on strain viability during alcoholic fermentation (Guo et al. 2011). Trehalose protects cells from stress and its intracellular concentration correlates with resistance to high temperatures and high concentrations of ethanol in the medium (Tao et al. 2012). Overexpression of the *TPS1* gene in *S. cerevisiae* resulted in increased thermotolerance, thereby allowing for the possible reduction in energy costs for cooling of fermentation vessels as well as for savings in energy used for heating due to reduced temperature difference between fermentation and distillation processes (An et al. 2011). Moreover,

high-temperature fermentation has advantages during the process of simultaneous saccharification and fermentation, as high temperature (around 50 °C) is optimal for hydrolytic enzymes involved in substrates saccharification (Shahsavarani et al. 2012).

The development of improved ethanol-producing strains can be achieved by applying traditional selection and adaptive evolution as a useful alternative to metabolic engineering approaches. The mutants that are resistant to toxic concentrations of oxythiamine, trehalose, 3-bromopyruvate, glyoxylic acid, and glucosamine have been isolated. Some of these are characterized by 5–8% increase in ethanol yield when compared to the parental industrial ethanol-producing strain (Table 1.1) (Dmytruk et al. 2016). By applying adaptive evolution, useful yeast strains with enhanced maltose utilization and osmotolerance (Higgins et al. 2001) and increased ethanol tolerance (Stanley et al. 2010) and yeast with improved ethanol production rate and decreased formation of acetate were selected (Cadiere et al. 2011).

In contrast to 1G, the 2G ethanol is an ethanol produced from nonfood feedstocks such as plant biomass. The utilization of lignocellulosic biomass for 2G ethanol production would be preferable over sugar and starch-based 1G ethanol production because of the absence of competition with food production and minimal changes to land use (Sánchez and Cardona 2008; Nigam and Singh 2011). 2G ethanol can utilize a range of different types of lignocellulosic materials substrates. Currently, a limited amount of 2G ethanol is produced at several demonstration plants around the world, but due to higher cost the large-scale ethanol production from lignocellulosics is not yet commercially feasible (Lennartsson et al. 2014).

The technologies to produce 2G ethanol do exist; however, there are many improvements that are needed. The production of 2G ethanol from lignocellulosics will require the development of robust microbial strains that can grow and produce ethanol from at least glucose and xylose, which are the major fermentable sugars produced from the hydrolysis of lignocellulosic biomass. Other sugars that are produce during hydrolysis include the hexoses fructose, mannose, galactose, and the pentose sugar arabinose.

The yeast *S. cerevisiae* efficiently ferments the hexoses glucose, fructose, and mannose but to a lesser extent galactose. In this yeast, galactose transporters are subjected to catabolic repression by glucose thereby limiting co-fermentation of these sugars. In contrast, mannose enters yeast cells using the glucose transport system allowing glucose/mannose co-fermentation to ethanol (Madhavan et al. 2012). The combined deletion of the genes *GAL6*, *GAL80*, and *MIG1* involved in negative regulation of galactose catabolism in a laboratory *S. cerevisiae* strain resulted in a partial co-consumption of glucose and galactose in aerobic batch cultures (Ostergaard et al. 2001). Normally, galactose metabolism in *S. cerevisiae* requires respiration. However, a *cox9Δ gal80Δ* double mutant has been isolated which effectively fermented galactose anaerobically (Quarterman et al. 2016). A natural strain of *S. cerevisiae* (NRRL Y-1528), which catabolizes galactose more effectively than glucose or mannose and able to simultaneous fermentation of all hexose presented in the hydrolyzed biomass was described. This strain may have a

Table 1.1 Growth rate, ethanol production, productivity, specific productivity, and yield of *S. cerevisiae* mutants resistant to oxythiamine, trehalose, bromopyruvate, glyoxylic acid, glucosamine, and initial industrial strain AS400 during alcoholic fermentation on YNB medium supplemented with 20% glucose and corn steep liquor (CSL) medium with hydrolyzed meal indicated in brackets

Strain	Selective agent	Specific growth rate, g $l^{-1}h^{-1}$	Ethanol, g l^{-1}	Ethanol productivity, g $l^{-1}h^{-1}$	Specific ethanol productivity, g g^{-1} biomass h^{-1}	Ethanol yield, g g^{-1} of consumed glucose
AS400	–	0.133 ± 0.003	80.3 ± 1.5 (84.6 ± 1.0)	4.46 ± 0.08 (3.53 ± 0.03)	1.86 ± 0.03	0.402 ± 0.007 (0.423 ± 0.005)
AS400-567	Oxythiamine	0.133 ± 0.004	85.2 ± 1.6 (90.5 ± 0.9)	4.73 ± 0.09 (3.77 ± 0.04)	1.97 ± 0.04	0.426 ± 0.008 (0.453 ± 0.004)
AS400-543	Trehalose	0.083 ± 0.002	84.3 ± 1.6 (88.8 ± 0.9)	4.68 ± 0.09 (3.70 ± 0.03)	2.75 ± 0.06	0.422 ± 0.008 (0.444 ± 0.005)
AS400-617	Bromopyruvate	0.133 ± 0.003	84.1 ± 1.5 (89.7 ± 1.0)	4.67 ± 0.09 (3.74 ± 0.03)	1.95 ± 0.03	0.421 ± 0.007 (0.449 ± 0.005)
AS400-510	Glyoxylic acid	0.133 ± 0.004	85.1 ± 1.6 (91.4 ± 0.9)	4.73 ± 0.09 (3.81 ± 0.03)	1.97 ± 0.04	0.426 ± 0.007 (0.457 ± 0.005)
AS400-128	Glucosamine	0.117 ± 0.003	84.6 ± 1.7 (89.6 ± 0.9)	4.70 ± 0.09 (3.73 ± 0.04)	2.24 ± 0.04	0.423 ± 0.008 (0.448 ± 0.005)
AS400-510-42	Glyoxylic acid, glucosamine	0.133 ± 0.004	86.7 ± 1.7 (93.0 ± 0.9)	4.82 ± 0.09 (3.88 ± 0.03)	2.01 ± 0.04	0.434 ± 0.008 (0.465 ± 0.004)
AS400-510-42-214	Glyoxylic acid, glucosamine, bromopyruvate	0.117 ± 0.003	88.4 ± 1.5 (94.8 ± 1.0)	4.91 ± 0.08 (3.95 ± 0.04)	2.34 ± 0.04	0.442 ± 0.008 (0.474 ± 0.005)

(±)—absolute error

For alcoholic fermentation, cells of AS400 strain and its derivatives were grown overnight in 100 mL of YPD medium in 300 mL Erlenmeyer flasks and then used to inoculate 20 mL aliquots of mineral YNB medium supplemented with 200 g/L glucose or CSL medium supplemented with hydrolyzed meal in 50 mL Erlenmeyer flasks. An initial biomass concentration of 8 g (dry weight)/L was used for fermentation in YNB medium. For CSL medium, an initial biomass concentration of 10 g (dry weight)/L was used. Fermentation was carried out at a temperature of 34 °C with limited aeration using a gyratory shaker at a setting of 120 rpm. Samples were taken every 3 h for YNB medium or 12 h for CSL medium

defect in carbon catabolic repression which alleviates glucose repression of galactose utilization (Keating et al. 2004).

Wild-type strains of *S. cerevisiae* are unable to catabolize and ferment pentoses (xylose or arabinose) which are major constituents of hydrolysates of plant biomass. This physiological feature of *S. cerevisiae* is due to the lack of enzymes that catalyze the initial stages of the pentose catabolism. Since the xylose content in the plant biomass hydrolysates is significantly higher than that of arabinose, most of the work has been directed to the construction of *S. cerevisiae* strains which are capable of xylose fermentation. These efforts have focused on the functional expression of heterologous genes for xylose catabolism. Some efforts have focused on the expression of the gene encoding xylose isomerase (XI) from different microbial origins. This enzyme does not require cofactors and catalyzes the isomerization of xylose in xylulose (Fig. 1.2). Several successful attempts to express XI from the bacteria *Thermus thermophilus* (Walfridsson et al. 1996), *Clostridium phytofermentans* (Brat et al. 2009), and *Bacteroides stercoris* (Ha et al. 2011) or from the anaerobic fungus *Piromyces* sp. E2 (Karhumaa et al. 2007) or from *Orpinomyces* sp. (Madhavan et al. 2009) were performed. Expression of the codons optimized XI of *C. phytofermentans* in *S. cerevisiae* resulted in a 46% increase in specific growth rate on xylose as compared to the strain expressing a non-optimized version of the gene (Brat et al. 2009).

Other research effort have focused on the expression of genes encoding xylose reductase (XR) and xylitol dehydrogenase (XDH), which catalyze the conversion of xylose into xylitol and subsequently to xylulose (Fig. 1.2). XR is encoded by gene *XYL1* and requires the cofactors NADH or NADPH with higher affinity to NADPH. XDH is an NAD-dependent enzyme that is encoded by *XYL2*. The co-expression of both genes results in an imbalance of cofactors with a deficiency in NAD and an excess of NADP. It is assumed that this imbalance causes the low efficiency of xylose alcoholic fermentation, primarily due to the xylitol accumulation (Krahulec et al. 2012). A genome-wide scale model of *S. cerevisiae* was used to predict the maximal achievable growth rate for cofactor-balanced xylose utilization pathway. By applying the use of dynamic modeling with experimental results, it was shown that a balanced cofactor system XR/XDH stimulated an increase in ethanol batch production of 24.7% while simultaneously reducing the predicted substrate utilization time by up to 70% (Ghosh et al. 2011). The combination of computational design (Khoury et al. 2009) and site-specific mutagenesis of domains responsible for binding these cofactors (Watanabe et al. 2005) resulted in a modification of the cofactor specificity of XR and XDH and for balanced action. The co-expression of NADH-dependent mutant forms of XR from *S. stipitis* (K270M, K270R, K270R/N272D, N272D/P275Q, R276H) in part with a native NAD-dependent XDH, increased productivity and ethanol yield with concomitant reduction of xylitol yield in recombinant strains of *S. cerevisiae* (Bengtsson et al. 2009; Lee et al. 2012). A decrease in xylitol yield and an increase in ethanol yield were also reported as a result of co-expression of the native XR with the modified NADP-dependent XDH (D207A/I208R/F209S/N211R). Xylose consumption was also 32% faster when compared to the strain expressing wild-type alleles of XR and

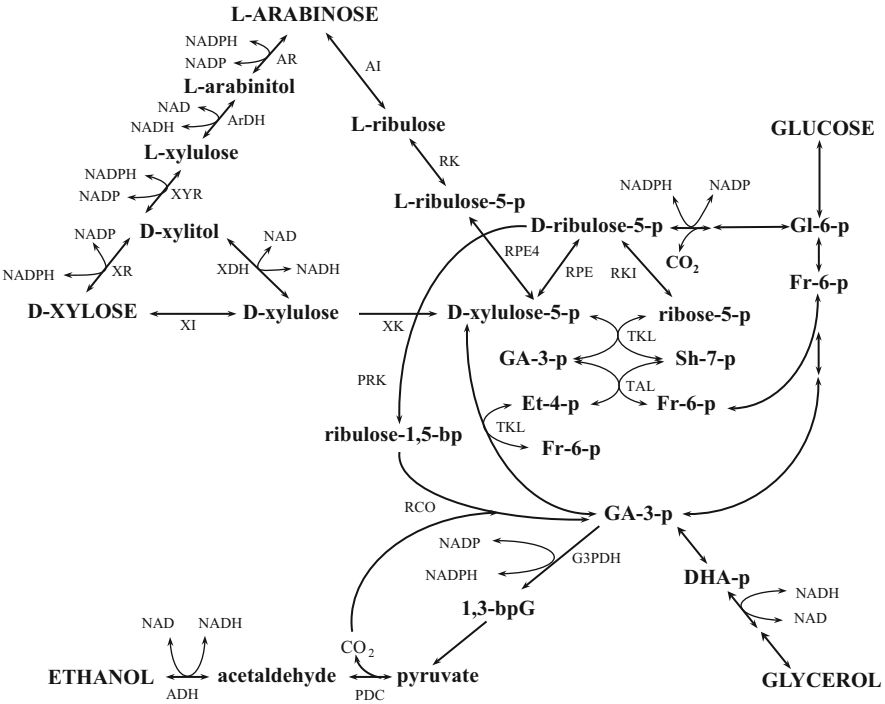

Fig. 1.2 Pathways of xylose, L-arabinose, and glucose fermentation to ethanol in yeasts. Gl-6-p is glucose-6-phosphate; Fr-6-p is fructose-6-phosphate; GA-3-p is glyceraldehyde-3-phosphate; SH-7-p is sedoheptulose 7-phosphate; ET-4-p is erythrose 4-phosphate; 1,3-bpG is 1,3-bisphospho-glycerate; DHA-p is dihydroxyacetone phosphate; XR is xylose reductase; XDH is xylitol dehydrogenase; XI is xylose isomerase; XK is xylulokinase; AI is arabinose isomerases; RK is ribulokinase; RPE4 is ribulose-5-phosphate-4-epimerase; AR is aldose reductase; ArDH is arabinitol dehydrogenase; XYR is xylulose reductase; RKI is ribose-5-phosphate ketol-isomerase; RPE is ribulose-5-phosphate-3-epimerase; TKL is transketolase; TAL is transaldolase; PRK is phosphoribulokinase; RCO is ribulose-1,5-bisphosphate carboxylase/oxygenase; G3PDH is glyceraldehyde-3-phosphate dehydrogenase; PDC is pyruvate decarboxylase; ADH is alcohol dehydrogenase

XDH (Matsushika and Sawayama 2008). The ratio between XR and XDH activities is essential to improve xylose alcoholic fermentation. The low level of *XYL2* gene expression is the main reason for the accumulation of xylitol in strains expressing *XYL1*, *XYL2*, and *XYL3* (Kim et al. 2013a).

Xylulokinase (XK) catalyzes the ATP-dependent phosphorylation of xylulose and is an important enzyme in xylose catabolism (Fig. 1.2). XK catalyzes the limiting step in xylose alcoholic fermentation as *S. cerevisiae* strains expressing XR/XDH or XI have a significant reduction in xylitol accumulation when XK was overexpressed (Parachin et al. 2011). The activation of XK was accomplished by the derepression of the homologous gene *XKS1 S. cerevisiae* or heterologous *XYL3 S. stipitis* (Jin et al. 2003). However, an extreme increase in XK activity has a

negative impact on the growth of recombinant strains on xylose, which is probably due to a rapid depletion of ATP that is used in xylulose phosphorylation (Du et al. 2010).

Two different L-arabinose catabolizing pathways were identified in bacteria (Wisselink et al. 2007) and fungi (Richard et al. 2001, 2002). In the bacterial pathway, arabinose is isomerized to ribulose by arabinose isomerase (*araA*), and then ribulose is phosphorylated to ribulose-5-phosphate with ribulokinase (*araB*) that in turn is converted to xylulose-5-phosphate by ribulose-5-phosphate-4-epimerase (*araD*) (Wisselink et al. 2007). Arabinose fermentation was observed when *araA*, *araB*, and *araD* from *Lactobacillus plantarum* were co-expressed in *S. cerevisiae* (Wisselink et al. 2007). In fungi, L-arabinose is reduced to L-arabitol by aldose reductase and then converted to L-xylulose with arabinitol dehydrogenase. L-xylulose is reduced to xylitol by xylulose reductase, which in turn is oxidized to xylulose by xylitol dehydrogenase. The expression of the fungal arabinose utilization pathway (aldose reductase Gre3 from *S. cerevisiae*, arabinitol dehydrogenase and xylulose reductase from *Trichoderma reesei*, XDH from *S. stipitis*, and XK from *S. cerevisiae*) in *S. cerevisiae* resulted in low ethanol with substantial arabinitol production, which is apparently due to the imbalance caused by the cofactor mismatch among used reductases/dehydrogenases (Richard et al. 2001, 2002; Bettiga et al. 2009). Based on published results, the expression of genes of the bacterial redox-independent pathway for L-arabinose utilization in *S. cerevisiae* is a more promising approach (Zhang et al. 2015a).

To increase the ethanol production, xylose-fermenting strains of *S. cerevisiae* that expressed the genes for xylose catabolism were subjected to other metabolic modifications. In particular, the heterologous expression of sugar transporters Sut1 and Sut2 from *S. stipitis* was carried out (Du et al. 2010; Hector et al. 2008). A system for the selection of glucose transporters with high affinity for xylose was also developed. As a result, a modified version of Gal2 *S. cerevisiae* transporter that is able to transport only xylose was selected (Farwick et al. 2014). It was also shown that endogenous *S. cerevisiae* hexose transporters (Hxt) can be engineered into specific xylose transporters (Farwick et al. 2014; Nijland et al. 2014). However, the expression of these genes remains subjected to glucose-regulated protein degradation. As a result, some Hxt proteins with high xylose transport capacity that are rapidly degraded in the absence of glucose or when glucose is exhausted from the medium (Nijland et al. 2016). The N-terminal lysine residues of the Hxt proteins were predicted to be the target of ubiquitination. The substitution of N-terminal lysine residues in the hexose transporters Hxt1 and Hxt36 that are subjected to catabolite degradation, resulted in an improved retention of these transporters at the cytoplasmic membrane in the absence of glucose and causes improved xylose fermentation upon the depletion of glucose and when cells are grown in xylose alone (Nijland et al. 2016).

In order to achieve efficient glucose/xylose co-fermentation, a modified xylose-specific, glucose-insensitive transporter Mgt05196 (N360F) from *Meyerozyma guilliermondii* was expressed on the background of a xylose-fermenting *S. cerevisiae* strain overproducing XI, xylulokinase Xks1 and the non-oxidative

pentose phosphate pathway (PPP), and the inactivation of the aldose reductase Gre3p and the alkaline phosphatase Pho13. These rationally designed genetic modifications, combined with alternating adaptive evolution in xylose and lignocellulosic hydrolysates, resulted in a final strain, with excellent xylose fermentation that had an enhanced resistance to inhibitors (Li et al. 2016).

The co-expression of genes of non-oxidative part of PPP (*RKI1*, *RPE1*, *TKL1*, and *TAL1*, encoding ribose-5-phosphate isomerase, ribulose-5-phosphate epimerase, transketolase, and transaldolase, respectively) (Fig. 1.2) on the background of a *S. cerevisiae* strain expressing XI and XK showed improved growth on xylose (Karhumaa et al. 2005). However, expression of genes of the non-oxidative part of PPP in a *S. cerevisiae* strain expressing XR, XDH, and XK did not improve its growth on a medium supplemented with xylose (Bera et al. 2011).

It is known that the regeneration of NADPH mainly occurs in the oxidative part of PPP. A series of studies were directed to reduce the activity of the enzymes participating in the oxidative branch of PPP. This approach will facilitate the reaction catalyzed by XR using NADH instead of NADPH, with a reduced production of CO_2. The deletion of genes *ZWF1* (encoding glucose-6-phosphate dehydrogenase) and/or *GND1* (decarboxylating 6-phosphogluconate dehydrogenase) with the simultaneous expression of the genes *XYL1* and *XYL2* increased ethanol yield during xylose fermentation (Jeppsson et al. 2002; Verho et al. 2003). Another successful approach describes the combination of fungal NADP-dependent glyceraldehyde-3-phosphate dehydrogenase overexpression in part with the deletion of the *ZWF1* gene, resulted in an increase in ethanol yield and productivity during xylose fermentation (Verho et al. 2003). To reduce xylitol production and increase ethanol yield during fermentation of glucose/xylose mixture, a modification of the redox balance of recombinant strains of *S. cerevisiae* was carried out by the deletion of gene *GDH1* (coding for NADPH-dependent glutamate dehydrogenase) and the overexpression of *GDH2* gene (NADH-dependent glutamate dehydrogenase) responsible for ammonium assimilation (Roca et al. 2003). The reduction of xylitol accumulation was achieved by the overexpression of the *noxE* gene from *L. lactis*, coding for the water forming NADH-oxidase (Zhang et al. 2012). The modification of the acetate biosynthetic pathway that involved the deletion of the *ALD6* gene encoding NADP-dependent aldehyde dehydrogenase, increased the efficiency of xylose alcohol fermentation (Lee et al. 2012).

To increase the production of ethanol during xylose fermentation, recombinant strains of *S. cerevisiae* were subjected to adaptive evolution and genome shuffling (Kim et al. 2013a; Jingping et al. 2012; Ren et al. 2016). Sequencing of the genomes of these strains after long-term culturing in xylose-containing media revealed new potential target genes for metabolic engineering approach, e.g., *XKS1*, *SOL3* (6-phosphogluconolactonase), *GND1* (6-phosphogluconate dehydrogenase), *TAL1*, *TKL1*, *YCR020C*, *YBR083W*, and *YPR199C* (Wahlbom et al. 2003). The genes for *TAL1* and *PHO13* (non-specific alkaline phosphatase) were identified by transposon mutagenesis as a new targets. The truncation of these genes increased production of ethanol during xylose alcoholic fermentation (Ni et al. 2007).

Carbon dioxide is a major by-product of carbohydrate alcoholic fermentation. It will be very attractive if the fermentation process becomes carbon conservative via re-assimilating the carbon from the carbon dioxide released from the decarboxylation step of pyruvate into acetaldehyde in last stages of the ethanol-producing pathway. This approach was recently described by the introduction a synthetic reductive PPP into a xylose-fermenting *S. cerevisiae* strain to achieve simultaneous lignocellulosic ethanol production and carbon dioxide recycling. The heterologous enzymes phosphoribulokinase from *Spinacia oleracea* and ribulose-1,5-bisphosphate carboxylase/oxygenase from *Rhodospirillum rubrum* were introduced into the *S. cerevisiae* strain harboring XR/XDH pathway, upregulated PPP, and knocked out *ALD6* and *PHO13* genes (Kim et al. 2013a). The phosphoribulokinase enzyme catalyzes ribulose-5-phosphate conversion to ribulose-1,5-bisphosphate, and the ribulose-1,5-bisphosphate carboxylase/oxygenase converts one ribulose-1,5-bisphosphate and CO_2 to two molecules of glycerate-3-phosphate (Fig. 1.2). The constructed strain with the synthetic reductive PPP revealed a higher yield of ethanol with lower yields of xylitol and glycerol than that of the parental strain. Moreover, a reduced release of CO_2 by the engineered strain was observed during xylose fermentation, suggesting that the carbon dioxide generated by pyruvate decarboxylase, was partially reassimilated through the synthetic reductive PPP (Xia et al. 2016). The strategy of carbon dioxide recycling from the ethanol fermentation pathway in yeast has a great potential in alleviating greenhouse gas emissions during the production of 2G ethanol.

Fermentation of biomass-derived hydrolysates is accompanied with the inhibition of ethanol production due to the presence of toxic compounds produced mainly during lignocelluloses pretreatment, e.g., furfural, hydroxymethylfurfural (HMF), weak acids, and phenols (Palmqvist and Hahn-Hägerdal 2000). To overcome this limitation, the strains of *S. cerevisiae* that are able to produce ethanol in the presence of these inhibitors have been selected through directed evolution and adaptation (Hawkins et al. 2013; Parreiras et al. 2014). Other alternative molecular approaches were also applied. Furfural and HMF were converted to less toxic furfuryl alcohol and furan dimethanol, respectively, by the overexpression of the endogenous *S. cerevisiae* oxidoreductases such as alcohol dehydrogenases (*ADH1*, *ADH6*, and *ADH7*) (Almeida et al. 2008; Liu et al. 2008; Petersson et al. 2006) and aldo-keto reductases (*GRE2*) (Moon and Liu 2012). It was shown (Gorsich et al. 2006) that tolerance to furfural-induced stress is associated with genes of the pentose phosphate pathway *ZWF1*, *GND1*, *RPE1*, and *TKL1*. The overexpression of the gene *PAD1* encoding phenylacrylic-acid decarboxylase in *S. cerevisiae* resulted in improved growth rate and ethanol productivity in dilute-acid hydrolysates (Larsson et al. 2001). An original approach to improve tolerance to fermentation inhibitors and ethanol was proposed by modulation of the polyamine (spermidine) content in *S. cerevisiae* (Kim et al. 2015). Intracellular spermidine contents was increased by a double gene deletion *OAZ1* (ornithine decarboxylase antizyme) and *TPO1* (polyamine transport protein) genes and the overexpression of *SPE3* (spermidine synthase) (Kim et al. 2015).

In summary, 1G ethanol production is a profitable technology that can be further improved by increasing ethanol yield and productivity by applying molecular and classical techniques to industrial ethanol-producing strains of *S. cerevisiae*. Despite significant efforts, existing technologies of 2G ethanol production are still non-profitable. Although recombinant *S. cerevisiae* strains capable of hexoses/pentoses co-fermentation were developed, none of the engineered strains are able to ferment glucose/xylose mixture as fast as the rate of glucose fermentation by the parental strain (Zhang et al. 2015a). Another important limitation is the sensitivity of ethanol-producing strains to inhibitors in lignocellulosic hydrolysates. Further studies are needed to develop recombinant *S. cerevisiae* strains that are capable of rapid fermentation of mixed sugars with improved resistance to fermentation inhibitors.

1.3 *Scheffersomyces (Pichia) stipitis*

Scheffersomyces (Pichia) stipitis belongs to the group of naturally xylose-fermenting yeasts that is characterized by rather low amounts of accumulated by-products from this fermentation-like xylitol (Kurtzman and Suzuki 2010). Other known representatives of the group of native xylose-fermenting yeasts are *Candida shehatae*, *Pachysolen tannophilus*, *Spathaspora passalidarum*, and *Ogataea (Hansenula) polymorpha* (Schneider et al. 1981; du Preez and van der Walt 1983; Ryabova et al. 2003; Nguyen et al. 2006). As a rule, most natural xylose-fermenting yeasts inhabit the guts of passalid beetles that degrade white-rotted hardwood (Nigam 2001; Nardi et al. 2006 with the exception of *O. polymorpha* strains that have been isolated from other natural habitats (Gellisen 2002). *S. stipitis* is closely related to other yeast endosymbionts of passalid beetles (Suh et al. 2003). This yeast has one of the highest native capacities for xylose fermentation among yeast species described so far (du Preez et al. 1989). In addition to utilizing xylose, *S. stipitis* has the capability to use all of the other major sugars found in wood. It also transforms low-molecular-weight lignin moieties, reduces acyclic enones to the corresponding alcohols, and forms various esters and aroma components. It has been recently engineered to produce lactic acid or xylitol in high yield (Lee et al. 1986; Targonski 1992; Kim et al. 2001; Conceicao et al. 2003; Ilmen et al. 2007). The published fermented sugar yields from lignocellulose hydrolysates by this yeast approach 80% of theoretical yield (Nigam 2001). The availability of genetic tools and capability for fermentation of hydrolysates has made *S. stipitis* an attractive microorganism for bioconversion of lignocellulose to fuels and chemicals. The major drawbacks of *S. stipitis* are the low fermentation rates, low ethanol tolerance, and the inability to grow anaerobically (du Preez et al. 1989; Grootjen et al. 1990; Shi and Jeffries 1998).

S. stipitis is a predominantly haploid, homothallic, hemiascomycetous yeast that forms buds along with pseudomycelia during vegetative growth and two hat-shaped ascospores from each ascus (Kurtzman 1990). Genetic manipulation of *S. stipitis* is much more complicated relative to those of *S. cerevisiae*. This is because *S. stipitis*

is resistant to most common antibiotics and the number of strains with convenient auxotrophic markers is limited. This yeast uses an alternative codon system that decodes CUG for serine instead of leucine as common in the classical genetic code. Thus, the expression of foreign proteins, including those used as drug resistance markers, requires codon modification. Unfortunately, random (nonhomologous) integration prevails in *S. stipitis* which makes targeted deletions much more difficult to obtain (Jeffries and Van Vleet 2009). Nevertheless, many efficient genetic methods have been developed for *S. stipitis* that include methods of sexual mating and sporulation (Melake et al. 1996). Auxotrophic strains have been created and methods for high-efficiency transformation have been developed for the auxotrophic mutants *ura3*, *leu2*, *trp5*, and *his3* of *S. stipitis* (Yang et al. 1994; Lu et al. 1998; Piontek et al. 1998). Genetic tools based on a *loxP*/*Cre* recombination system and the dominant marker for zeocin resistance have been developed for *S. stipitis* (Laplaza et al. 2006). Deletion of the *KU80* gene that is responsible for nonhomologous end joining significantly increases the fraction of homologous recombinant transformants, albeit at the expense of transformation frequency (Maassen et al. 2008).

The 15.4-Mbp genome of *S. stipitis* was sequenced (Jeffries et al. 2007). *S. stipitis* CBS 6054 is known to have eight chromosomes, of which two pairs are very similar in size, accounting for the earlier results that suggested the presence only of six chromosomes (Passoth et al. 1992). *S. stipitis* genome annotation predicted that 5841 genes are present in this of which a majority of 72% have a single exon. The average gene density is 56% and the average gene, transcript, and protein lengths are 1.6 kb, 1.5 kb, and 493 amino acids, respectively. Expressed sequence tags (ESTs) confirmed the identity of 40% of the predicted genes with 84% showing strong similarity to proteins in other fungi (Jeffries et al. 2007). So far, protein function can be tentatively assigned to about 70% of the genes. Synteny analysis of *P. stipitis* with its nearest completely sequenced yeast genome neighbor, *Debaryomyces hansenii*, shows extensive recombination and shuffling of the chromosomes, which appear to be a common feature. *S. stipitis* and *D. hansenii* share 151 gene families that are not found in the other genomes. The *S. stipitis* gene set is missing 81 gene families (442 proteins) relative to the other yeast genomes in the analysis.

The most frequent domains characterized so far include protein kinases, helicases, transporters (sugar and MFS), and domains involved in transcriptional regulation (fungal-specific transcription factors, RNA recognition motifs, and WD40 domains). A majority of these is shared with other hemiascomycota. These range from 1534 domains in common with *Schizosaccharomyces pombe* and 1639 domains with *D. hansenii*. One of the few *S. stipitis*-specific domains belongs to one of glycosyl hydrolase families, a subgroup of cellulases and xylanases. All of the genes for xylose assimilation, including the oxidative PPP, glycolytic cycle, the tricarboxylic acid cycle (TCA), and ethanol production, were present in isoforms similar to those found in other yeasts (Jeffries et al. 2007). Genes of first three enzymes of xylose metabolism, *XYL1*, *XYL2*, and *XYL3*, and that of PPP (*ZWF1*, *GND1*, *TKL1*, *TAL1*, *RPI1*) are also induced by xylose, and the

expression of *XYL2* yields one of the most abundant transcripts in xylose-grown cells (Jeffries and Van Vleet 2009). Another interesting pattern of the regulation of gene expression was found by oxygen limitation. Such limitation led to strong derepression of some glycolytic genes that include two genes of glyceraldehyde-3-phosphate dehydrogenase *TDH1* and *TDH2*, of pyruvate decarboxylase *PDC1* and *PDC2*, and of the secondary alcohol dehydrogenase *SAD2* (the function of the last gene/enzyme in metabolism is not known) (Jeffries and Van Vleet 2009). Genome-displayed genes for sensing and regulatory proteins (<200 putative Zn-finger regulatory proteins) are similar to that in *S. cerevisiae*. The genome *S. stipitis* also revealed many gene clusters representing either pairs/clusters of nonhomologous genes in which each cluster has a single function such as galactose metabolism, or tandem repeats of paralogous genes. Gene clusters seem to be particularly abundant in *S. stipitis* as there are at least 35 clusters of functionally related genes (Jeffries et al. 2007). The studies of structure, function, and regulation in *S. stipitis* genome are likely to have important impact on understanding its physiology and could be used for metabolic engineering of this organism.

The study of *S. stipitis* has attracted scientists and technologists primarily due to its natural ability to produce large amounts of ethanol during xylose fermentation with small or no production of xylitol. It was found that fed-batch cultures of *S. stipitis* produce around 47 g/L of ethanol with yields of 0.36 g/g xylose at 30 °C (van Dijken et al. 1986). However, *S. stipitis* fermentation rate on xylose is low relative to that of *S. cerevisiae* on glucose, so increasing the rate of fermentation of xylose by *S. stipitis* could greatly improve its usefulness in commercial applications (Jeffries and Van Vleet 2009). Another drawback to using *S. stipitis* is that it is much more susceptible to ethanol inhibition relative to *S. cerevisiae* (Shi and Jeffries 1998).

There are many detailed publications on the physiology of *S. stipitis*. Oxygen plays an important role in cell growth, redox balance, functioning of the mitochondria, and generation of energy for xylose transport in *S. stipitis* (Skoog and Hahn-Hagerdahl 1990). Fermentation in *S. stipitis* is activated by oxygen limitation (Passoth et al. 1996, 2003; Klinner et al. 2005). It is interesting to note that *S. stipitis* can metabolize xylose anaerobically, even though it does not grow under anaerobic conditions (Wijsman et al. 1985). The optimal temperature for *S. stipitis* fermentation is between 25 and 33 °C, and the optimal pH is in the range of 4.5–5.5 (du Preez et al. 1986). The nutrients in the fermentation media play an important part in the growth and ethanol production in *S. stipitis*. Ethanol production increased with the addition of amino acids and nitrogen was required for non-growth associated ethanol production (Slininger et al. 2006). Ammonium salts increased the ethanol productivity and the ethanol to biomass yield in *S. stipitis* (Guebel et al. 1992; Agbogbo and Wenger 2006). Magnesium has also been shown to play an important role in redox balance and therefore has an effect on xylitol production (Mahler and Nudel 2000). Low levels of Mg^{2+} resulted in xylitol accumulation and a high intracellular NADH content. Corn steep liquor is a viable nutrient source for *S. stipitis* fermentation when used as a sole nitrogen source compared to amino acids, vitamins, and other nutrients (Amartey and Jeffries

1994). The initial xylose concentration has an effect on the fermentation parameters of *S. stipitis* with maximum ethanol productivities occurring at a xylose concentration of 50 g/L (du Preez et al. 1985).

The conversion of xylose to ethanol in *P. stipitis* consists of three stages: (1) xylose transport and the initial reaction to enter the PPP, (2) non-oxidative reactions of PPP, and (3) glycolysis (Fig. 1.2). Little is known about xylose transport in *S. stipitis*. The low-affinity transport system is shared between glucose and xylose for sugar transport. Glucose inhibits xylose transport by noncompetitive inhibition in the low-affinity proton symport system (Kilian and van Uden 1988). The low-affinity transport is used when sugar concentrations are high, and the high-affinity systems are used when sugar concentrations are low. Repression of xylose uptake occurs in fermentation media containing glucose and xylose. Therefore, glucose is the preferred sugar by *S. stipitis* in ethanol production. The rate of glucose consumption is higher than xylose under similar growth conditions (Agbogbo et al. 2006). The transport of sugars into the cells is the rate-limiting step in the utilization of sugars for ethanol production in *S. stipitis* (Legthelm et al. 1988. A high-affinity xylose-transporting system has been described that is specific for xylose in this yeast (Hahn-Hägerdal et al. 2001). Three genes, *SUT1*, *SUT2*, and *SUT3*, have been characterized that encode glucose transporters in *S. stipitis* (Weierstall et al. 1999). Sut2 and Sut3 are highly similar to the *S. cerevisiae* glucose transporter family, and the Sut2 and Sut3 transporters have a higher affinity for glucose than for xylose. Transcription of *SUT1* is induced in *S. stipitis* independently of oxygen supply, whereas *SUT2* and *SUT3* are expressed only under aerobic conditions but independently of the carbon source. Disruption of *SUT1* eliminates the low-affinity xylose transport system in *S. stipitis* (Weierstall et al. 1999).

Initial metabolism of xylose in *S. stipitis* is similar to other natural xylose-fermenting yeasts (Fig. 1.2). Xylose first is reduced by xylose reductase (aldose reductase, gene *XYL1*) to xylitol (Veduyn et al. 1985; Hahn-Hagerdal and Pamment 2004). This enzyme has affinity to both NADH and NADPH but shows much higher affinity toward NADPH. By comparison, *S. stipitis* xylitol dehydrogenase (gene *XYL2*), which converts xylitol to xylulose, has affinity only for NAD (Metzger and Hollenberg 1995). The third reaction of xylose metabolism is catalyzed by xylulokinase (gene *XYL3*), which converts xylulose to the PPP intermediate xylulose-5-phosphate (Jin et al. 2002). The cofactor unbalance, resulting from the first two reactions involving xylose reductase and xylitol dehydrogenase, leads to xylitol accumulation in most natural xylose-fermenting yeasts but not in *S. stipitis* (Skoog and Hahn-Hagerdahl 1990). In *P. stipitis* there are efficient systems for NADH reoxidation to NAD and NADPH regeneration (Balagurunathan et al. 2012). One particular pathway that has been observed for xylose metabolism that is induced under oxygen limited conditions is effective in tackling the cofactor imbalance caused by the first two steps in xylose utilization (Jeffries et al. 2007; Jeffries and Van Vleet 2009). This pathway involves the four enzymes: NAD-dependent glutamate dehydrogenase (*GDH2*), which converts 2-oxoglutarate to L-glutamate consuming NADH; glutamate decarboxylase (*GAD2*), which decarboxylates L-glutamate to 4-aminobutyrate; 4-aminobutyrate

aminotransferase (*UGA1*), which transaminates 4-aminobutyrate to succinate semialdehyde; and succinate semialdehyde dehydrogenase (*UGA2*), which oxidizes succinate semialdehyde to succinate using NADP. The net result of the four reactions is the conversion of NADH to NADPH.

The PPP plays an important role in xylose fermentation (Fig. 1.2), and the corresponding genes (*ZWF1*, *GND1*, *TKL1*, *TAL1*, *RPI1*) are known to be induced by xylose (Jeffries and Van Vleet 2009). Alcohol dehydrogenase is also an important enzyme in ethanol production with the deletion of either *ADH1* or *ADH2* gene significantly reducing ethanol formation, and the deletion of both entirely abolishes ethanol production (Cho and Jeffries 1998; Passoth et al. 1998).

Relatively little is known about the rate-limiting steps in ethanol production from xylose. It has been shown that XR and XDH are repressed by glucose and are induced during growth on xylose. Xylose is generally not consumed in the presence of glucose; hence, under glucose repression, these activities, along with xylose transport, are rate limiting (Bicho et al. 1988). The XK does not, however, appear to be rate limiting in *S. stipitis* once its activity is induced by xylose (Jin et al. 2002).

In addition to the ultimate goal of using *S. stipitis* for ethanol production from lignocellulosic feedstock, this yeast can also be successfully used for xylitol production from xylose using a mutant defective in *XYL2* that codes for xylitol dehydrogenase (Kim et al. 2001; Rodrigues et al. 2008). Efficient producers of lactic acid have also been constructed on *S. stipitis* after expression of the lactate dehydrogenase *LDH* gene from *Lactobacillus helveticus* under control of the yeast *ADH1* promoter. It is interesting to note that xylose was more efficient substrate for lactate synthesis than glucose (Ilmen et al. 2007). A strain of *S. stipitis* hyperaccumulating *S*-adenosylmethionine has been isolated (Križanović et al. 2015). Other *S. stipitis* recombinant strains have been constructed that produce fumaric acid from xylose after acquiring heterologous reductive pathway from *Rhizopus oryzae* (Wei et al. 2008).

In spite of the fact that *S. stipitis* is one of the best natural xylose-fermenting yeasts with no xylitol accumulation, it has several drawbacks which include a low rate of fermentation, low tolerance to ethanol, and requirement of oxygen for growth. When grown on lignocellulosic hydrolysates, other limitations to the use of this yeast are observed. Among these is the need for simultaneous fermentation of glucose and xylose and the poor tolerance to inhibitors that are present in hydrolysates. Additionally as oxygen is required, *S. stipitis* has a tendency to utilize the ethanol produced with a considerable amount of xylose remaining in the medium (Harner et al. 2015). One of the most popular methods used to increase *S. stipitis* tolerance toward inhibitors is adaptation by repeated subculturing or recycled of yeast cells while increasing concentrations of the inhibitor(s) in a stepwise fashion by adding more concentrated lignocellulosic hydrolysate solutions (Amartey and Jeffries 1996; Huang et al. 2009; Watanabe et al. 2011; Yang et al. 2011). Strains adapted to inhibitors present in a specific hydrolysate may exhibit cross-tolerance to other hydrolysates. Adaptation has been done to individual inhibitor(s) as well as to mixtures of inhibitors, with the latter approach being most common. Ability to tolerate inhibitory compounds in lignocellulose

hydrolysates could reduce the need for detoxification procedures, and this can decrease the overall production cost of ethanol. Adaptation is frequently substituted by random mutagenesis (Bajwa et al. 2009) or by using genome shuffling via protoplast fusion of strains with different genotypes (Bajwa et al. 2010, 2011). To improve ethanol tolerance and production, similar approaches of random mutagenesis and genome shuffling were combined and used (Harner et al. 2015). Selection of higher ethanol tolerance *P. stipitis* strains has been accomplished by using UV mutagenesis has also led to improved ethanol production (Watanabe et al. 2011). Protoplast fusion of *S. stipitis* with *S. cerevisiae* has allowed the isolation of a hybrid strain with higher ethanol productivity from xylose relative to the *S. stipitis* parental strain; however, such hybrid was unstable (Yoon et al. 1996). A popular method for genetic improvement that consist of genome shuffling uses transformation of a yeast strain with total DNA isolated from another strain of the same species or even from different yeast species. In one series of experiments, total DNA of *S. stipitis* was introduced in *S. cerevisiae* by electroporation, and the best xylose-fermenting strain isolated was used as the source of DNA for the next round of transformation. This strain showed improvement in ethanol production from xylose and higher ethanol tolerance (Zhang and Geng 2012). To isolate *S. stipitis* strains with improved growth and fermentation characteristics on the xylose/glucose mixture, several fast-growing mutants were isolated on a xylose medium with respiration inhibitors antimycin A and salicyl hydroxamate. Several other mutants have also been isolated which produced more ethanol on xylose/glucose mixture (Sreenath and Jeffries 1997). In another approach, mutants of *S. stipitis* which grow anaerobically on xylose plates were isolated. In contrast to the wild-type strain, these isolated mutants grew and fermented xylose and glucose anaerobically though very slowly (Hughes et al. 2012).

Glucose prevents xylose utilization as it competes with xylose for transport, and its use is subject to glucose catabolite repression. To obtain strains of *S. stipitis* that can ferment simultaneously glucose and xylose, 2-deoxyglucose-resistant mutants were isolated (Sreenath and Jeffries 1999). This was also accomplished by deletion of the gene *HXK1* coding for hexokinase I (Dashtban et al. 2015). No strains with glucose-insensitive xylose transport have been reported for *S. stipitis*. The last experiment could be considered as the only one in which a metabolic engineering approach was applied (Dashtban et al. 2015). As a rule, experiments in metabolic engineering of *S. stipitis* are hampered by our limited knowledge of the limiting steps of the fermentation process. In another approach, the mutant of *S. stipitis* with disruption of cytochrome *c* was isolated. Due to defects in respiration, this mutant appeared to be superior when compared to the wild-type strain in xylose alcoholic fermentation as it accumulated elevated amounts of ethanol (Shi et al. 1999).

In order to determine the gene(s) affecting *S. stipitis* fermentative capabilities, insertional mutants with altered ethanol production from glucose and xylose have been isolated (V. Passoth, K. Berezka, K. Dmytruk, A. Sibirny, unpublished). Mutants obtained by random insertional mutagenesis were screened for their growth abilities on solid media with different sugars and for resistance to the glycolysis inhibitor, 3-bromopyruvate. Fermentations in shake flask agitated

cultures were carried out in order to measure sugar consumption and ethanol formation rates. Subsequently, the most interesting strains were analyzed to determine the genetic background of the observed alterations. Of more than 1300 screened mutants, 17 were identified that have significantly changed ethanol yields during the fermentation. In one of the best fermenting strains, a single insertion event resulted in the enhancement of ethanol formation in the media with both glucose and xylose. This strain had to have within the ORF a gene homologous to *S. cerevisiae* gene YDL119C, that encoded for a not yet described mitochondrial transporter and was designated *TMI1* (*T*ransport to *MI*tochondria). Wild-type phenotype was restored via complementation of the insertional mutation of the wild allele of *TMI1* gene Thus, gene *TMI1* is apparently involved in the control of hexose and pentose alcoholic fermentation in *S. stipitis*.

In summary, *S. stipitis* remains as one of the most efficient organisms for xylose and in general, lignocellulose fermentation. Access to genome sequence and the development of methods of molecular genetics have been used to alleviate some of the shortcomings of this yeast (low tolerance to ethanol, glucose inhibition of xylose metabolism, inability to grow anaerobically). This can be accomplished by using the combination of rational design methods of metabolic engineering and random selection. Once the aforementioned shortcomings have been addressed, this organism yeast has the potential for use in industrial fermentation.

1.4 *Ogataea polymorpha*

Ogataea (Hansenula) polymorpha is one of the most thermotolerant species of yeast known. This yeast has the ability to grow up to a temperature of 50 °C. Wild-type strains of this yeast are able to ferment glucose, xylose, mannose, maltose, and cellobiose into ethanol but are not able to ferment galactose and L-arabinose (Ryabova et al. 2003). Sugar fermentation in this yeast is most efficient under conditions of limited aeration, and even at 45–48 °C it is still sufficiently vigorous. At industrial scale high fermentation temperature can reduce cooling costs and the risk of contamination with more energy efficient removal of ethanol. Several successful attempts have been made to improve this yeast thermotolerance even further (Ishchuk et al. 2009). It is known that, similar to other fungi, *O. polymorpha* accumulates trehalose and expresses heat shock proteins (Hsps) under heat shock conditions (Guerra et al. 2005). The increase in the intracellular level of trehalose in *O. polymorpha* following the knockout of acid trehalase gene *ATH1* resulted in a sixfold higher ethanol production of xylose fermentation at 50 °C. The overexpression of the heat shock proteins Hsp16 and Hsp104 have also led to three to six times improved ethanol production at 50 °C (Ishchuk et al. 2009).

High ethanol tolerance is another important feature of ethanol producers for industrial applications. *O. polymorpha* appear to be more resistant to the toxic concentrations of ethanol when compared to *S. stipitis*; but it was more sensitive than *S. cerevisiae* (Dmytruk and Sibirny 2013). The overexpression of the endogenous *ETT1* gene (a homolog of *S. cerevisiae MPE1* gene) significantly increased the

resistance of *H. polymorpha* to ethanol, resulting in ten- and threefold improvements in the growth on agar and in liquid media with ethanol, respectively. The resistance of *O. polymorpha* to ethyl alcohol was also enhanced by the overexpression of the heterologous *S. cerevisiae MPR1* gene that codes for acetyltransferase (Ishchuk et al. 2010).

Direct microbial conversion of carbon polymers into ethanol is a promising technology for the production of alcohols from lignocellulosic raw material. The optimal temperature for the activity of hydrolytic enzymes used during microbial conversion of polymers into ethanol is about 50 °C. Recombinant *O. polymorpha* strains that fermented starch were constructed by the expression of heterologous secretory α-amylase and glucoamylase that code for the *SWA2* and *GAM1* genes of *Schwanniomyces occidentalis* (Wang et al. 1989). The heterologous expression of *XYN2* gene of *Trichoderma reesei* and *xlnD* of *Aspergillus niger* that code for secretory endoxylanase and secretory β-xylosidase was successfully performed in *O. polymorpha* resulting in strains with ability of direct xylan fermentation at high temperature (Voronovsky et al. 2009).

O. polymorpha has a good potential to be used for the efficient process of simultaneous saccharification and fermentation (SSF) due to its temperature tolerance and ability to ferment xylose to ethanol (Radecka et al. 2015). However, the ethanol yield and productivity from xylose of wild-type strains of *O. polymorpha* are very low. Identification of rate-limiting enzymes for xylose conversion to ethanol is necessary for rational strain modification to improve the fermenting efficiency.

The molecular tools for this yeast species are well developed (Gellisen 2000), and a complete genome sequence of the strain NCYC495 of *O. polymorpha* is publically available (http://genome.jgi-psf.org/Hanpo2/Hanpo2.home.html). As a consequence, a combination of metabolic engineering and classical selection approaches were successfully used for improvement of the pathway for xylose alcoholic fermentation in *O. polymorpha* (Kurylenko et al. 2014).

The difference in cofactor specificity in the first steps of xylose metabolism results in cofactor imbalance causing a substantially reduced ethanol production with accumulation of xylitol as a by-product in yeasts. The problem arises during consecutive action of NADPH-dependent XR and NAD-dependent XDH that catalyze the reduction of xylose to xylitol and the oxidation of xylitol to xylulose (Fig. 1.2). To avoid the cofactor imbalance, XR and XDH were replaced with bacterial XI, which immediately converts xylose into xylulose with no cofactors required. The bacterial gene *xylA* from *E. coli* or *Streptomyces coelicolor* coding for XI was successfully expressed in *O. polymorpha* strain with a knockout of *XYL1* gene coding for XR and two paralogs *XYL2A* and *XYL2B* of XDH. The recombinant strains were able to grow on xylose as carbon source; however, the amount of accumulated ethanol remained at the level of the wild-type strain CBS4732 (0.15 g/L) (Voronovsky et al. 2005). The overexpression of *E. coli xylA* together with *O. polymorpha XYL3* that codes for XK has led to fourfold increase of ethanol production, but still the maximal ethanol accumulation did not exceed 0.6 g/L at 48 °C (Dmytruk et al. 2008b).

The XR of *O. polymorpha* can use both NADPH and NADH as cofactors. However, the affinity of XR to NADH is significantly lower than that to NADPH. Therefore, another approach to eliminate the imbalance of these two cofactors consists of engineering of the actions of XR and XDH using an *O. polymorpha* XR with reduced affinity toward NADPH. Using site-specific mutagenesis, a modified XR was constructed by the substitution of lysine and asparagine for arginine and aspartic acid at amino acid positions 341 and 343. As a result of the modification of the primary structure of the protein, the affinity of XR to NADPH was 17-fold decreased as compared to the native enzyme, while the affinity of modified XR to NADH remained unchanged. A recombinant strain of *O. polymorpha* with enhanced expression of modified XR and a native XDH and XK was characterized by a fivefold decrease of xylitol accumulation as compared to the wild-type strain and twofold higher ethanol production reaching 1.3 g of ethanol/L (Dmytruk et al. 2008a).

The wild-type *O. polymorpha* strain NCYC495 was shown to be a more efficient xylose fermenter relative to the strain CBS4732 as this strain can accumulate up to 0.5 g of ethanol/L during high-temperature xylose alcoholic fermentation. Pyruvate decarboxylase (PDC) is one of the key enzymes of the final steps of alcoholic fermentation, catalyzing the conversion of pyruvate into acetaldehyde and CO_2. Subsequently, acetaldehyde is reduced to ethanol by alcohol dehydrogenase (ADH) that is coded by the *ADH1* gene (Fig. 1.2). Under conditions of limited aeration, a sufficient activity of PDC is important for redirection of pyruvate toward ethanol formation instead of respiration. Overexpression of endogenous *PDC1* gene under control of the strong constitutive promoter using a plasmid with multicopy integration has led to a twofold increase in ethanol production from xylose in the wild-type strain NCYC495 (Ishchuk et al. 2008; Sohn et al. 1999). Overexpression of both *PDC1* and *ADH1* genes in *O. polymorpha* have resulted in an additional twofold activation of xylose alcoholic fermentation when compared to the strain expressing solely *PDC1* (Kurylenko et al. 2016b).

The *O. polymorpha* mutant 2EthOH$^-$ unable to utilize ethanol as a sole carbon source was isolated from strain NCYC495 and characterized by a threefold increase in ethanol production from xylose. Subsequently, the overexpression of the gene *PDC1* in this mutant$^-$ resulted in further improvement of ethanol accumulation, reaching 2.5 g of ethanol/L at 48 °C (Ishchuk et al. 2008). To achieve higher amount of ethanol from xylose, several successful metabolic engineering approaches were combined to modify the genome of the 2EthOH$^-$ mutant. The overexpression of the genes *XYL1m*, *XYL2*, and *XYL3* that code for the modified XR and native XDH and XK, on the background of non-identified mutation in the strain 2EthOH$^-$, led to a substantial increase in ethanol accumulation during xylose fermentation (7.4 g/L at 45 °C relative to 0.6 g/L in the wild-type strain NCYC495) (Kurylenko et al. 2014). The additional activation of PDC did not lead to any further improvement of xylose conversion to ethanol though the overexpression of *PDC1* on the background of *XYL1m* and *XYL2* overexpressed strain, increased ethanol production. The impact of XK on ethanol production during xylose alcoholic fermentation is more pronounced relative to PDC

suggesting that PDC does not limit xylose conversion in strain with higher activities of XR, XDH, and XK.

An additional increase in ethanol production from xylose (to 10 g/L at 45 °C) was obtained in mutants selected on a medium supplemented with toxic concentrations of 3-bromopyruvate (BrPA), as described for ethanol accumulation from glucose in *S. cerevisiae* (Dmytruk et al. 2015).

While mutation(s) causing resistance to BrPA in the ethanol overproducing strain remain to be identified, a corresponding mutation was mapped in the strain with the wild-type background. Insertional mutagenesis was used for NCYC495 strain of *O. polymorpha* with subsequent selection of transformants using a mineral medium supplemented with 25 mM of BrPA. Sequencing of the flanking regions revealed that the insertional cassette disrupted the ORF of a gene homologous to the *S. cerevisiae* autophagy-related gene *ATG13*. This gene encodes a regulatory subunit of the Atg1 signaling complex, stimulating Atg1 kinase activity, which is required for vesicle formation during autophagy and the cytoplasm-to-vacuole targeting pathway. Identified mutation led to a 40% increase in ethanol production from xylose as compared to the parental strain. However, the mechanism of such regulation remains unknown (Kurylenko et al. 2016a).

Further possible increase in ethanol yield and productivity from xylose in *O. polymorpha* is hampered by the lack of the knowledge of the regulation of xylose metabolism and fermentation. Therefore, it is important to identify the corresponding genes and, depending on their functions, activate or repress them. Xylose is a unique carbon source that can be fermented to ethanol, similarly to glucose, and simultaneously able to be converted to glucose and other hexoses, mostly in PPP though partial contribution of gluconeogenesis in hexose synthesis from xylose cannot be neglected.

The *CAT8* gene codes for a zinc-finger cluster protein that regulates at least 30 genes that are involved in gluconeogenesis, ethanol utilization, glyoxylate cycle, and diauxic shift from fermentation to respiration (Haurie et al. 2001). The roles of *CAT8* gene in the regulation of cell metabolism are well understood in *S. cerevisiae*. It was shown that the deletion of this gene in *S. cerevisiae* and *P. guilliermondii* activated glucose alcoholic fermentation though maximally achieved level of ethanol in the latter species was still very low (Watanabe et al. 2013; Qi et al. 2014). The role of *CAT8* in regulation of xylose metabolism was poorly understood. Transcriptome analysis of the natural xylose-metabolizing yeast *O. polymorpha* did not find changes in *CAT8* expression between xylose- and glucose-containing media (Kim et al. 2013b).

To define the role of Cat8 transcriptional factor in xylose fermentation, *cat8Δ* knockout mutants in *O. polymorpha* were constructed and analyzed from either wild-type or ethanol overproducing (from xylose) (Kurylenko et al. 2016a). In *O. polymorpha*, *CAT8* deletion did not lead to any significant changes in ethanol production from glucose, while a considerable increase in xylose alcoholic fermentation was observed (Fig. 1.3). The cell respiration of *cat8Δ* mutants on xylose was impaired to a much higher extent relative to that on glucose as a substrate. Moreover, the impaired ethanol and glycerol utilization in *cat8Δ* mutants were

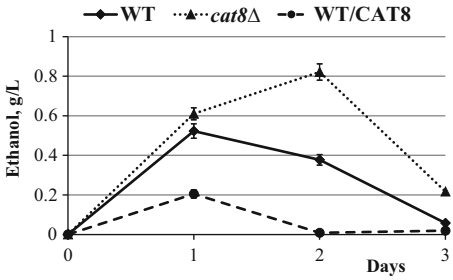

Fig. 1.3 The ethanol production during xylose fermentation of *O. polymorpha* WT, *cat8Δ*, and WT/CAT8 strains. Alcoholic fermentation of yeast strains was fulfilled by cultivation in liquid mineral medium at oxygen-limited conditions at 45 °C. The conditions were provided by agitation at 140 rpm. Nine percent xylose was added into the medium used for the fermentation. The cells were pregrown in 100 mL of liquid YPX medium (1% yeast extract, 2% peptone, and 4% xylose) in 300 mL Erlenmeyer flasks at 220 rpm till the mid-exponential growth phase. Then the cells were precipitated by centrifugation, washed by water, and inoculated into 40 mL of the fermentation medium in 100 mL Erlenmeyer flasks covered with cotton plugs. The initial biomass concentration for fermentation experiments was 2 g (dry weight)/L

observed, suggesting the involvement of *CAT8* in the regulation of gluconeogenesis in *O. polymorpha*, similar to that described for *S. cerevisiae*. Remarkably, growth on xylose of the *cat8Δ* mutant strain was also partially impaired which suggests that xylose can be considered, at least partially, as a gluconeogenic substrate. The slight decrease in the specific activity of fructose-1,6-bisphosphatase in *cat8Δ* mutants was observed suggesting differences in Cat8 action between *S. cerevisiae* and *O. polymorpha*. The drop in other enzymes of gluconeogenesis, e.g., phosphoenolpyruvate carboxykinase, quite possibly determines growth impairment of *O. polymorpha cat8Δ* mutants on gluconeogenic substrates. Overexpression of *CAT8* had opposite effect on xylose alcoholic fermentation as compared to that in *cat8Δ* mutants, as transformants overexpressing *CAT8* gene were characterized by a decrease in ethanol production from xylose (Fig. 1.3).

Thus, the *CAT8* gene is one of the first identified genes that is specifically involved in the regulation of xylose alcoholic fermentation in natural xylose-fermenting yeasts. Mutant *O. polymorpha cat8Δ* isolated from an advanced ethanol producer accumulated 30% more ethanol relative to the parental strain, reaching 12.5 g ethanol/L at 45 °C, which is the highest ethanol titer for high-temperature xylose fermentation (Kurylenko et al. 2016a). The ethanol yield of the constructed *O. polymorpha* recombinant strain (0.34 g/g xylose) is close to that described for *S. stipitis* (0.35–0.44 g/g xylose) (Jeffries et al. 2007) and *Spathaspora passalidarum* (0.42 g/g xylose) (Long et al. 2012). However, this was achieved for *O. polymorpha* at 45 °C, whereas the compared organisms are mesophilic and, therefore, unable to grow and ferment at such a high temperature. Among the thermotolerant ethanol-producing strains, the most promising one is engineered *Kluyveromyces marxianus* strain with ethanol yield 0.38 g/g xylose at 42 °C but lower yield at 45 °C (0.27 g/g xylose). The additional advantage for *O. polymorpha*

recombinant strain in contrast to recombinant *K. marxianus* was that no xylitol accumulation can be observed (Zhang et al. 2015b).

O. polymorpha belongs to the methylotrophic yeasts that are capable for growth on methanol as sole source of carbon and energy. To enable methanol utilization these organisms have evolved highly specialized metabolic pathways that are partly compartmentalized in peroxisomes. The first enzyme of methanol catabolism, alcohol oxidase, catalyzes oxidation of methanol into the two reactive compounds, formaldehyde and hydrogen peroxide. Alcohol oxidase is localized in peroxisomes together with catalase, which decomposes hydrogen peroxide into water and oxygen. A third peroxisomal enzyme of methanol metabolism is dihydroxyacetone synthase (DHAS). DHAS is a component of the xylulose-5-phosphate cycle and catalyzes the transfer of two-carbon moieties from xylulose-5-phosphate to formaldehyde with the production of glyceraldehyde-3-phosphate (an intermediate of glycolysis) and dihydroxyacetone, which after phosphorylation is converted to a glycolytic intermediate (van der Klei et al. 2006). DHAS can also display classical transketolase activity using aldose phosphates (such as ribose 5-phosphate) as the acceptors for the glycolyl group from the donor substrate xylulose 5-phosphate, therefore playing role in xylose utilization through PPP (Lindley et al. 1981). Screening for other enzymes that are putatively involved in xylose utilization in *O. polymorpha* have revealed a peroxisomal transaldolase coded for the gene *TAL2*. The functional roles of peroxisome localized transaldolase and the specific peroxisomal transketolase in xylose utilization and fermentation in *O. polymorpha* remain unclear. To investigate the role of these enzymes in ethanol production during xylose fermentation, the corresponding genes *DAS1* and *TAL2* were overexpressed in *O. polymorpha* NCYC495 strain under control of the strong constitutive promoter of *GAP1* gene (encodes glycerol-3-phospate dehydrogenase) using a plasmid for multicopy integration pGLG61 (van der Klei et al. 2006). The recombinant strains overexpressing *DAS1* and *TAL2* revealed 4.6- and 1.5-fold increase in the specific activity of the corresponding enzymes. The overexpression of *TAL2* gene resulted in a 1.5-fold increase in ethanol production at fourth day of xylose fermentation as compared to the wild-type strain (Fig. 1.4). The effect of the overexpression of *DAS1* gene was more pronounced. A strain overexpressing *DAS1* gene synthesized 2.3-fold higher amount of ethanol than that of the parental strain after 4 days of xylose fermentation (Fig. 1.4) (Kurylenko et al. 2015). Both *das1Δ* and *tal2Δ* mutants did not show growth retardation on xylose as carbon source but were impaired in xylose alcoholic fermentation as compared to the wild-type strain (Fig. 1.4). Overexpression of *DAS1* and *TAL2* genes in *O. polymorpha* in an advanced ethanol producer increased ethanol production by 40% up to 16 g ethanol/L during xylose alcoholic fermentation at 45 °C. As a consequence, it was shown for the first time that peroxisomal enzymes Das1 and Tal2 are involved in the xylose alcoholic fermentation in *O. polymorpha*; however, the functions of peroxisomes during alcoholic fermentation of xylose requires further investigation.

Nevertheless, the ethanol yield in the best obtained *O. polymorpha* advanced ethanol producer obtained by metabolic engineering and classical selection approaches is not high enough for economic feasibility. Further improvement of

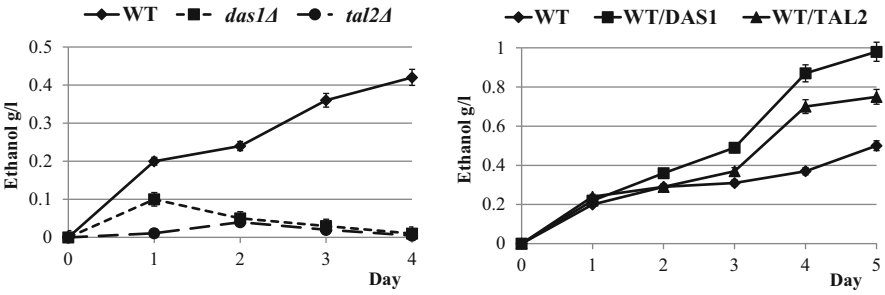

Fig. 1.4 The ethanol production during fermentation of *H. polymorpha* WT, *das1Δ*, *tal2Δ*, WT/DAS1, and WT/TAL2 strains on xylose-containing medium. The fermentation conditions were the same as described in Fig. 1.3

the parameters of alcoholic fermentation of xylose in *O. polymorpha* could be obtained by optimization of the transport of this pentose into cells, amplification of the limiting genes of glycolysis and the PPP, as well as that of the genes determining the resistance to toxic and inhibitory compounds derived after pretreatment of lignocellulosic biomass. At present, the resistance of *H. polymorpha* to toxic products (aldehydes, phenols, acetic, and formic acids) accumulated in lignocellulose hydrolysates under the conditions of acidic hydrolysis has not been precisely studied.

1.5 Concluding Remarks

Commercial strains of *S. cerevisiae* which are characterized by increased ethanol yield from glucose and sucrose (1G ethanol) have been deployed extensively in industrial production. However, apparently all of these strains have some drawbacks. Strains of *S. cerevisiae*, which accumulate more ethanol by cost of biomass, typically show lower robustness and cannot compete with wild-type contaminants during non-sterile production process. Strains that accumulate more ethanol due to lower glycerol production display worse performance being more susceptible to osmotic stress relative to the wild-type strains. Such shortcomings could be overcome by additional metabolic changes.

S. cerevisiae strains constructed for production of 2G ethanol efficiently ferment, in addition to glucose, abundant pentose sugars of lignocellulosic hydrolyzates, xylose, and L-arabinose. Strains fermenting galactose have also been isolated. There are also known strains which could ferment different sugars of hydrolyzates simultaneously due to elaboration of specific xylose transporters and expression of genes responsible for xylose catabolism under control of strong constitutive promoters. Strains resistant to inhibitors of lignocellulosic hydrolyzates are also known. Pilot-plant production of 2G ethanol using engineered *S. cerevisiae* strains has been started.

S. stipitis strains with further improvements of xylose fermentation on lignocellulosic hydrolyzates have been constructed. Such strains have defects in glucose catabolite repression and are more resistant to inhibitors present in hydrolyzates. Still low ethanol tolerance and the need in oxygen for growth are major drawbacks of *S. stipitis* that need to still be addressed. Plans to start pilot plants for 2G ethanol production based on *S. stipitis* are known. If so, such plant could be good platform for further development of *S. stipitis* strains.

O. polymorpha also looks as another promising organism for 2G ethanol production especially as it could ferment at temperatures of 45 °C and higher which is compatible with SSF process. However, level of ethanol production from xylose is still low, glucose inhibits xylose utilization, and fermentation characteristics of glucose and xylose from the real lignocellulosic hydrolyzates are not known. L-arabinose and galactose are not metabolized or fermented by *O. polymorpha*. These questions and problems need to be addressed prior to commercial deployment of this yeast.

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References

Agbogbo FK, Coward-Kelly G, Torry-Smith M, Wenger KS (2006) Fermentation of glucose/xylose mixtures using *P. stipitis*. Process Biochem 41(11):2333–2336

Agbogbo FK, Wenger KS (2006) Effect of pretreatment chemicals on xylose fermentation by *P. stipitis*. Biotechnol Lett 28(24):2065–2069

Almeida JR, Roder A, Modig T, Laadan B, Liden G, Gorwa-Grauslund MF (2008) NADH- vs NADPH-coupled reduction of 5-hydroxymethyl furfural (HMF) and its implications on product distribution in *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 78(6):939–945

Amartey S, Jeffries T (1996) An improvement in *Pichia stipitis* fermentation of acid-hydrolysed hemicellulose achieved by overliming (calcium hydroxide treatment) and strain adaptation. World J Microbiol Biotechnol 12(3):281–283

Amartey SA, Jeffries TW (1994) Comparison of corn steep liquor with other nutrients in the fermentation of D-xylose by *Pichia stipitis* CBS6054. Biotechnol Lett 16(2):211–214

An MZ, Tang YQ, Mitsumasu K, Liu ZS, Shigeru M, Kenji K (2011) Enhanced thermotolerance for ethanol fermentation of *Saccharomyces cerevisiae* strain by overexpression of the gene coding for trehalose-6-phosphate synthase. Biotechnol Lett 33(7):1367–1374

Ansell R, Granath K, Hohmann S, Thevelein JM, Adler L (1997) The two isoenzymes for yeast NAD^+-dependent glycerol 3-phosphate dehydrogenase encoded by *GPD1* and *GPD2* have distinct roles in osmoadaptation and redox regulation. EMBO J 16(9):2179–2187

Bafuelos M, Gancedo C (1978) In situ study of the glycolytic pathway in *Saccharomyces cerevisiae*. Arch Microbiol 117(2):197–201

Bajwa PK, Phaenark C, Grant N, Zhang X, Paice M, Martin VJ, Trevors JT, Lee H (2011) Ethanol production from selected lignocellulosic hydrolysates by genome shuffled strains of *Scheffersomyces stipitis*. Bioresour Technol 102(21):9965–9969

Bajwa PK, Pinel D, Martin VJ, Trevors JT, Lee H (2010) Strain improvement of the pentose-fermenting yeast *Pichia stipitis* by genome shuffling. J Microbiol Methods 81:179–186

Bajwa PK, Shireen T, D'Aoust F, Pinel D, Martin VJ, Trevors JT, Lee H (2009) Mutants of the pentose-fermenting yeast *Pichia stipitis* with improved tolerance to inhibitors in hardwood spent sulfite liquor. Biotechnol Bioeng 104(5):892–900

Balagurunathan B, Jonnalagadda S, Tan L, Srinivasan R (2012) Reconstruction and analysis of a genome-scale metabolic model for *Scheffersomyces stipitis*. Microb Cell Fact 11:27

Basso TO, de Kok S, Dario M, do Espirito-Santo JC, Müller G, Schlölg PS, Silva CP, Tonso A, Daran JM, Gombert AK, van Maris AJ, Pronk JT, Stambuk BU (2011) Engineering topology and kinetics of sucrose metabolism in *Saccharomyces cerevisiae* for improved ethanol yield. Metab Eng 13(6):694–703

Bengtsson O, Hahn-Hägerdal B, Gorwa-Grauslund MF (2009) Xylose reductase from *Pichia stipitis* with altered coenzyme preference improves ethanolic xylose fermentation by recombinant *Saccharomyces cerevisiae*. Biotechnol Biofuels 2:9

Benisch F, Boles E (2014) The bacterial Entner-Doudoroff pathway does not replace glycolysis in *Saccharomyces cerevisiae* due to the lack of activity of iron-sulfur cluster enzyme 6-phosphogluconate dehydratase. J Biotechnol 171:45–55

Bera AK, Ho NW, Khan A, Sedlak M (2011) A genetic overhaul of *Saccharomyces cerevisiae* 424A(LNH-ST) to improve xylose fermentation. J Ind Microbiol Biotechnol 38(5):617–626

Bettiga M, Bengtsson O, Hahn-Hagerdal B, Gorwa-Grauslund MF (2009) Arabinose and xylose fermentation by recombinant *Saccharomyces cerevisiae* expressing a fungal pentose utilization pathway. Microb Cell Fact 8:40

Bicho PA, Runnals PL, Cunningham JD, Lee H (1988) Induction of xylose reductase and xylitol dehydrogenase activities in *Pachysolen tannophilus* and *Pichia stipitis* on mixed sugars. Appl Environ Microbiol 54(1):50–54

Brat D, Boles E, Wiedemann B (2009) Functional expression of a bacterial xylose isomerase in *Saccharomyces cerevisiae*. Appl Environ Microbiol 75(8):2304–2311

Cadiere A, Ortiz-Julien A, Camarasa C, Dequin S (2011) Evolutionary engineered *Saccharomyces cerevisiae* wine yeast strains with increased in vivo flux through the pentose phosphate pathway. Metab Eng 13(3):263–271

Cho JY, Jeffries TW (1998) *Pichia stipitis* genes for alcohol dehydrogenase with fermentative and respiratory functions. Appl Environ Microbiol 64:1350–1358

Clifton D, Fraenkel DG (1981) The gcr (glycolysis regulation) mutation of *Saccharomyces cerevisiae*. J Biol Chem 256(24):13074–13078

Conceicao GJA, Moran PJS, Rodrigues JAR (2003) Highly efficient extractive biocatalysis in the asymmetric reduction of an acyclic enone by the yeast *Pichia stipitis*. Tetrahedron-Asymmetry 14:43–45

Dashtban M, Wen X, Bajwa PK, Ho CY, Lee H (2015) Deletion of *hxk1* gene results in derepression of xylose utilization in *Scheffersomyces stipitis*. J Ind Microbiol Biotechnol 42 (6):889–896

De Kok S, Kozak B, Pronk J, Van Maris A (2012) Energy coupling in *Saccharomyces cerevisiae*: selected opportunities for metabolic engineering. FEMS Yeast Res 12:387–397

Della-Bianca BE, Basso TO, Stambuk BU, Basso LC, Gombert AK (2013) What do we know about the yeast strains from the Brazilian fuel ethanol industry? Appl Microbiol Biotechnol 97:979–991

Dmytruk K, Kshanovska B, Abbas C, Sibirny A (2016) New methods for positive selection of yeast ethanol overproducing mutants. Bioethanol 2:24–31

Dmytruk K, Kshanovska B, Sibirny A (2015) New methods for selection of *Saccharomyces cerevisiae* ethanol overproducing mutants resistant to oxythiamine, trehalose, bromopyruvate, glyoxylic acid and glucosamine. In: Sibirny A, Fedorovyvh D, Gonchar M, Grabek-Lejko D (eds) Living organisms and bioanalytical approaches for detoxification and monitoring of toxic compounds. University of Rzeszow, Rzeszow, pp 237–246

Dmytruk KV, Sibirny AA (2013) Metabolic engineering of yeast *Hansenula polymorpha* for construction of efficient ethanol producers. Tsitol Genet 47(6):3–21

Dmytruk OV, Dmytruk KV, Abbas CA, Voronovsky AY, Sibirny AA (2008a) Engineering of xylose reductase and overexpression of xylitol dehydrogenase and xylulokinase improves xylose alcoholic fermentation in the thermotolerant yeast *Hansenula polymorpha*. Microb Cell Fact 7:21

Dmytruk OV, Voronovsky AY, Abbas CA, Dmytruk KV, Ishchuk OP, Sibirny AA (2008b) Overexpression of bacterial xylose isomerase and yeast host xylulokinase improves xylose alcoholic fermentation in the thermotolerant yeast *Hansenula polymorpha*. FEMS Yeast Res 8 (1):165–173

Du J, Li S, Zhao H (2010) Discovery and characterization of novel D-xylose-specific transporters from *Neurospora crassa* and *Pichia stipitis*. Mol Biosyst 6(11):2150–2156

du Preez JC, Bosch M, Prior B (1986) The fermentation of hexose and pentose sugars by *Candida shehatae* and *Pichia stipitis*. Appl Microbiol Biotechnol 23(3):228–233

du Preez JC, Bosch M, Prior BA (1985) Xylose fermentation by *Candida shehatae* and *Pichia stipitis*: effects of pH, temperature and substrate concentration. Enzyme Microb Technol 8:360–364

du Preez JC, van der Walt JP (1983) Fermentation of D-xylose to ethanol by a strain of *Candida shehatae*. Biotechnol Lett 5:357–362

du Preez JC, van Driessel B, Prior BA (1989) Ethanol tolerance of *Pichia stipitis* and *Candida shehatae* strains in fed-batch cultures at controlled low dissolved-oxygen levels. Appl Microbiol Biotechnol 30:53–58

Farwick A, Bruder S, Schadeweg V, Oreb M, Boles E (2014) Engineering of yeast hexose transporters to transport D-xylose without inhibition by D-glucose. Proc Natl Acad Sci U S A 111(14):5159–5164

Gellisen G (ed) (2002) *Hansenula polymorpha* – biology and applications. Wiley VCH, Weinheim. 347 p

Gellisen G (2000) Heterologous protein production in methylotrophic yeasts. Appl Microbiol Biotechnol 54:741–750

Ghosh A, Zhao H, Price ND (2011) Genome-scale consequences of cofactor balancing in engineered pentose utilization pathways in *Saccharomyces cerevisiae*. PLoS One 6(11): e27316

Gombert AK, van Maris AJ (2015) Improving conversion yield of fermentable sugars into fuel ethanol in 1st generation yeast-based production processes. Curr Opin Biotechnol 33:81–86

Gorsich SW, Dien BS, Nichols NN, Slininger PJ, Liu ZL, Skory CD (2006) Tolerance to furfural-induced stress is associated with pentose phosphate pathway genes *ZWF1*, *GND1*, *RPE1*, and *TKL1* in *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 71(3):339–349

Grootjen DRJ, van der Lans RGJM, Luyben KCA (1990) Effects of the aeration rate on the fermentation of glucose and xylose by *Pichia stipitis* CBS 5773. Enzyme Microb Technol 12:20–23

Guadalupe Medina V, Almering MJH, van Maris AJA, Pronk JT (2010) Elimination of glycerol production in anaerobic cultures of a *Saccharomyces cerevisiae* strain engineered to use acetic acid as an electron acceptor. Appl Environ Microbiol 76(1):190–195

Guebel DV, Cordenons A, Cascone O, Giulietti AM, Nudel C (1992) Influence of the nitrogen source on growth and ethanol production by *Pichia stipitis* NRRL Y-7124. Biotechnol Lett 14 (12):1193–1198

Guerra E, Chye PP, Berardi E, Piper PW (2005) Hypoxia abolishes transience of the heat-shock response in the methylotrophic yeast *Hansenula polymorpha*. Microbiology 151:805–811

Guo ZP, Zhang L, Ding ZY, Shi GY (2011) Minimization of glycerol synthesis in industrial ethanol yeast without influencing its fermentation performance. Metab Eng 13(1):49–59

Ha SJ, Wei Q, Kim SR, Galazka JM, Cate JH, Jin YS (2011) Cofermentation of cellobiose and galactose by an engineered *Saccharomyces cerevisiae* strain. Appl Environ Microbiol 77 (16):5822–5825

Hahn-Hägerdal B, Galbe M, Gorwa-Grauslund MF, Lidén G, Zacchi G (2006) Bio-ethanol–the fuel of tomorrow from the residues of today. Trends Biotechnol 24(12):549–556

Hahn-Hagerdal B, Pamment N (2004) Microbial pentose metabolism. Appl Biochem Biotechnol 113–116:1207–1209

Hahn-Hägerdal B, Wahlbom CF, Gárdonyi M, van Zyl WH, Cordero Otero RR, Jönsson LJ (2001) Metabolic engineering of *Saccharomyces cerevisiae* for xylose utilization. Adv Biochem Eng Biotechnol 73:53–84

Harner NK, Wen X, Bajwa PK, Austin GD, Ho CY, Habash MB, Trevors JT, Lee H (2015) Genetic improvement of native xylose-fermenting yeasts for ethanol production. J Ind Microbiol Biotechnol 42(1):1–20

Haurie V, Perrot M, Mini T, Jenö P, Sagliocco F, Boucherie H (2001) The transcriptional activator Cat8p provides a major contribution to the reprogramming of carbon metabolism during the diauxic shift in *Saccharomyces cerevisiae*. J Biol Chem 276:76–85

Hawkins GM, Ghose D, Russel J, Peterson J (2013) Production of ethanol from high dry matter of pretreated loblolly pine by an evolved strain of *Saccharomyces cerevisiae*. J Bioremed Biodegr 4:195

Hector RE, Qureshi N, Hughes SR, Cotta MA (2008) Expression of a heterologous xylose transporter in a *Saccharomyces cerevisiae* strain engineered to utilize xylose improves aerobic xylose consumption. Appl Microbiol Biotechnol 80(4):675–684

Higgins VJ, Bell PJL, Dawes IW, Attfield PV (2001) Generation of a novel *Saccharomyces cerevisiae* strain that exhibits strong maltose utilization and hyperosmotic resistance using nonrecombinant techniques. Appl Environ Microbiol 67:4346–4348

Hohmann S (2002) Osmotic stress signaling and osmoadaptation in yeasts. Microbiol Mol Biol Rev 66(2):300–372

Hou L (2010) Improved production of ethanol by novel genome shuffling in *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 160(4):1084–1093

Huang CF, Lin TH, Guo GL, Hwang WS (2009) Enhanced ethanol production by fermentation of rice straw hydrolysate without detoxification using a newly adapted strain of *Pichia stipitis*. Bioresour Technol 100:3914–3920

Hughes SR, Gibbons WR, Bang SS, Pinkelman R, Bischoff KM, Slininger PJ, Qureshi N, Kurtzman CP, Liu S, Saha BC, Jackson JS, Cotta MA, Rich JO, Javers JE (2012) Random UV-C mutagenesis of *Scheffersomyces* (formerly *Pichia*) *stipitis* NRRL Y-7124 to improve anaerobic growth on lignocellulosic sugars. J Ind Microbiol Biotechnol 39:163–173

Ilmen M, Koivuranta K, Ruohonen L, Suominen P, Penttilä M (2007) Efficient production of L-lactic acid from xylose by *Pichia stipitis*. Appl Environ Microbiol 73:117–123

Ingledew WM. Alcohol production by *Saccharomyces cerevisiae*: a yeast primer. In: Lyons TP, Kelsall DR, editos. The alcohol textbook, 3rd ed. Nottingham University Press: Nottingham. 1999, pp. 49–87.

Ishchuk OP, Abbas CA, Sibirny AA (2010) Heterologous expression of *Saccharomyces cerevisiae MPR1* gene confers tolerance to ethanol and L-azetidine-2-carboxylic acid in *Hansenula polymorpha*. J Ind Microbiol Biotechnol 37(2):213–218

Ishchuk OP, Voronovsky AY, Abbas CA, Sibirny AA (2009) Construction of *Hansenula polymorpha* strains with improved thermotolerance. Biotechnol Bioeng 104(5):911–919

Ishchuk OP, Voronovsky AY, Stasyk OV, Gayda GZ, Gonchar MV, Abbas CA, Sibirny AA (2008) Overexpression of pyruvate decarboxylase in the yeast *Hansenula polymorpha* results in increased ethanol yield in high-temperature fermentation of xylose. FEMS Yeast Res 8 (7):1164–1174

Jeffries TW, Grigoriev IV, Grimwood J, Laplaza JM, Aerts A, Salamov A, Schmutz J, Lindquist E, Dehal P, Shapiro H, Jin YS, Passoth V, Richardson PM (2007) Genome sequence of the lignocellulose-bioconverting and xylose-fermenting yeast *Pichia stipitis*. Nat Biotechnol 25 (3):319–326

Jeffries TW, Van Vleet JR (2009) *Pichia stipitis* genomics, transcriptomics, and gene clusters. FEMS Yeast Res 9(6):793–807

Jensen P, Snoep J, Westerhoff H 2006 Method of improving the production of biomass or a desired product from a cell. US Patent, 20060094078, A1

Jeppsson M, Johansson B, Hahn-Hägerdal B, Gorwa-Grauslund MF (2002) Reduced oxidative pentose phosphate pathway flux in recombinant xylose-utilizing *Saccharomyces cerevisiae* strains improves the ethanol yield from xylose. Appl Environ Microbiol 68(4):1604–1609

Jin YS, Jones S, Shi NQ, Jeffries TW (2002) Molecular cloning of *XYL3* (D-xylulokinase) from *Pichia stipitis* and characterization of its physiological function. Appl Environ Microbiol 68:1232–1239

Jin YS, Ni H, Laplaza JM, Jeffries TW (2003) Optimal growth and ethanol production from xylose by recombinant *Saccharomyces cerevisiae* require moderate D-xylulokinase activity. Appl Environ Microbiol 69:495–503

Jingping G, Hongbing S, Gang S, Hongzhi L, Wenxiang P (2012) A genome shuffling-generated *Saccharomyces cerevisiae* isolate that ferments xylose and glucose to produce high levels of ethanol. J Ind Microbiol Biotechnol 39(5):777–787

Kaliterna J, Weusthuis RA, Castrillo JI, van Dijken JP, Pronk JT (1995) Coordination of sucrose uptake and respiration in the yeast *Debaryomyces yamadae*. Microbiology 141:1567–1574

Karhumaa K, Hahn-Hägerdal B, Gorwa-Grauslund MF (2005) Investigation of limiting metabolic steps in the utilization of xylose by recombinant *Saccharomyces cerevisiae* using metabolic engineering. Yeast 22:359–368

Karhumaa K, Garcia Sanchez R, Hahn-Hägerdal B, Gorwa-Grauslund MF (2007) Comparison of the xylose reductase-xylitol dehydrogenase and the xylose isomerase pathways for xylose fermentation by recombinant *Saccharomyces cerevisiae*. Microb Cell Fact 6:5

Keating JD, Robinson J, Cotta MA, Saddler JN, Mansfield SD (2004) An ethanologenic yeast exhibiting unusual metabolism in the fermentation of lignocellulosic hexose sugars. J Ind Microbiol Biotechnol 31(5):235–244

Khoury GA, Fazelinia H, Chin JW, Pantazes RJ, Cirino PC, Maranas CD (2009) Computational design of *Candida boidinii* xylose reductase for altered cofactor. Protein Sci 18(10):2125–2138

Kilian SG, van Uden N (1988) Transport of xylose and glucose in the xylose fermenting yeast *Pichia stipitis*. Appl Microbiol Biotechnol 27:545–548

Kim MS, Chung YS, Seo JH, Jo DH, Park YH, Ryu YW (2001) High-yield production of xylitol from xylose by a xylitol dehydrogenase defective mutant of *Pichia stipitis*. J Microbiol Biotechnol 11:564–569

Kim SR, Skerker JM, Kang W, Lesmana A, Wei N, Arkin AP, Jin Y-S (2013a) Rational and evolutionary engineering approaches uncover a small set of genetic changes efficient for rapid xylose fermentation in *Saccharomyces cerevisiae*. PLoS One 8:e57048

Kim OC, Suwannarangsee S, Oh DB, Kim S, Seo JW, Kim CH, Kang HA, Kim JY, Kwon O (2013b) Transcriptome analysis of xylose metabolism in the thermotolerant methylotrophic yeast *Hansenula polymorpha*. Bioprocess Biosyst Eng 36:1509–1518

Kim SK, Jin YS, Choi IG, Park YC, Seo JH (2015) Enhanced tolerance of *Saccharomyces cerevisiae* to multiple lignocellulose-derived inhibitors through modulation of spermidine contents. Metab Eng 29:46–55

Klinner U, Fluthgraf S, Freese S, Passoth V (2005) Aerobic induction of respiro-fermentative growth by decreasing oxygen tensions in the respiratory yeast *Pichia stipitis*. Appl Microbiol Biotechnol 67:247–253

Krahulec S, Klimacek M, Nidetzky B (2012) Analysis and prediction of the physiological effects of altered coenzyme specificity in xylose reductase and xylitol dehydrogenase during xylose fermentation by *Saccharomyces cerevisiae*. J Biotechnol 158(4):192–202

Križanović S, Butorac A, Mrvčić J, Krpan M, Cindrić M, Bačun-Družina V, Stanzer D (2015) Characterization of a S-adenosyl-l-methionine (SAM)-accumulating strain of *Scheffersomyces stipitis*. Int Microbiol 18(2):117–125

Kurtzman CP, Suzuki M (2010) Phylogenetic analysis of ascomycete yeasts that form coenzyme Q-9 and the proposal of the new genera *Babjeviella*, *Meyerozyma*, *Millerozyma*, *Priceomyces*, and *Scheffersomyces*. Mycoscience 51:2–14

Kurtzman CP (1990) *Candida shehatae* – genetic diversity and phylogenetic relationships with other xylose-fermenting yeasts. Antonie Van Leeuwenhoek 57:215–222

Kurylenko O, Ruchala J, Dmytruk K, Sibirny A (2015) New targets for improvement of xylose alcoholic fermentation in the methylotrophic yeast *Hansenula polymorpha*. In: Sibirny A, Fedorovyvh D, Gonchar M, Grabek-Lejko D (eds) Living organisms and bioanalytical approaches for detoxification and monitoring of toxic compounds. University of Rzeszow, Rzeszow, pp 247–257

Kurylenko O, Semkiv M, Ruchala J, Kshanovska B, Abbas C, Dmytruk K, Sibirny A (2016a) New approaches for improving the production of the 1st and 2nd generation ethanol by yeast. Acta Biochim Pol 63(1):1156

Kurylenko OO, Ruchala J, Hryniv OB, Abbas CA, Dmytruk KV, Sibirny AA (2014) Metabolic engineering and classical selection of the methylotrophic thermotolerant yeast *Hansenula polymorpha* for improvement of high-temperature xylose alcoholic fermentation. Microb Cell Fact 13:122

Kurylenko O, Vasylyshyn R, Kata I, Ruchala J, Semkiv M, Dmytruk K, Sibirny A (2016b) Improvement of high-temperature xylose and glucose alcoholic fermentation in methylotrophic yeast *Ogataea (Hansenula) polymorpha* by overexpression of *PDC1* and *ADH1* genes. Visnyk Lviv Univ 73:147–153

Lancashire WE, Dickinson JR, Malloch RA (1998) DNA encoding enzymes of the glycolytic pathway for use in alcohol producing yeast. US Patent, 5786186 A

Laplaza JM, Torres BR, Jin YS, Jeffries TW (2006) *Sh ble* and Cre adapted for functional genomics and metabolic engineering of *Pichia stipitis*. Enzyme Microb Technol 38:741–747

Larsson S, Nilvebrant NO, Jonsson LJ (2001) Effect of overexpression of *Saccharomyces cerevisiae* Pad1p on the resistance to phenylacrylic acids and lignocellulose hydrolysates under aerobic and oxygen limited conditions. Appl Microbiol Biotechnol 57:167–174

Lee H, James AP, Zahab DM, Mahmourides G, Maleszka R, Schneider H (1986) Mutants of *Pachysolen tannophilus* with improved production of ethanol d-xylose. Appl Environ Microbiol 51:1252–1258

Lee SH, Kodaki T, Park YC, Seo JH (2012) Effects of NADH-preferring xylose reductase expression on ethanol production from xylose in xylose-metabolizing recombinant *Saccharomyces cerevisiae*. J Biotechnol 158(4):184–191

Legthelm ME, Prior JC, du Preez JC, Brandt V (1988) An investigation of D-xylose metabolism in *Pichia stipitis* under aerobic and anaerobic conditions. Appl Microbiol Biotechnol 28:293–296

Lennartsson PR, Erlandsson P, Taherzadeh MJ (2014) Integration of the first and second generation bioethanol processes and the importance of by-products. Bioresour Technol 165:3–8

Li H, Shen Y, Wu M, Hou J, Jiao C, Li Z, Liu X, Bao X (2016) Engineering a wild-type diploid *Saccharomyces cerevisiae* strain for second-generation bioethanol production. Bioresour Bioprocess 3(1):51

Lindley ND, Waites MJ, Quayle JR (1981) Simultaneous assay of dihydroxyacetone synthase and transketolase in a methylotrophic yeast grown in continuous culture. A cautionary note. J Gen Microbiol 126(2):253–259

Liu ZL, Moon J, Andersh BJ, Slininger PJ, Weber S (2008) Multiple gene-mediated NAD(P)H-dependent aldehyde reduction is a mechanism of in situ detoxification of furfural and 5-hydroxymethylfurfural by *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 81 (4):743–753

Long TM, Su YK, Headman J, Higbee A, Willis LB, Jeffries TW (2012) Cofermentation of glucose, xylose and cellobiose by the beetle-associated yeast *Spathaspora passalidarum*. Appl Environ Microbiol 78(16):5492–5500

Lu P, Davis BP, Hendrick J, Jeffries TW (1998) Cloning and disruption of the beta-isopropylmalate dehydrogenase gene (*LEU2*) of *Pichia stipitis* with *URA3* and recovery of the double auxotroph. Appl Microbiol Biotechnol 49:141–146

Maassen N, Freese S, Schruff B, Passoth V, Klinner U (2008) Nonhomologous end joining and homologous recombination DNA repair pathways in integration mutagenesis in the xylose-fermenting yeast *Pichia stipitis*. FEMS Yeast Res 8:735–743

Madhavan A, Srivastava A, Kondo A, Bisaria VS (2012) Bioconversion of lignocellulose-derived sugars to ethanol by engineered *Saccharomyces cerevisiae*. Crit Rev Biotechnol 32(1):22–48

Madhavan A, Tamalampudi S, Ushida K, Kanai D, Katahira S, Srivastava A, Fukuda H, Bisaria VS, Kondo A (2009) Xylose isomerase from polycentric fungus *Orpinomyces*: gene sequencing, cloning, and expression in *Saccharomyces cerevisiae* for bioconversion of xylose to ethanol. Appl Microbiol Biotechnol 82(6):1067–1078

Mahler G, Nudel C (2000) Effect of magnesium ions on the fermentative and respirative functions in *Pichia stipitis* under oxygen-restricted growth. Microbiol Res 155:31–35

Matsushika A, Sawayama S (2008) Efficient bioethanol production from xylose by recombinant *Saccharomyces cerevisiae* requires high activity of xylose reductase and moderate xylulokinase activity. J Biosci Bioeng 106:306–309

Melake T, Passoth V, Klinner U (1996) Characterization of the genetic system of the xylose-fermenting yeast *Pichia stipitis*. Curr Microbiol 33:237–242

Metzger MH, Hollenberg CP (1995) Amino acid substitutions in the yeast *Pichia stipitis* xylitol dehydrogenase coenzyme-binding domain affect the coenzyme specificity. Eur J Biochem 228 (1):50–54

Moon J, Liu ZL (2012) Engineered NADH-dependent *GRE2* from *Saccharomyces cerevisiae* by directed enzyme evolution enhances HMF reduction using additional cofactor NADPH. Enzyme Microb Technol 50(2):115–120

Nardi JB, Bee CM, Miller LA, Nguyen NH, Suh SO, Blackwell M (2006) Communities of microbes that inhabit the changing hindgut landscape of a subsocial beetle. Arthropod Struct Dev 35:57–68

Navas MA, Cerdan S, Gancedo JM (1993) Futile cycles in *Saccharomyces cerevisiae* strains expressing the gluconeogenic enzymes during growth on glucose. Proc Natl Acad Sci U S A 90:1290–1294

Navas MA, Gancedo JM (1996) The regulatory characteristics of yeast fructose-1,6-bisphosphatase confer only a small selective advantage. J Bacteriol 178:1809–1812

Nguyen NH, Suh SO, Marshall CJ, Blackwell M (2006) Morphological and ecological similarities: wood-boring beetles associated with novel xylose-fermenting yeasts, *Spathaspora passalidarum gen. sp. nov*. and *Candida jeffriesii sp. nov*. Mycol Res 110:1232–1241

Ni H, Laplaza JM, Jeffries TW (2007) Transposon mutagenesis to improve the growth of recombinant *Saccharomyces cerevisiae* on D-xylose. Appl Environ Microbiol 73:2061–2066

Nigam JN (2001) Development of xylose-fermenting yeast *Pichia stipitis* for ethanol production through adaptation on hardwood hemicellulose acid prehydrolysate. J Appl Microbiol 90:208–215

Nigam PS, Singh A (2011) Production of liquid biofuels from renewable resources. Prog Energy Combust Sci 37:52–68

Nijland JG, Shin HY, de Jong RM, de Waal PP, Klaassen P, Driessen AJ (2014) Engineering of an endogenous hexose transporter into a specific d-xylose transporter facilitates glucose-xylose co-consumption in *Saccharomyces cerevisiae*. Biotechnol Biofuels 7:168

Nijland JG, Vos E, Shin HY, de Waal PP, Klaassen P, Driessen AJ (2016) Improving pentose fermentation by preventing ubiquitination of hexose transporters in *Saccharomyces cerevisiae*. Biotechnol Biofuels 9:158

Nissen TL, Kielland-Brandt MC, Nielsen J, Villadsen J (2000) Optimization of ethanol production in *Saccharomyces cerevisiae* by metabolic engineering of the ammonium assimilation. Metab Eng 2(1):69–77

Ostergaard S, Walloe KO, Gomes SG, Olsson L, Nielsen J (2001) The impact of *GAL6*, *GAL80*, and *MIG1* on glucose control of the GAL system in *Saccharomyces cerevisiae*. FEMS Yeast Res 1:47–55

Palmqvist E, Hahn-Hägerdal B (2000) Fermentation of lignocellulosic hydrolysates. I: Inhibition and detoxification. Bioresour Technol 74:17–24

Parachin NS, Bergdahl B, van Niel EWJ, Gorwa-Grauslund MF (2011) Kinetic modelling reveals current limitations in the production of ethanol from xylose by recombinant *Saccharomyces cerevisiae*. Metab Eng 13:508–517

Parreiras LS, Breuer RJ, Avanasi Narasimhan R, Higbee AJ, La Reau A, Tremaine M, Qin L, Willis LB, Bice BD, Bonfert BL, Pinhancos RC, Balloon AJ, Uppugundla N, Liu T, Li C, Tanjore D, Ong IM, Li H, Pohlmann EL, Serate J, Withers ST, Simmons BA, Hodge DB, Westphall MS, Coon JJ, Dale BE, Balan V, Keating DH, Zhang Y, Landick R, Gasch AP, Sato TK (2014) Engineering and two-stage evolution of a lignocellulosic hydrolysate-tolerant *Saccharomyces cerevisiae* strain for anaerobic fermentation of xylose from AFEX pretreated corn stover. PLoS One 9(9):e107499

Passoth V, Cohn M, Schafer B, Hahn-Hägerdal B, Klinner U (2003) Analysis of the hypoxia-induced *ADH2* promoter of the respiratory yeast *Pichia stipitis* reveals a new mechanism for sensing of oxygen limitation in yeast. Yeast 20:39–51

Passoth V, Hansen M, Klinner U, Emeis CC (1992) The electrophoretic banding patterns of the chromosomes of *Pichia stipitis* and *Candida shehatae*. Curr Genet 22:429–431

Passoth V, Schafer B, Liebel B, Weierstall T, Klinner U (1998) Molecular cloning of alcohol dehydrogenase genes of the yeast *Pichia stipitis* and identification of the fermentative *ADH*. Yeast 14:1311–1325

Passoth V, Zimmermann M, Klinner U (1996) Peculiarities of the regulation of fermentation and respiration in the crabtree-negative, xylose-fermenting yeast *Pichia stipitis*. Appl Biochem Biotechnol 57/58:201–212

Petersson A, Almeida JR, Modig T, Karhumaa K, Hahn-Hägerdal B, Gorwa-Grauslund MF, Lidén G (2006) A 5-hydroxymethyl furfural reducing enzyme encoded by the *Saccharomyces cerevisiae ADH6* gene conveys HMF tolerance. Yeast 23(6):455–464

Piontek M, Hagedorn J, Hollenberg CP, Gellissen G, Strasser AW (1998) Two novel gene expression systems based on the yeasts *Schwanniomyces occidentalis* and *Pichia stipitis*. Appl Microbiol Biotechnol 50:331–338

Qi K, Zhong JJ, Xia XX (2014) Triggering respirofermentative metabolism in the Crabtree-negative yeast *Pichia guilliermondii* by disrupting the *CAT8* gene. Appl Environ Microbiol 80:3879–3887

Quarterman J, Skerker JM, Feng X, Liu IY, Zhao H, Arkin AP, Jin YS (2016) Rapid and efficient galactose fermentation by engineered *Saccharomyces cerevisiae*. J Biotechnol 229:13–21

Radecka D., Mukherjee V, Mateo RQ, Stojiljkovic M, Foulquié-Moreno MR, Thevelein JM (2015) Looking beyond Saccharomyces: the potential of non-conventional yeast species for desirable traits in bioethanol fermentation. FEMS Yeast Res 15(6). pii: fov053

Ren X, Wang J, Yu H, Peng C, Hu J, Ruan Z, Zhao S, Liang Y, Peng N (2016) Anaerobic and sequential aerobic production of high-titer ethanol and single cell protein from NaOH-pretreated corn stover by a genome shuffling-modified *Saccharomyces cerevisiae* strain. Bioresour Technol 218:623–630

Richard P, Londesborough J, Putkonen M, Kalkkinen N, Penttila M (2001) Cloning and expression of a fungal L-arabinitol-4-dehydrogenase gene. J Biol Chem 276(44):40631–40637

Richard P, Putkonen M, Vaananen R, Londesborough J, Penttila M (2002) The missing link in the fungal L-arabinose catabolic pathway, identification of the L-xylulose reductase gene. Biochemistry 41(20):6432–6437

Roca C, Nielsen J, Olsson L (2003) Metabolic engineering of ammonium assimilation in xylose-fermenting *Saccharomyces cerevisiae* improves ethanol production. Appl Environ Microbiol 69(8):4732–4736

Rodrigues R, Lu CF, Lin B, Jeffries TW (2008) Fermentation kinetics for xylitol production by a *Pichia stipitis* D-xylulokinase mutant previously grown in spent sulfite liquor. Appl Biochem Biotechnol 148:199–209

Rogers DT, Szostak JW (1993) Strains of yeast with increased rates of glycolysis. US Patent, 5268285, A1

Ryabova OB, Chmil OM, Sibirny AA (2003) Xylose and cellobiose fermentation to ethanol by the thermotolerant methylotrophic yeast *Hansenula polymorpha*. FEMS Yeast Res 4(2):157–164

Sánchez ÓJ, Cardona CA (2008) Trends in biotechnological production of fuel ethanol from different feedstocks. Bioresour Technol 99(13):5270–5295

Schneider H, Wang PY, Chan YK, Kurtzman CP (1981) Conversion of d-xylose into ethanol by the yeast *Pachysolen tannophilus*. Biotechnol Lett 3:89–92

Semkiv MV, Dmytruk KV, Abbas CA, Sibirny AA (2016) Activation of futile cycles as an approach to increase ethanol yield during glucose fermentation in *Saccharomyces cerevisiae*. Bioengineered 7(2):106–111

Semkiv MV, Dmytruk KV, Abbas CA, Sibirny AA (2014) Increased ethanol accumulation from glucose via reduction of ATP level in a recombinant strain of *Saccharomyces cerevisiae* overexpressing alkaline phosphatase. BMC Biotechnol 14:42

Shahsavarani H, Sugiyama M, Kaneko Y, Chuenchit B, Harashima S (2012) Superior thermotolerance of *Saccharomyces cerevisiae* for efficient bioethanol fermentation can be achieved by overexpression of *RSP5* ubiquitin ligase. Biotechnol Adv 30(6):1289–1300

Shi NQ, Davis B, Sherman F, Cruz J, Jeffries TW (1999) Disruption of the cytochrome c gene in xylose-utilizing yeast *Pichia stipitis* leads to higher ethanol production. Yeast 15:1021–1030

Shi NQ, Jeffries TW (1998) Anaerobic growth and improved fermentation of *Pichia stipitis* bearing a *URA1* gene from *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 50 (3):339–345

Skoog K, Hahn-Hagerdahl B (1990) Effect of oxygenation on xylose fermentation by *Pichia stipitis*. Appl Environ Microbiol 56(11):3389–3394

Slininger PJ, Dien BS, Gorsick SW, Liu ZL (2006) Nitrogen source and mineral optimization enhances D-xylose conversion to ethanol by the yeast *Pichia stipitis* NRRL Y-7124. Appl Microbial Cell Physiol 72(6):1285–1296

Sohn J, Choi E, Kang H, Rhee J, Agaphonov M, Ter-Avanesyan M, Rhee S (1999) A dominant selection system designed for copy-number-controlled gene integration in *Hansenula polymorpha* DL-1. Appl Microbiol Biotechnol 51:800–807

Sprenger GA (1996) Carbohydrate metabolism in *Zymomonas mobilis*: a catabolic highway with some scenic routes. FEMS Microbiol Lett 145:301–307

Sreenath HK, Jeffries TW (1999) 2-deoxyglucose as a selective agent for derepressed mutants of *Pichia stipitis*. Appl Biochem Biotechnol 77:211–222

Sreenath HK, Jeffries TW (1997) Diminished respirative growth and enhanced assimilative sugar uptake result in higher specific fermentation rates by the mutant *Pichia stipitis* FPL-061. Appl Biochem Biotechnol 63–65:109–116

Stanley D, Fraser S, Chambers PJ, Rogers P, Stanley GA (2010) Generation and characterisation of stable ethanol-tolerant mutants of *Saccharomyces cerevisiae*. J Ind Microbiol Biotechnol 37:139–149

Suh SO, Marshall CJ, McHugh JV, Blackwell M (2003) Wood ingestion by passalid beetles in the presence of xylose-fermenting gut yeasts. Mol Ecol 12(11):3137–3145

Tao X, Zheng D, Liu T, Wang P, Zhao W, Zhu M, Jiang X, Zhao Y, Wu X (2012) A novel strategy to construct yeast *Saccharomyces cerevisiae* for very high gravity fermentation. PLoS One 7 (2):e31235

Targonski Z (1992) Biotransformation of lignin-related aromatic-compounds by *Pichia stipitis* Pignal. Zentralbl Mikrobiol 147:244–249

Valadi H, Larsson C, Gustafsson L (1998) Improved ethanol production by glycerol-3-phosphate dehydrogenase mutants of *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 50 (4):434–439

van der Klei IJ, Yurimoto H, Sakai Y, Veenhuis M (2006) The significance of peroxisomes in methanol metabolism in methylotrophic yeast. Biochim Biophys Acta 1763(12):1453–1462

van Dijken JP, van den Bosch E, Hermans JJ, de Miranda LR, Scheffers WA (1986) Alcoholic fermentation by 'nonfermentative' yeasts. Yeast 2:123–127

Veduyn C, Van Kleef R, Frank J, Schreuder H, Van Dijken JP, Scheffers WA (1985) Properties of the NAD(P)H-dependent xylose reductase from the xylose-fermenting yeast *Pichia stipitis*. Biochem J 226(3):669–677

Verho R, Londesborough J, Penttilä M, Richard P (2003) Engineering redox cofactor regeneration for improved pentose fermentation in *Saccharomyces cerevisiae*. Appl Environ Microbiol 69 (10):5892–5897

Voronovsky AY, Rohulya OV, Abbas CA, Sibirny AA (2009) Development of strains of the thermotolerant yeast *Hansenula polymorpha* capable of alcoholic fermentation of starch and xylan. Metab Eng 11:234–242

Voronovsky AY, Ryabova OB, Verba OV, Ishchuk OP, Dmytruk KV, Sibirny AA (2005) Expression of *xylA* genes encoding xylose isomerases from *Escherichia coli* and *Streptomyces coelicolor* in the methylotrophic yeast *Hansenula polymorpha*. FEMS Yeast Res 5 (11):1055–1062

Wahlbom CF, Cordero Otero RR, van Zyl WH, Hahn-Hägerdal B, Jönsson LJ (2003) Molecular analysis of a *Saccharomyces cerevisiae* mutant with improved ability to utilize xylose shows enhanced expression of proteins involved in transport, initial xylose metabolism, and the pentose phosphate pathway. Appl Environ Microbiol 69(2):740–746

Walfridsson M, Bao X, Anderlund M, Lilius G, Bülow L, Hahn-Hägerdal B (1996) Ehanolic fermentation of xylose with *Saccharomyces cerevisiae* harboring the *Thermus thermophilus xylA* gene, which expresses an active xylose (glucose) isomerase. Appl Environ Microbiol 62 (12):4648–4651

Wang PM, Zheng DQ, Liu TZ, Tao XL, Feng MG, Min H, Jiang XH, Wu XC (2012) The combination of glycerol metabolic engineering and drug resistance marker-aided genome shuffling to improve very-high-gravity fermentation performances of industrial *Saccharomyces cerevisiae*. Bioresour Technol 108:203–210

Wang TT, Lin LL, Hsu WH (1989) Cloning and expression of a *Schwanniomyces occidentalis* α-amylase gene in *Saccharomyces cerevisiae*. Appl Environ Microbiol 55:3167–3172

Watanabe D, Hashimoto N, Mizuno M, Zhou Y, Akao T, Shimoi H (2013) Accelerated alcoholic fermentation caused by defective gene expression related to glucose derepression in *Saccharomyces cerevisiae*. Biosci Biotechnol Biochem 77:2255–2262

Watanabe S, Kodaki T, Makino K (2005) Complete reversal of coenzyme specificity of xylitol dehydrogenase and increase of thermostability by the introduction of structural zinc. J Biol Chem 280(11):10340–10349

Watanabe T, Watanabe I, Yamamoto M, Ando A, Nakamura T (2011) A UV-induced mutant of *Pichia stipitis* with increased ethanol production from xylose and selection of a spontaneous mutant with increased ethanol tolerance. Bioresour Technol 102:1844–1848

Wei P, Li Z, He P, Lin Y, Jiang N (2008) Genome shuffling in the ethanologenic yeast *Candida krusei* to improve acetic acid tolerance. Biotechnol Appl Biochem 49:113–120

Weierstall T, Hollenberg CP, Boles E (1999) Cloning and characterization of three genes (*SUT1-3*) encoding glucose transporters of the yeast *Pichia stipitis*. Mol Microbiol 31:871–883

Weusthuis RA, Adams H, Scheffers WA, van Dijken JP (1993) Energetics and kinetics of maltose transport in *Saccharomyces cerevisiae*: a continuous culture study. Appl Environ Microbiol 59 (9):3102–3109

Wijsman MR, Bruinenberg PM, van Diken JP, Scheffers WA (1985) Incapacity for anaerobic growth in xylose-fermenting yeasts. Antonie Van Leeuwenhoek 51:563–564

Wilson AJ, Bhattacharjee JK (1986) Regulation of phosphoenolpyruvate carboxykinase and pyruvate kinase in *Saccharomyces cerevisiae* grown in the presence of glycolytic and gluconeogenic carbon sources and the role of mitochondrial function on gluconeogenesis. Can J Microbiol 32(12):969–972

Wisselink HW, Toirkens MJ, del Rosario Franco Berriel M, Winkler AA, van Dijken JP, Pronk JT, van Maris AJ (2007) Engineering of *Saccharomyces cerevisiae* for efficient anaerobic alcoholic fermentation of L-arabinose. Appl Environ Microbiol 73(15):4881–4891

Xia PF, Zhang GC, Walker B, Seo SO, Kwak S, Liu JJ, Kim H, Ort DR, Wang SG, Jin YS (2016) Recycling carbon dioxide during xylose fermentation by engineered *Saccharomyces cerevisiae*. ACS Synth Biol 6(2):276–283

Yang VW, Marks JA, Davis BP, Jeffries TW (1994) High efficiency transformation of *Pichia stipitis* based on its *URA3* gene and a homologous autonomous replication sequence, *ARS2*. Appl Environ Microbiol 60:4245–4254

Yang XS, Zhang SJ, Zuo Z, Men X, Tian S (2011) Ethanol production from the enzymatic hydrolysis of non-detoxified steamexploded corn stalk. Bioresour Technol 102:7840–7844

Yoon GS, Lee TS, Kim C, Seo JH, Ryu YW (1996) Characterization of alcohol fermentation and segregation of protoplast fusant of *Saccharomyces cerevisiae* and *Pichia stipitis*. J Microbiol Biotechnol 6:286–291

Zhang GC, Liu JJ, Kong II, Kwak S, Jin YS (2015a) Combining C6 and C5 sugar metabolism for enhancing microbial bioconversion. Curr Opin Chem Biol 29:49–57

Zhang GC, Liu JJ, Ding WT (2012) Decreased xylitol formation during xylose fermentation in *Saccharomyces cerevisiae* due to overexpression of water-forming NADH oxidase. Appl Environ Microbiol 78(4):1081–1086

Zhang J, Zhang B, Wang D, Gao X, Sun L, Hong J (2015b) Rapid ethanol production at elevated temperatures by engineered thermotolerant *Kluyveromyces marxianus* via the NADP(H)-preferring xylose reductase-xylitol dehydrogenase pathway. Metab Eng 31:140–152

Zhang L, Tang Y, Guo ZP, Ding ZY, Shi GY (2011) Improving the ethanol yield by reducing glycerol formation using cofactor regulation in *Saccharomyces cerevisiae*. Biotechnol Lett 33 (7):1375–1380

Zhang W, Geng AL (2012) Improved ethanol production by a xylose-fermenting recombinant yeast strain constructed through a modified genome shuffling method. Biotechnol Biofuels 5:46–57

High-Temperature Bioethanol Fermentation by Conventional and Nonconventional Yeasts

2

Hisashi Hoshida and Rinji Akada

Abstract

Fuel ethanol production has dramatically increased in the world. Ethanol fermentation for alcohol beverage is a traditional and mature technology. However, the fermentation technology for fuel ethanol should be reconsidered because it must be large scale and low cost. High-temperature fermentation is the most promising approach for efficient fuel ethanol production from the viewpoints of cost reduction and robustness. In addition, high temperature makes it possible to establish simultaneous saccharification and fermentation process because enzyme activities can be enhanced. For the high-temperature fermentation, thermotolerant ethanol-producing yeasts are necessary. In this chapter, yeast strains suitable for high-temperature bioethanol fermentation are reviewed. Among these, *Kluyveromyces marxianus* achieved sufficient ethanol production in a pilot-scale high-temperature fermentation from starch contained in waste cassava pulp. Studies of the thermotolerant mechanisms will help to construct genetically engineered yeasts for further cost-effective high-temperature ethanol fermentation.

Contents

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2.1 Introduction

Fermentation is a biological process of microorganisms to convert carbohydrates to ethanol or other chemical compounds. Humans have used yeasts for fermentation since ancient times to produce beer, wine, and bread; however, only since the late nineteenth century has the nature and mechanism of yeast fermentation been started to be understood. Louis Pasteur's and Eduard Buchner's findings led to a complete understanding of the enzymatic reactions in the fermentation pathway. Glucose is metabolized to pyruvate by the enzymes in the glycolysis pathway, and the pyruvate is used by the fermentation pathway to produce ethanol under anaerobic conditions or in the presence of high concentrations of sugar. These ethanol fermentation studies have been performed unexceptionally using the yeast *Saccharomyces cerevisiae* because *S. cerevisiae* is commonly used in traditional yeast fermentation.

In addition to the traditional fermentation, ethanol has become one of the most important industrial products in these days because ethanol can be used as an alternative fuel for automobiles instead of gasoline. Ethanol produced via fermentation from biomass is called bioethanol. The bioethanol is a renewable energy because plant biomass is produced by fixation of carbon dioxide. The carbon dioxide generated by combustion of bioethanol is the material originally fixed by plants. Therefore, bioethanol will be the best alternative liquid fuel. Ethanol fermentation has been studied based on brewing and bread fermentation, which require taste and flavor considerations. Instead, fermentation of bioethanol as fuel only requires efficient production. Therefore, bioethanol fermentation for fuel should be reconsidered from the viewpoint of cost, speed, type of biomass, location, and operation conditions. New technologies are being developed in order to increase ethanol production in more economically and environmentally favorable manners, in which nonconventional yeasts will play important roles in addition to the conventional yeast *S. cerevisiae*. Especially, in the bioethanol production process, fermentation at high-temperature environment will be the most desirable process. In this chapter, we describe the advantage of high-temperature

fermentation, the yeasts capable of high-temperature fermentation, simultaneous saccharification and fermentation (SSF) at high temperature, genetic engineering for consolidated bioprocess (CBP), and development of more thermotolerant strains for further efficient high-temperature fermentation.

2.2 Advantages of High-Temperature Fermentation

2.2.1 Cooling Cost

During fermentation, heat is released from microorganisms and reactors. As a result, the fermentation temperature of the broth raises above the optimal temperature as fermentation progresses. Optimal temperatures for various kinds of fermentation depend on the microorganism used in the reactors. In the case of *S. cerevisiae*, the optimal temperature for ethanol fermentation is 30–35 °C. For stable and efficient ethanol production, the reactor must be cooled to maintain the temperature below 35 °C. The biomass used for ethanol fermentation is rich in tropical and subtropical areas, where environmental temperature is not cold enough for cooling. Therefore, fermentation plants require an expensive refrigerant system using additional energy. High-temperature fermentation is defined as fermentation conducted at 5–10 °C higher than the conventional fermentation temperatures. If the bioethanol fermentation is conducted at 45 °C, various costs in fermentation process could be reduced, including investment for refrigerant systems and the cooling energy.

2.2.2 Contamination and Sterilization

Most microorganisms in the environment prefer 25–35 °C for growth, which is also the conventional fermentation temperature for *S. cerevisiae*. Contamination of other microorganisms leads to loss of ethanol production. Complete sterilization of huge amounts of biomass and fermentation facilities for bio-ethanol production is usually impractical. The high temperature in bioethanol fermentation will reduce contamination risk.

2.2.3 Viscosity of Fermentation Broth

Some types of biomass containing high concentrations of carbohydrate, such as starch and cellulose, show high viscosity. This decreases thermal diffusion and dissipation and requires more energy for mixing and cooling. By fermenting at a higher temperature, the viscosity of the broth can be reduced compared to the conventional fermentation.

2.2.4 Robustness Against Accidental Temperature Elevation

During longtime fermentation process, unexpected problems, such as an electricity outage and operation mistake, may happen. Generally, the fermentation system is operated at the maximum temperature to achieve optimal production. Therefore, accidental temperature elevation impairs yeast cell activity and decreases or halts ethanol production. High-temperature fermentation using a thermotolerant microorganism would have a higher limiting temperature than using general *S. cerevisiae* strains, resulting in the robust fermentation process.

2.2.5 Enzyme Activity

Temperature affects enzyme activities. Biomass consisting of polysaccharides, such as starch and cellulose, must be degraded to glucose by enzymes. In the case of starch, it is digested by α-amylases and glucoamylases and the resulting glucose is used for fermentation. In the case of cellulose, end-type cellulases and β-glucosidases are used to generate glucose. Generally, the optimal temperatures for these enzymes are more than 50 °C, which is much higher than the optimal fermentation temperature of the yeast *S. cerevisiae*. The degradation process of oligosaccharides to glucose is called saccharification. To conduct saccharification and fermentation in the optimal conditions, these processes are generally separated. In contrast to this, saccharification and fermentation can be performed simultaneously in the same reactor, which is called simultaneous saccharification and fermentation (SSF). However, the ability of the enzymes to catalyze saccharification is suboptimal because activities of the enzymes are generally about half of their maximal activity at 30–35 °C compared with their optimal temperature. In high-temperature fermentation, the temperature is closer to the optimal temperature for the sugar-degrading enzymes. In high-temperature SSF, saccharification proceeds faster or needs a lower concentration of enzyme (Matsushita et al. 2015). Increased enzymatic activity at high temperatures provides additional value.

The concept of high-temperature fermentation for ethanol production was proposed by Hacking et al. (1984), but it is not yet used in commercial ethanol fermentation. To establish high-temperature fermentation, thermotolerant and ethanol-producing yeasts are essential.

2.3 Isolation of Natural Thermotolerant Ethanol-Producing Yeasts

Many natural isolates, including strains from culture collections, have been tested for thermotolerance and high-temperature ethanol fermentation. In these studies, primary screening conditions to select strains were categorized into four groups: thermotolerant yeast, ethanol-producing yeast, ethanol-producing yeast at high temperature, and one of these with a specific condition. Regardless of the primary

condition, strains of *K. marxianus* and *S. cerevisiae* were often isolated as ethanol-producing yeast at high temperatures, likely because *K. marxianus* is a highly thermotolerant yeast and *S. cerevisiae* is an efficient ethanol-producing yeast. We summarized the ethanol production of thermotolerant ethanol-producing yeasts in Table 2.1.

Hacking et al. (1984) compared 55 efficient ethanol-producing strains from seven genera and found that *K. marxianus* was more thermotolerant, but *S. cerevisiae*, *Saccharomyces uvarum*, and *Candida pseudotropicalis* produced much higher amounts of ethanol at 40 °C. Hughes et al. (1984) evaluated ethanol fermentation ability of a *K. marxianus* strain at 30–48 °C because *K. marxianus* was found to be a thermotolerant strain. The strain showed maximum ethanol production (approximately 65 g/L from approximately 150 g/L glucose) at 30–40 °C. Anderson et al. (1986) isolated *K. marxianus* strains from sugarcane mills, and these strains produced 5.50–6.26% (w/v) ethanol at 45 °C. Szczodrak and Targoński (1988) isolated *K. marxianus* strain (former *Fabospora fragilis*) CCY51–1-1 as the best ethanol producer at high temperatures from 58 yeast strains belonging to the genera *Aureobasidium*, *Candida*, *Cryptococcus*, *Pichia*, *Schizosaccharomyces*, *Hanseniaspora*, *Fabospora*, *Pachysolen*, *Saccharomyces*, *Trichosporon*, and *Torulopsis*. The strain produced 5.6% (w/v) ethanol at 40 and 43 °C from 14% (w/v) glucose. On the basis of these studies, *K. marxianus* is considered a good thermotolerant ethanol-producing yeast. Limtong et al. isolated *K. marxianus* strain DMKU 3–1042 in Thailand and found to produce ethanol well at 40 °C (2007). In the study, strains were enriched in a condition supplemented with 4% ethanol (v/v) as a first step of screening. The ethanol fermentation ability of *K. marxianus* DMKU 3–1042 was compared with those of *K. marxianus* strains NCYC587, NCYC1429, and NCYC2791 (Nonklang et al. 2008). Among the four strains, DMKU 3–1042 was the best ethanol producer at 45 °C. Kumar et al. (2009) isolated *Kluyveromyces* sp. IIPE453 as an ethanol-producing yeast at high temperatures from a soil sample in a sugar mill. The strain showed highest ethanol production rate (g/g/h) at 45–50 °C. The strain was reported to produce ethanol from various types of biomasses, such as sugarcane juice, molasses, and bagasse hydrolysate at 50 °C (Kumar et al. 2010). Rodrussamee et al. (2011) and Nonklang et al. (2008) reported that *K. marxianus* grows by utilizing various types of sugar. However, there was little ethanol production from xylose and none from arabinose as carbon sources.

Several *S. cerevisiae* strains were also isolated for high-temperature fermentation. D'Amore et al. (1989) screened 65 yeast strains containing *S. cerevisiae*, *Saccharomyces byanus*, *K. marxianus*, *Kluyveromyces lactis*, *Candida shehatae*, *Hansenula wingei*, *Saccharomyces rouxii*, and *Phichia stipitis*. The fermentation ability was tested at 40 °C, and a *S. cerevisiae* (former *Saccharomyces diastaticus*) strain showed the highest ethanol production at 40 °C. Kiran Sree et al. (2000) isolated four *S. cerevisiae* strains from a thermal power plant in India. Among the strains, VS_1 and VS_3 grew at 44 °C. Ethanol production by the strains was highest at 30 and 35 °C but decreased over 40 °C. Nuanpeng et al. (2016) isolated *S. cerevisiae* strain DBKKU Y-53 in Thailand. In the screening process, yeast strains were first

Table 2.1 Ethanol production of yeast species at high temperature

Species	Strain	Initial sugar conc. % (w/v)	Type of sugars	Ethanol production[a] (%) Temperature (°C)													Unit of ethanol	Refs.
				35	36	37	38	39	40	41	42	43	44	45	46	50		
Kluyveromyces marxianus																		
	NCYC587	14.5	Glc						**6.3**								v/v	Hacking et al. (1984)
	974	15	Glc											**6.3**			w/v	Anderson et al. (1986)
	FT23[b]	14	Glc						5.4			**5.6**			3.2		w/v	Szczodrak and Targoński (1988)
	FT5[b]	14	Glc						**5.0**			3.6			2.4		w/v	Szczodrak and Targoński (1988)
	CCY51–1-1[c]	14	Glc						**5.6**			**5.6**			3.5		w/v	Szczodrak and Targoński (1988)
	2713	5	Glc						2.1		**2.2**			2.0			w/v	Ballesteros et al. (1991)
	2671[b]	5	Glc						**2.1**		**2.1**			**2.1**			w/v	Ballesteros et al. (1991)
	DMKU3–1042	18	Suc[d]			**7.4**			7.2					4.9			w/v	Limtong et al. (2007)
	NBRC1777	10	Glc						(4.5)		**(4.6)**			(4.2)		(2.0)	w/v	Yanase et al. (2010)
	IIPE453[e]	20	Glc													**8.2**	w/v	Kumar et al. (2009)

Saccharomyces cerevisiae																		
	YSa86	13.5	Glc						**8.0**								v/v	Hacking et al. (1984)
	FTJa(a)	14	Glc						**4.3**			3.7			1.9		v/v	Szczodrak and Targoński (1988)
	LB62[f]	15	Glc						**6.4**			(4.8)		(1.2)			w/v	D'Amore et al. (1989)
	VS1	15	Glc	**6.6**					5.5		5.0		4.0				w/v	Kiran Sree et al. (2000)
	VS3	15	Glc	**7.5**					6.0		5.8		5.8				w/v	Kiran Sree et al. (2000)
	DBKKU Y-53	22	SSJ[g]			**7.1**			5.8		3.2			0.91			w/v	Nuanpeng et al. (2016)
	C3867	10	Glc			3.0				**3.9**							w/v	Auesukaree et al. (2012)
Saccharomyces uvarum																		
	YSa85	14.5	Glc						**8.2**								v/v	Hacking et al. (1984)
Saccharomyces carlsbergensis																		
	FT14	14	Glc						**5.4**			2.1			1.5		w/v	Szczodrak and Targoński (1988)
Ogataea polymorpha																		
	356	4	Glc			**1.3**			**1.3**			0.79			0.24		w/v	Ryabova et al. (2003)

(continued)

Table 2.1 (continued)

Species	Strain	Initial sugar conc. % (w/v)	Type of sugars	Ethanol production[a] (%) Temperature (°C) 35	36	37	38	39	40	41	42	43	44	45	46	50	Unit of ethanol	Refs.
Candida pseudotropicalis																		
	YCa9	14.2	Glc						**8.6**								v/v	Hacking et al. (1984)
Candida acidothermophilum																		
	ATCC 20381	5	Glc			(2.2)			**(2.5)**					(2.3)		(0.6)	w/v	Kadam and Schmidt (1997)
Pichia kudriavzevii																		
	KVMP10	10	Mixed[h]			3.9			4.0		**5.4**			1.0			w/v	Koutinas et al. (2015)
	DMKU 3-ET15	18	Glc	7.5					**7.9**		7.7			3.8			w/v	Yuangsaard et al. (2013)
Unknown																		
	RND13	15	Glc						**6.6**			6.0					w/v	Ueno et al. (2002)

[a]Bold—maximum ethanol production was achieved at the temperature, parentheses—value was read from a graph, underlined—data was shown only at the temperature
[b]*K. fragilis*
[c]*Fabospora fragilis*
[d]Sugarcane juice supplemented with sucrose
[e]*Kluyveromyces* sp.
[f]*Saccharomyces diastaticus*, Labatt Brewing culture number
[g]Sweet sorghum juice
[h]Galactose, fructose, glucose, and sucrose

enriched at 35 °C in the presence of 4% ethanol. The strain grew well and produced ethanol at ~42 °C, but maximum production was achieved at 37 °C in sweet sorghum juice.

Several other thermotolerant ethanol-producing strains were also identified from culture collections or isolated from various environments. *Ogataea polymorpha* (former *Hansenula polymorpha*) is a thermotolerant yeast. From six *O. polymorpha* strains tested, several strains produced over 9 g/L ethanol from 2% sucrose at 37 °C (Ryabova et al. 2003). *Candida* species also produced ethanol at relatively high temperatures. *C. tropicalis* strain TH4 was isolated from an Indonesian traditional beverage as an ethanol-producing yeast and produced 4.6% (v/v) ethanol at 42 °C. However, the production was decreased by 30% compared with fermentation at 30 °C (Hermansyah et al. 2015). *Candida acidothermophilum* is reported as a thermotolerant yeast and produced ethanol at 70–80% of the theoretical yield from 5% glucose at 37–45 °C (Kadam and Schmidt 1997). *Pichia kudriavzevii* strains were recently isolated as a thermotolerant ethanol-producing yeast. Strain DMKU 3-ET15 was isolated from traditional fermented pork sausage in Thailand through an enriched process at 40 °C in a medium with 4% ethanol. The strain produced ethanol at approximately 7.7% (w/v) from 18% glucose at 35–42 °C (Yuangsaard et al. 2013). Strain KVMP10 was isolated for orange peel fermentation and was reported to produce 5.4% (w/v) ethanol at 42 °C in a model medium containing ~10% total sugars (Koutinas et al. 2015). From a hot spring drainage in Japan, a thermotolerant yeast was isolated and produced ethanol 6.6% (w/v) from 15% glucose at 40 °C (Ueno et al. 2002). The species was not determined, but rDNA sequence suggested that the strain was similar to *Candida glabrata* and *Kluyveromyces delphensis*. Strains of *Kluyveromyces lactis*, *Cryptococcus neoformans*, *Candida albicans*, and *Debaryomyces hansenii* are reported to be thermotolerant yeasts (Nedeva et al. 1993; Arguelled 1997; Takashima et al. 2009; Prakash et al. 2011). However, these species have little or no ethanol fermentation ability and have not been studied with regard to high-temperature ethanol fermentation. However, resources in the world remain to be investigated for the isolation of thermotolerant ethanol-producing yeasts.

In Table 2.1, optimum ethanol production temperatures of each strain were shown in bold numbers for comparison between species. The optimum fermentation temperature for the strains is approximately 40 °C. Some *K. marxianus* strains showed best ethanol production ability or kept fermentation ability at higher temperatures than 40 °C, suggesting that *K. marxianus* is a better species for high-temperature ethanol fermentation.

2.4 Yeast Immobilization for High-Temperature Fermentation

To perform high-temperature fermentation, cell immobilization has been studied. A thermotolerant strain (RA-74-2) of *S. cerevisiae* was immobilized in alginate gel beads. Ethanol production of the immobilized cells was higher than that of free cells at 26–42 °C (Sohn et al. 1997). The immobilized cells also produced ethanol

efficiently in three rounds of fermentation at 40 °C. Interestingly, some cells in beads were lost during 40 h of fermentation at 43 °C, but the number of cells increased during the following fermentation at 37 °C. By decreasing the fermentation temperature, fermentation activity of the immobilized cells can be recovered. Ylitervo et al. (2011) encapsulated *S. cerevisiae* CBS 8066 into alginate-chitosan capsules. The encapsulated cells consumed almost 3% of glucose in the medium and converted it to ethanol at more than 85% yield at 42 °C over seven repeated fermentation cycles, although free cells completely lost glucose-consuming activity after two rounds. In addition, the maximum temperature of ethanol fermentation increased from 42 to 45 °C by encapsulation. In another study, *S. cerevisiae* AXAZ-1 was immobilized in brewer's spent grains, and the immobilized cells were used for fermentation 37 times repeatedly at various temperatures (Kopsahelis et al. 2009). For comparison, free cells were also tested in repeated fermentations. In the repeated fermentations, molasses at 12% (w/v) of initial sugar concentration was used as a substrate. Ethanol concentration and conversion rate in each batch fermentation were similar between immobilized cells and free cells at 30–35 °C. In contrast, fermentation time required to consume the sugars with immobilized cells was around half that of free cells. In addition, the effect of immobilization against temperature was also observed. Immobilized cells produced 5.5–6.8% (v/v) ethanol during 14 rounds of fermentation at 38 and 40 °C. In free cells, ethanol production decreased and fermentation time was extended during repeated fermentations at 38 °C. These reports indicate that immobilization protects yeast cells in high-temperature fermentation.

2.5 High-Temperature SSF

SSF can make ethanol in more simple way and shorter time, but the different optimal temperatures between saccharification and fermentation is a barrier to its industrial application. Attempt of high-temperature SSF is summarized in Table 2.2.

S. cerevisiae NKU5377 produced ethanol from waste paper in SSF, and ethanol production was higher at 40 °C than at 30 and 37 °C (Park et al. 2010). Liu et al. (2014) demonstrated that ethanol production and fermentation speed in a SSF system using corn stover increased on elevating the temperature from 30 to 39 °C. However, a high concentration of glucose remained at 36 and 39 °C, suggesting that saccharification was enhanced but glucose consumption by *S. cerevisiae* is not fast enough at high temperatures. In SSF with *S. cerevisiae* VS_3 strain using starchy materials as feedstock, ethanol production at 42 °C was lower than that at 37 °C, likely due to the lower thermotolerance of *S. cerevisiae* at 42 °C (Sree et al. 1999).

SSF with *K. marxianus* L. G. strain at 42 °C produced 37.6 g/L ethanol from 10% of a microcrystalline cellulose in 78 h, but later reducing sugars released by enzyme hydrolysis started to accumulate in the medium (Ballesteros et al. 1991). Castro and Roberto (2014) compared ethanol production (g/L/h) between

Table 2.2 Ethanol production in high-temperature SSF

Strain	Biomass		Temp. (°C)	Ethanol production		Enzyme		Remarks	Refs.
Saccharomyces cerevisiae									
80000012[a]	Corn stover		39	33.1	g/L	30	FPU/g glucan	With pre-hydrolysis	Liu et al. (2014)
KNU5377	Waste newsprint	250 g/L	50→40	8.4	% (v/v)	140	FPU/g paper	With pre-hydrolysis	Park et al. (2010)
VS_3	Sweet Sorghum	100 g	37	8.2	g/g substrate	1300	U/g enzyme		Sree et al. (1999)
Kluyveromyces marxianus									
L. G.	Solka-floc	10%	42	37.6	g/L	15	FPU/g		Ballesteros et al. (1991)
NRRL Y-6860	Rice straw	8%	45	11.5	g/L	6.25	FPU	+6.25 U β-glucosidase/g substrate	Castro and Roberto (2014)
NCIM 3358	Solka-floc	10%	43	3.0	% (w/v)	40	FPU/g		Hari Krishna et al. (2001)
Y. 01070	Spruce chip	5%	42	7.5	g/L	37	FPU/g	+381 U β-glucosidase/g cellulose	Bollók et al. (2000)
IMB4	Switchgrass	41 g/L	45	16.6	g/L	15	FPU/g glucan		Suryawati et al. (2008)
Candida glabrata									
NFRI3164	Avicel	5%	42	14	g/L	500	CMC U/g Avicel		Watanabe et al. (2010)
Candida acidothermophilum									
ATCC 20381	Poplar	6% (3.5% cellulose)	42	18	g/L	25	FPU/g		Kadam and Schmidt (1997)

[a]Angel Yeast Co. Ltd

K. marxianus and *S. cerevisiae* in SSF of rice straw. Maximum ethanol concentration was similar, but only in the case of *K. marxianus*, productivity was enhanced at 45 °C than at 30 °C. In addition, glucose remained in the medium of *S. cerevisiae* at 45 °C. Higher temperature is preferred for SSF but 45 °C is too high for *S. cerevisiae*. *K. marxianus* IMB4 strain was used for SSF of switchgrass at 37, 41, and 45 °C. Ethanol production increased by elevating the temperature, and the highest ethanol production was achieved at 45 °C with initial pH at 5.5 (Suryawati et al. 2008). *K. marxianus* NCIM 3358 also showed better ethanol production than *S. cerevisiae* NRRL-Y-132 in high-temperature SSF (Hari Krishna et al. 2001). NCIM 3358 produced 3.0% (w/v) ethanol from microcrystalline cellulose at 43 °C in the presence of cellulase while NRRL-Y-132 produced 2.3% (w/v) ethanol. In addition, ethanol production was enhanced by the addition of β-glucosidase to 3.5% with the *K. marxianus* strain, suggesting that efficient conversion to glucose is required for a higher ethanol yield.

Candida yeasts were also examined for high-temperature SSF. A *Candida glabrata* wild-type strain produced 14 g/L ethanol in SSF at 42 °C using 5% microcrystalline cellulose as a substrate with agitation at 150 rpm. A respiratory-deficient strain produced 17 g/L ethanol in the same condition (Watanabe et al. 2010). *C. acidothermophilum* produced ethanol faster at 40 °C than 37 °C from acid-treated poplar (Kadam and Schmidt 1997). In the study, it was estimated that cellulase-related cost reduction by increasing temperature in SSF was 16% at 40 °C, 35% at 45 °C, and (if the yeast produces ethanol efficiently) 51% at 50 °C compared with 37 °C.

A xylose-fermenting genetically engineered *S. cerevisiae* (strain RWB222) was used for SSF of paper sludge containing a significant amount of xylan as well as glucan. The ethanol production was 45.2 g/L at 37 °C and 4.01 g/L at 30 °C. The paper sludge used in the study contained 82 g/L glucan and 22 g/L xylan. Final ethanol yield was higher than the theoretical complete conversion of the glucan, indicating that xylose released from xylan was also utilized for ethanol production (Zhang and Lynd 2010). High-temperature SSF has been studied in many thermotolerant yeast strains. However, if the final production level was low, the following distillation cost becomes much higher. Therefore, high performance of yeasts and suitable process are required for practical high-temperature SSF.

2.6 Pilot-Scale High-Temperature SSF Using *K. marxianus*

These days, cornstarch and sugarcane are major feedstock for bioethanol production. However, consumption of these "food" biomass for ethanol production causes an economic problem. Therefore, lignocellulosic material has received much attention as feedstock because it is nonedible. However, industrially efficient ethanol production from lignocellulose is still under development.

Cassava pulp is nonedible waste in tapioca starch factories. It is very cheap and used as a fertilizer and is an additive in feed. However, cassava pulp contains approximately 50% starch and has the potential to be used for fermentation. In

Thailand, up to 2.6 million tons of cassava pulp is produced per year. It accounts for approximately 10% of the total cassava production in Thailand. Cassava is also cultivated in tropical areas, such as Nigeria and Indonesia. Annual production in the world is more than 200 million tons. If 10% remains as cassava pulp by-product, as is the case in Thailand, approximately 10 million tons of starch is remained to be used as a feedstock in the world. To produce ethanol from cassava pulp efficiently, we developed a high-temperature SSF process using the thermotolerant yeast *K. marxianus*.

First, we isolated thermotolerant yeast strains (Hoshida et al. 2012). Samples such as fruits, flowers, waste from alcoholic beverages, and a filter cake in a sugarcane factory were collected in Thailand. After enrichment in sugarcane broth at 45 °C, thermotolerant yeasts growing at 50 °C were isolated. Among the isolates, seven strains showed better growth compared with *K. marxianus* DMKU 3–1042, which was previously isolated as a good ethanol-producing yeast at high temperatures (Limtong et al. 2007; Nonklang et al. 2008). Ethanol production abilities of the seven strains at 45 °C in molasses were also comparable with DMKU 3–1042. As a result, strain UBU 1-1 was selected. The draft genome sequence of UBU 1-1 strongly suggested that it is a *K. marxianus* strain.

In laboratory-scale experiments, minimum amounts of enzymes for efficient ethanol production from cassava pulp in SSF were determined. Cassava pulp was hydrated, treated with α-amylase (0.50 g/kg-pulp of Liquozyme® SC, Novozyme), and autoclaved. The liquefied cassava pulp was mixed with 0.23 g glucoamylase/kg pulp (Spirizyme® Fuel, Novozyme) and 6.67 g cellulase/kg pulp of (Accellerase® DUET, Genecor). Cellulase was added mainly to loosen cellulosic fibers and to improve the access of glucoamylase to starch. The amounts of glucoamylase and cellulase were lower than the levels recommended in the instructions, and increased amounts of glucoamylase and cellulase to twofold and tenfold, respectively, did not enhance ethanol production speed or final ethanol concentration. By using *K. marxianus* UBU 1-1 strain, cassava pulp (25% w/w water) was fermented in a 2-L SSF system at 40 °C. As a result, 7.8% ethanol was produced in 42 h. In a 20 L scale, 8.5% (w/v) was produced in 48 h of fermentation at 40 °C. The viscosity of the cassava pulp slurry was very high (24,000–200,000 mPa s) at the fermentation start point. During fermentation, the viscosity of the fermentation broth became lower than we expected. In addition to the degradation of starch and cellulose, polygalacturonase activity produced by *K. marxianus* helped to lower viscosity by hydrolyzing pectin in the cassava pulp. In a larger scale fermentation, high viscosity of the fermentation broth in the early phase might have been a problem, causing low heat diffusion and inefficient mixing of enzymes.

To demonstrate that high-temperature SSF of cassava pulp by using *K. marxianus* UBU 1-1 is possible in more large-scale fermentation, a 10 kL size reactor was built in Thailand. The average maximum temperature in the environment is approximately 33 °C in Thailand. Even in such a climate, temperature of the reactor was controlled via circulating water, which was circulated through a cooling tower but not chilled. Wet cassava pulp is easily utilized for fermentation, but its starch contents were not enough for an economically enough concentration of

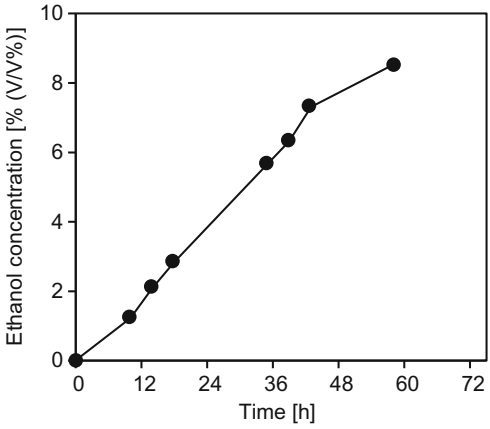

Fig. 2.1 Ethanol production in a 10 kL scale SSF at 40 °C using *K. marxianus* and cassava pulp as feedstock

ethanol production. Therefore, wet and dry cassava pulp were mixed and used as a substrate. Utilization of dried pulp is preferable for whole-year production because wet pulp is easily spoiled by contamination of microorganisms. Starch contents of cassava pulp used in the pilot tests were sometimes lower than 50% (w/dry weight). In such cases, dried cassava contents were increased. We conducted over 68 rounds of batch fermentations to optimize ethanol fermentation conditions and pretreatment. Optimum fermentation conditions were determined as follows: Dry and wet cassava pulp was pulverized to an average particle size of approximately 200 μm. Pulverized pulp was mixed with water and steamed at 100–105 °C for hydration. After cooling, glucoamylase and cellulase were added, and fermentation was carried out at 40 °C with *K. marxianus* UBU 1-1. At the end of fermentation, the viscosity of the broth decreased to 100 mPa s. Typical ethanol production during the high-temperature SSF is shown in Fig. 2.1. More than 8% ethanol, which was the target concentration for this proof-of-concept study, was produced within 60 h.

2.7 Consolidated Bioprocessing by Genetically Engineered Yeasts

The cost of enzymes in SSF is a significant consideration, especially for cellulose utilization. The fermentation process, in which polysaccharides are degraded by enzymes secreted by the organism for fermentation and resulting sugars are used for fermentation, is called consolidated bioprocessing (CBP). The concept of CBP may be first used in *K. marxianus*, because *K. marxianus* produces a large amount of inulinase, which degrades inulin to fructose (Margaritis and Bajpai 1982). Inulin is a polysaccharide mainly composed of fructoses, which bind via β2,1 glycosyl bonds. Inulinase degrades the β2,1 glycosyl bonds and releases fructose efficiently from inulin. The Jerusalem artichoke is a plant that accumulates inulin in its tuber. Margaritis and Bajpai (1982) reported CBP producing 46 g/L ethanol from Jerusalem artichoke juice (10% total sugar) using *K. marxianus* at 35 °C. This

report was published prior to the identification of *K. marxianus* as a thermotolerant yeast for high-temperature fermentation in 1984 by Hacking et al. Charoensopharat et al. (2015) reported high-temperature CBP of Jerusalem artichoke by a newly isolated *K. marxianus* DBKKU Y-102. The strain produced 90.6 and 85.4 g/L ethanol from 230 g/L of total sugar of tuber mash at 37 and 40 °C, respectively. To produce hydrolyzing enzymes by yeasts, genetic engineering was applied. First, *S. cerevisiae* was engineered to express a glucoamylase gene. A strain produced 19.6 g/L ethanol from 58.5 g/L carbohydrate in feed and with a yield 0.39 g/g consumed glucose from soluble starch in continuous fermentation at 30 °C (Inlow et al. 1988).

Genetic engineering for high-temperature CBP has often been performed in thermotolerant yeasts. In *K. marxianus*, three genes encoding thermostable secretory cellulases, endo-β-1,4-glucanase, cellobiohydrolase, and β-glucosidase, were introduced. The resulting strain secreted these cellulases but resulted in little ethanol production from carboxymethyl cellulose (CMC). On the other hand, cellobiose was a good sugar for the strain to produce ethanol; a maximum ethanol concentration of 43.4 g/L was achieved from 10% cellobiose at 45 °C (Hong et al. 2007). Yanase et al. (2010) expressed cellulases on *K. marxianus* cell membrane by fusing the 3′ region of the α-agglutinin gene. As a result, the resulting K1 strain produced 4.25 g/L ethanol from 10 g/L β-glucan at 48 °C in 12 h by using a large number of pre-cultured cells. Similar amounts of ethanol were also produced at 40 and 45 °C in 24 h fermentation.

Other thermotolerant yeasts were also engineered for CBP at high temperatures. An α-amylase gene and a glucoamylase gene of yeast *Schwanniomyces occidentalis* were expressed in *O. polymorpha* in order to utilize starchy material directly. An engineered strain produced 10 g/L ethanol from 3% soluble starch at 47 °C (Voronovsky et al. 2009). A strain of *O. polymorpha* expressing xylan-hydrolyzing enzymes was also constructed. It grew by utilizing xylan as a sole carbon source, but ethanol production was low (Voronovsky et al. 2009). Kitagawa et al. (2010) developed a transformation and heterologous expression system and expressed a β-glucosidase in *Issatchenkia orientalis*. The engineered strain produced 29 g/L ethanol in a CBP at 40 °C using 10% microcrystalline cellulose as a substrate.

S. cerevisiae has also been engineered for CBP, but it is not practically high temperature. Engineered strains expressing endo-/exo-glucanase and β-glucosidase were reported by Cho and Yoo (1999), Den Haan et al. (2007), Wen et al. (2010), and Hu et al. (2016) for CBP using cellulosic substrates. However, CBP temperature of the studies were lower than 38 °C, therefore we do not describe them in this chapter.

2.8 Strain Improvement for High-Temperature Fermentation

S. cerevisiae is used for industrial ethanol fermentation due to its high ethanol production in conventional fermentation. Taking advantage of this ability in high-temperature fermentation, strain improvements of *S. cerevisiae* strains have been conducted (Table 2.3). Sridhar et al. (2002) treated *S. cerevisiae* VS_1 and VS_3

Table 2.3 *S. cerevisiae* strain improvement by mutagenesis and laboratory evolution

Strain name		Mutagenesis	Screening condition	Improvement of high-temperature ethanol production			Refs.
Parent	Mutant			Parent (g/L)	Mutant (g/L)	Condition	
VS_1	UV-VS_1 40	UV	42 °C	50	58	42 °C, 250 g/L glucose	Sridhar et al. (2002)
ATCC 26602	MT15	UV	45 °C	55[a]	72	40 °C, 150 g/L glucose	Rajoka et al. (2005)
FAS	MLD10	UV, gray	45 °C, deoxy-glucose	48	85	43 °C, 180 g/L sugars	Arshad et al. (2014)
S288C	Y43	HEPEB[b]	45 °C	0	79	43 °C, 200 g/L sugars	Zhang et al. (2011)
IR2	IR2-9a	Evolution	42 °C	16	28	40 °C, SSF of 10% substrate	Edgardo et al. (2008)
p1 (D3)	S1	Evolution, UV, EMS	50, 55 °C	–	46	40 °C, 100 g/L glucose	Balakumar et al. (2001)
WW	AT	Evolution, mating	42 °C	52	66	41 °C, 150 g/L glucose	Mitsumasu et al. (2014)

[a]Value was read from a graph
[b]High-energy-pulse-electron beam

strains with ultraviolet (UV) radiation and selected mutants growing well at 42 °C. Growth of a mutant named UV-VS_1 100 was improved under high concentrations of glucose, and the strain produced 5.8% (w/v) ethanol from 250 g/L glucose at 42 °C. UV mutagenesis of *S. cerevisiae* ATCC 26602 generated a mutant showing 1.45-fold higher productivity (7.2 g L^{-1} h^{-1}) compared with the parental strain (4.4 g L^{-1} h^{-1}) (Rajoka et al. 2005). Zhang et al. (2011) used high-energy-pulse-electron beam for mutagenesis of *S. cerevisiae* S288C. Strain S288C did not grow or produce ethanol at 43 °C, but a mutant strain Y43 produced approximately 8% (w/v) ethanol. Arshad et al. (2014) combined and repeated mutagenesis with UV and γ-ray to obtain thermotolerant and catabolite-resistant mutants. Ethanol production of the most productive mutant strain (MLD10) reached 8.5% (w/v) from 18% fermentable sugars at 42 °C, while the parental strain produced 4.8% (w/v) in the same condition.

Another approach is laboratory evolution at high temperatures. Edgardo et al. (2008) conducted laboratory evolution by culturing yeast cells repeatedly from 40 to 48 °C. As a result, a mutant strain was obtained that showed higher ethanol yield at 40 °C in SSF. Balakumar et al. (2001) combined UV and ethyl methanesulfonate (EMS) mutagenesis with laboratory evolution. The finally obtained mutant strain (S1) produced ethanol 4.6% (w/v) at 40 °C within 36 h from 10% glucose. Mitsumasu et al. (2014) used a strain for laboratory evolution at high temperature and low pH, and evolved strains were mated. The resulting thermo- and acid-tolerant strain (AT) produced 6.58 g/L of ethanol at 41 °C within 24 h, though the parental WW strain produced 5.19 g/L.

UV mutagenesis was also conducted on *K. marxianus*. The *K. marxianus* strain 8–1 was obtained from NRRLY-1109 after UV-C radiation and produced 2.25% (w/v) ethanol at 46 °C, though the parental strain produced 1.75% (Hughes et al. 2013). Sakanaka et al. (1996) applied protoplast fusion of *K. marxianus* and *S. cerevisiae*. Ethanol production by the strain F-1 obtained was higher than that by the parental *K. marxianus* strain at 43 °C; at this temperature, the parental *S. cerevisiae* did not grow and produce ethanol. These approaches have enhanced high-temperature ethanol fermentation ability of yeast strains, but the improvement has not been drastic.

2.9 Thermotolerant Mechanism of Yeasts

It is well known that heat shock proteins protect proteins and cells from stress conditions, including high temperature. Trehalose accumulation and its protective effect against heat is also known in yeast (De Virgilio et al. 1994). In *O. polymorpha*, a significant increase of ethanol production from xylose at 50 °C is achieved by overexpression of *HSP104* and disruption of the trehalase gene *ATH1* (Ishchuk et al. 2009). *S. cerevisiae* overexpressing the trehalose-6-phosphate synthase gene accumulated larger amounts of trehalose and produced a twofold higher concentration of ethanol (60 g/L) at 38 °C compared with nonengineered strains (An et al. 2011).

Thermotolerance is a quantitative trait which determined by several alleles called quantitative trait locus (QTL). A QTL analysis by repeated mating and sporulation of thermotolerant and thermosensitive strains of *S. cerevisiae* found alleles involved in growth at high temperature (Steinmetz et al. 2002). An allele of *END3*, a gene involved in actin cytoskeletal organization, contributes to the thermotolerance. Benjaphokee et al. (2012) revealed that an allele in the *CDC19* gene of a thermotolerant strain is required for thermotolerance. Recent whole-genome sequencing by next-generation sequencers has become an alternative strategy for thermotolerance analysis. Caspeta et al. (2014) found ergosterol composition change as a common phenotype among high-temperature evolved strains of *S. cerevisiae* based on genome sequencing. As another approach, transposon insertion was used to isolate thermotolerant disruptants in *S. cerevisiae*. However, the relation between the transposon-inserted positions and thermotolerance is not clear (Kim et al. 2011).

Genome-wide analysis using microarray and sets of yeast knockout strains have been conducted to elucidate the genes involved in heat shock of *S. cerevisiae*. When we analyzed the growth of diploid nonessential-gene knockout strains at 37 °C (high-temperature growth) and viability after exposure to 49 °C for 60 min (heat shock), the identified genes were not overlapped with each other (unpublished results). For high-temperature growth, genes involved in vesicle and protein transport, RNA metabolism, cell cycle and cell division, and DNA repair account for 63% of the total genes identified. In contrast, mitochondrial genes account for 50% of genes identified in the heat shock condition. Auesukaree et al. (2009) identified 178 genes required for high-temperature growth using a *S. cerevisiae* knockout collection. Chen et al. (2013) analyzed gene expression in high-temperature fermentation and identified four upregulated and five downregulated genes at 40 °C compared with 30 °C. Three heat shock genes were upregulated, and *ERG9*, which affects cell membrane structure and integrity, was downregulated. In *K. marxianus*, transcription start sites analysis revealed transcriptional differences between growth at 45 and 30 °C. The number of up- and downregulated genes at 45 °C compared with 30 °C were 199 and 508, respectively, and grouping the genes in GO terms suggested that *K. marxianus* copes with high temperature by reducing central metabolism and increasing protein turnover and DNA repair (Lertwattanasakul et al. 2015). To establish strains suitable for high-temperature fermentation via genetic engineering, intensive studies to reveal the genes required for robustness under fermentation conditions at high temperature are desired.

2.10 Conclusion

Many thermotolerant yeast species have been isolated. However, it seems that *K. marxianus* is the practical high-temperature ethanol fermentation species at present. In our high-temperature fermentation using molasses as well as cassava pulp, a *K. marxianus* strain repeatedly produced more than 8% ethanol at 40 °C within 24 h. High-temperature ethanol fermentation has the potential to be used in

commercial ethanol production in the near future. For nonconventional and conventional yeast species, fermentation conditions and genetic improvements should be further considered for high-temperature SSF using nonedible biomass. To enhance the performance of ethanol fermentation at high temperatures, understanding of thermotolerance mechanisms is essential. Various genetic approaches must be employed to elucidate thermotolerance mechanisms and to improve thermotolerance of yeasts.

References

An MZ, Tang YQ, Mitsumasu K, Liu ZS, Shigeru M, Kenji K (2011) Enhanced thermotolerance for ethanol fermentation of *Saccharomyces cerevisiae* strain by overexpression of the gene coding for trehalose-6-phosphate synthase. Biotechnol Lett 33:1367–1374

Anderson PJ, McNeil K, Watson K (1986) High-efficiency carbohydrate fermentation to ethanol at temperatures above 40 °C by *Kluyveromyces marxianus* var. *marxianus* isolated from sugar mills. Appl Environ Microbiol 51:1314–1320

Arguelled JC (1997) Thermotolerance and trehalose accumulation induced by heat shock in yeast cell of *Candida albicans*. FEMS Microbiol Lett 146:65–71. doi:10.1016/S0378-1097(96)00455-7

Arshad M, Ahmed S, Zia MA, Rajoka MI (2014) Kinetics and thermodynamics of ethanol production by *Saccharomyces cerevisiae* MLD10 using molasses. Appl Biochem Biotechnol 172:2455–2464. doi:10.1007/s12010-013-0689-x

Auesukaree C, Damnernsawad A, Kruatrachue M, Pokethitiyook P, Boonchird C, Kaneko Y, Harashima S (2009) Genome-wide identification of genes involved in tolerance to various environmental stresses in *Saccharomyces cerevisiae*. J Appl Genet 50:301–310. doi:10.1007/BF03195688

Auesukaree C, Koedrith P, Saenpayavai P, Asvarak T, Benjaphokee S, Sugiyama M, Kaneko Y, Harashima H, Boonchird C (2012) Characterization and gene expression profiles of thermotolerant *Saccharomyces cerevisiae* isolates from Thai fruits. J Biosci Bioeng 114:144–149. doi:10.1016/j.jbiosc.2012.03.012

Balakumar S, Arasaratnam V, Balasubramaniam K (2001) Isolation and improvement of a thermotolerant *Saccharomyces cerevisiae* strain. World J Microbiol Biotechnol 17:739–746. doi:10.1023/A:1012952809273

Ballesteros I, Ballesteros M, Cabañas A, Carrasco J, Martín C, Negro MJ, Saez F, Saez R (1991) Selection of thermotolerant yeasts for simultaneous saccharification and fermentation (SSF) of cellulose to ethanol. Appl Biochem Biotechnol 28–29:307–315. doi:10.1007/BF02922610

Benjaphokee S, Koedrith P, Auesukaree C, Asvarak T, Sugiyama M, Kaneko Y, Boonchird C, Harashima S (2012) *CDC19* encoding pyruvate kinase is important for high-temperature tolerance in *Saccharomyces cerevisiae*. N Biotechnol 29:166–176. doi:10.1016/j.nbt.2011.03.007

Bollók M, Réczey K, Zacchi G (2000) Simultaneous saccharification and fermentation of steam-pretreated spruce to ethanol. Appl Biochem Biotechnol 84–86:69–80. doi:10.1385/ABAB:84-86:1-9:69

Caspeta L, Chen Y, Ghiaci P, Feizi A, Buskov S, Hallström BM, Petranovic D, Nielsen J, Caspeta L, Buijs NAA, Nielsen J, Chu S, Majumdar A, Abreu-Cavalheiro A, Monteiro G, Nielsen J, Larsson C, van Maris A, Pronk J, Peralta-Yahya PP, Zhang F, del Cardayre SB, Keasling JD, Caspeta L, Nielsen J, Abdel-Banat BMA, Hoshida H, Ano A, Nonklang S, Akada R, Kristensen JB, Felby C, Jørgensen H, Caspeta L, Caro-Bermúdez MA, Ponce-Noyola T, Martinez A, Piper PW, Lindquist S, Dettman JR, Rodrigue N, Melnyk AH, Wong A, Bailey SF, Kassen R, Yona AH, Manor YS, Herbst RH, Romano GH, Mitchell A,

Kupiec M, Pilpel Y, Dahan O, Piper PW, Ortiz-Calderon C, Holyoak C, Coote P, Cole M, Moy TI, Boettner D, Rhodes JC, Silver PA, Askew DS, Tkach JM, Yimit A, Lee AY, Riffle M, Costanzo M, Jaschob D, Hendry JA, Ou J, Moffat J, Boone C, Davis TN, Nislow C, Brown GW, Smith RL, Johnson AD, Lingwood D, Simons K, Simons K, Gerl MJ, Dufourc EJ, Parks LW, Smith SJ, Crowley JH, Thompson ED, Parks LW, Watson K, Arthur H, Morton H, Yčas M, Sherman F, Özcan S, Johnston M, Sabina J, Johnston M, Koga Y, Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, Ellis B, Gautier L, Ge Y, Gentry J, Hornik K, Hothorn T, Huber W, Iacus S, Irizarry R, Leisch F, Li C, Maechler M, Rossini AJ, Sawitzki G, Smith C, Smyth G, Tierney L, Yang JY, Zhang J, Benjamini Y, Hochberg Y, Väremo L, Nielsen J, Nookaew I, Chen H, Boutros PC, McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernytsky A, Garimella K, Altshuler D, Gabriel S, Daly M, DePristo MA, Solis-Escalante D, Kuijpers NG, Bongaerts N, Bolat I, Bosman L, Pronk JT, Daran JM, Daran-Lapujade P, Agren R, Liu L, Shoaie S, Vongsangnak W, Nookaew I, Nielsen J, Bordel S, Agren R, Nielsen J (2014) Altered sterol composition renders yeast thermotolerant. Science 346:75–78. doi:10.1126/science.1258137

Castro RCA, Roberto IC (2014) Selection of a thermotolerant *Kluyveromyces marxianus* strain with potential application for cellulosic ethanol production by simultaneous saccharification and fermentation. Appl Biochem Biotechnol 172:1553–1564. doi:10.1007/s12010-013-0612-5

Charoensopharat K, Thanonkeo P, Thanonkeo S, Yamada M (2015) Ethanol production from Jerusalem artichoke tubers at high temperature by newly isolated thermotolerant inulin-utilizing yeast *Kluyveromyces marxianus* using consolidated bioprocessing. Antonie van Leeuwenhoek Int J Gen Mol Microbiol. doi:10.1007/s10482-015-0476-5

Chen Q, Fang Y, Zhao H, Zhang G, Jin Y (2013) Transcriptional analysis of *Saccharomyces cerevisiae* during high-temperature fermentation. Ann Microbiol. doi:10.1007/s13213-013-0606-0

Cho KM, Yoo YJ (1999) Novel SSF process for ethanol production from microcrystalline cellulose using δ-integrated recombinant yeast, *Saccharomyces cerevisiae* L2612δGC. J Microbiol Biotechnol 9:340–345

D'Amore T, Celotto G, Russell I, Stewart GG (1989) Selection and optimization of yeast suitable for ethanol production at 40 °C. Enzyme Microb Technol 11:411–416. doi:10.1016/0141-0229(89)90135-X

De Virgilio C, Hottiger T, Dominguez J, Boller T, Wiemken A (1994) The role of trehalose synthesis for the acquisition of thermotolerance in yeast. I. Genetic evidence that trehalose is a thermoprotectant. Eur J Biochem 219:179–186. doi:10.1111/j.1432-1033.1994.tb19928.x

Den Haan R, Rose SH, Lynd LR, van Zyl WH (2007) Hydrolysis and fermentation of amorphous cellulose by recombinant *Saccharomyces cerevisiae*. Metab Eng 9:87–94. doi:10.1016/j.ymben.2006.08.005

Edgardo A, Carolina P, Manuel R, Juanita F, Baeza J (2008) Selection of thermotolerant yeast strains *Saccharomyces cerevisiae* for bioethanol production. Enzyme Microb Technol. doi:10.1016/j.enzmictec.2008.02.007

Hacking AJ, Taylor IWF, Hanas CM (1984) Selection of yeast able to produce ethanol from glucose at 40 °C. Appl Microbiol Biotechnol 19:361–363. doi:10.1007/BF00253786

Hari Krishna S, Janardhan Reddy T, Chowdary GV (2001) Simultaneous saccharification and fermentation of lignocellulosic wastes to ethanol using a thermotolerant yeast. Bioresour Technol 77:193–196. doi:10.1016/S0960-8524(00)00151-6

Hermansyah N, Sugiyama M, Harashima S (2015) *Candida tropicalis* isolated from Tuak, a North Sumatera-Indonesian traditional beverage, for bioethanol production. Korean J Microbiol Biotechnol 43:241–248. doi:10.4014/mbl.1506.06002

Hong J, Wang Y, Kumagai H, Tamaki H (2007) Construction of thermotolerant yeast expressing thermostable cellulase genes. J Biotechnol 130:114–123. doi:10.1016/j.jbiotec.2007.03.008

Hoshida H, Phong TT, Nonklang S, Ano A, Yarimizu T, Tokuda S, Yagi Y, Shite Y, Murashige S, Nakagawa T, Pukahuta C, Sutthikhampa S, Lorroengsil S, Hung LT, Cha-aim K, Akada R (2012) Isolation of thermotolerant ethanologenic yeasts and improvement of fermentation

ability by using breeding and genetic engineering. In: Yamada M et al (eds) Summary Book Asia Core Program (2008–2013) on "Capacity building and development of microbial potential and fermentation technology towards new era". International exchange core program in Yamaguchi Univeristy, isbn 978–4–9906826-0-6

Hu ML, Zha J, He LW, Lv YJ, Shen MH, Zhong C, Li BZ, Yuan YJ (2016) Enhanced bioconversion of cellobiose by industrial *Saccharomyces cerevisiae* used for cellulose utilization. Front Microbiol 7:241. doi:10.3389/fmicb.2016.00241

Hughes SR, Bang SS, Cox EJ, Schoepke A, Ochwat K, Pinkelman R, Nelson D, Qureshi N, Gibbons WR, Kurtzman CP, Bischoff KM, Liu S, Cote GL, Rich JO, Jones MA, Cedeno D, Doran-Peterson J, Riano-Herrera NM, Rodriguez-Valencia N, Lopez-Nunez JC (2013) Automated UV-C mutagenesis of *Kluyveromyces marxianus* NRRL Y-1109 and selection for microaerophilic growth and ethanol production at elevated temperature on biomass sugars. J Lab Autom 18:276–290. doi:10.1177/2211068213480037

Hughes DB, Tudroszen NJ, Moye CJ (1984) The effect of temperature on the kinetics of ethanol production by a thermotolerant strain of *Kluveromyces marxianus*. Biotechnol Lett 6:1–6. doi:10.1007/BF00128221

Inlow D, McRae J, Ben-Bassat A (1988) Fermentation of corn starch to ethanol with genetically engineered yeast. Biotechnol Bioeng 32:227–234. doi:10.1002/bit.260320213

Ishchuk OP, Voronovsky AY, Abbas CA, Sibirny AA (2009) Construction of *Hansenula polymorpha* strains with improved thermotolerance. Biotechnol Bioeng 104:911–919. doi:10.1002/bit.22457

Kadam KL, Schmidt SL (1997) Evaluation of *Candida acidothermophilum* in ethanol production from lignocellulosic biomass. Appl Microbiol Biotechnol 48:709–713. doi:10.1007/s002530051120

Kim HS, Kim NR, Yang J, Choi W (2011) Identification of novel genes responsible for ethanol and/or thermotolerance by transposon mutagenesis in *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 91:1159–1172. doi:10.1007/s00253-011-3298-z

Kiran Sree N, Sridhar M, Suresh K, Banat IM, Venkateswar Rao L (2000) Isolation of thermotolerant, osmotolerant, flocculating *Saccharomyces cerevisiae* for ethanol production. Bioresour Technol 72:43–46. doi:10.1016/S0960-8524(99)90097-4

Kitagawa T, Tokuhiro K, Sugiyama H, Kohda K, Isono N, Hisamatsu M, Takahashi H, Imaeda T (2010) Construction of a β-glucosidase expression system using the multistress-tolerant yeast *Issatchenkia orientalis*. Appl Microbiol Biotechnol 87:1841–1853. doi:10.1007/s00253-010-2629-9

Kopsahelis N, Nisiotou A, Kourkoutas Y, Panas P, Nychas GJE, Kanellaki M (2009) Molecular characterization and molasses fermentation performance of a wild yeast strain operating in an extremely wide temperature range. Bioresour Technol 100:4854–4862. doi:10.1016/j.biortech.2009.05.011

Koutinas M, Patsalou M, Stavrinou S, Vyrides I (2015) High temperature alcoholic fermentation of orange peel by the newly isolated thermotolerant *Pichia kudriavzevii* KVMP10. Lett Appl Microbiol 62:75–83. doi:10.1111/lam.12514

Kumar S, Singh SP, Mishra IM, Adhikari DK (2009) Ethanol and xylitol production from glucose and xylose at high temperature by *Kluyveromyces* sp. IIPE453. J Ind Microbiol Biotechnol 36:1483–1489. doi:10.1007/s10295-009-0636-6

Kumar S, Singh SP, Mishra IM, Adhikari DK (2010) Feasibility of ethanol production with enhanced sugar concentration in bagasse hydrolysate at high temperature using *Kluyveromyces* sp. IIPE453. Biofuels 1:697–704. doi:10.4155/bfs.10.51

Lertwattanasakul N, Kosaka T, Hosoyama A, Suzuki Y, Rodrussamee N, Matsutani M, Murata M, Fujimoto N, Suprayogi, Tsuchikane K, Limtong S, Fujita N, Yamada M (2015) Genetic basis of the highly efficient yeast *Kluyveromyces marxianus*: complete genome sequence and transcriptome analyses. Biotechnol Biofuels 8:47. doi:10.1186/s13068-015-0227-x

Limtong S, Sringiew C, Yongmanitchai W (2007) Production of fuel ethanol at high temperature from sugar cane juice by a newly isolated *Kluyveromyces marxianus*. Bioresour Technol 98:3367–3374. doi:10.1016/j.biortech.2006.10.044

Liu Z-H, Qin L, Zhu J-Q, Li B-Z, Yuan Y-J (2014) Simultaneous saccharification and fermentation of steam-exploded corn stover at high glucan loading and high temperature. Biotechnol Biofuels 7:167. doi:10.1186/s13068-014-0167-x

Margaritis A, Bajpai P (1982) Continuous ethanol production from Jerusalem artichoke tubers. I. Use of free cells of *Kluyveromyces marxianus*. Biotechnol Bioeng 24:1483–1493

Matsushita K, Azuma Y, Kosaka T, Yakushi T, Hoshida H, Akada R, Yamada M (2015) Genomic analyses of thermotolerant microorganisms used for high-temperature fermentations. Biosci Biotechnol Biochem 8451:1–14. doi:10.1080/09168451.2015.1104235

Mitsumasu K, Liu Z-S, Tang Y-Q, Akamatsu T, Taguchi H, Kida K (2014) Development of industrial yeast strain with improved acid- and thermo-tolerance through evolution under continuous fermentation conditions followed by haploidization and mating. J Biosci Bioeng 118:689–695. doi:10.1016/j.jbiosc.2014.05.012

Nedeva TS, Savov VA, Kujumdzieva-Savova AV, Davidov ER (1993) Screening of thermotolerant yeasts as producers of superoxide dismutase. FEMS Microbiol Lett 107:49–52

Nonklang S, Abdel-Banat BMA, Cha-aim K, Moonjai N, Hoshida H, Limtong S, Yamada M, Akada R (2008) High-temperature ethanol fermentation and transformation with linear DNA in the thermotolerant yeast *Kluyveromyces marxianus* DMKU3-1042. Appl Environ Microbiol 73:1090–1095. doi:10.1128/AEM.01854-08

Nuanpeng S, Thanonkeo S, Yamada M, Thanonkeo P (2016) Ethanol production from sweet sorghum juice at high temperatures using a newly isolated thermotolerant yeast *Saccharomyces cerevisiae* DBKKU Y-53. Energies 9:253. doi:10.3390/en9040253

Park I, Kim I, Kang K, Sohn H, Rhee I, Jin I, Jang H (2010) Cellulose ethanol production from waste newsprint by simultaneous saccharification and fermentation using *Saccharomyces cerevisiae* KNU5377. Process Biochem 45:487–492. doi:10.1016/j.procbio.2009.11.006

Prakash G, Varma AJ, Prabhune A, Shouche Y, Rao M (2011) Microbial production of xylitol from D-xylose and sugarcane bagasse hemicellulose using newly isolated thermotolerant yeast *Debaryomyces hansenii*. Bioresour Technol 102:3304–3308. doi:10.1016/j.biortech.2010.10.074

Rajoka MI, Ferhan M, Khalid AM (2005) Kinetics and thermodynamics of ethanol production by a thermotolerant mutant of *Saccharomyces cerevisiae* in a microprocessor-controlled bioreactor. Lett Appl Microbiol 40:316–321. doi:10.1111/j.1472-765X.2005.01663.x

Rodrussamee N, Lertwattanasakul N, Hirata K, Suprayogi, Limtong S, Kosaka T, Yamada M (2011) Growth and ethanol fermentation ability on hexose and pentose sugars and glucose effect under various conditions in thermotolerant yeast *Kluyveromyces marxianus*. Appl Microbiol Biotechnol 90:1573–1586. doi:10.1007/s00253-011-3218-2

Ryabova OB, Chmil OM, Sibirny AA (2003) Xylose and cellobiose fermentation to ethanol by the thermotolerant methylotrophic yeast *Hansenula polymorpha*. FEMS Yeast Res 4:157–164. doi:10.1016/S1567-1356(03)00146-6

Sakanaka K, Yan W, Kishida M, Sakai T (1996) Breeding a fermentative yeast at high temperature using protoplast fusion. J Ferment Bioeng 81:104–108. doi:10.1016/0922-338X(96)87585-4

Sohn HY, Park W, Jin I, Seu JH (1997) Characterization of ethanol fermentation using alginate immobilized thermotolerant yeast cells. J Microbiol Biotechnol 7:62–67

Sree NK, Sridhar M, Suresh K, Rao LV (1999) High alcohol production by solid substrate fermentation from starchy substrates using thermotolerant *Saccharomyces cerevisiae*. Bioprocess Eng 20:561–563. doi:10.1007/s004490050629

Sridhar M, Kiran Sree N, Venkateswar Rao LV (2002) Effect of UV radiation on thermotolerance, ethanol tolerance and osmotolerance of *Saccharomyces cerevisiae* VS_1 and VS_3 strains. Bioresour Technol 83:199–202. doi:10.1016/S0960-8524(01)00221-8

Steinmetz LM, Sinha H, Richards DR, Spiegelman JI, Oefner PJ, McCusker JH, Davis RW (2002) Dissecting the architecture of a quantitative trait locus in yeast. Nature 416:326–330. doi:10.1038/416326a

Suryawati L, Wilkins MR, Bellmer DD, Huhnke RL, Maness NO, Banat IM (2008) Simultaneous saccharification and fermentation of Kanlow switchgrass pretreated by hydrothermolysis using *Kluyveromyces marxianus* IMB4. Biotechnol Bioeng 101:894–902. doi:10.1002/bit.21965

Szczodrak J, Targoński Z (1988) Selection of thermotolerant yeast strains for simultaneous saccharification and fermentation of cellulose. Biotechnol Bioeng 31:300–303. doi:10.1002/bit.260310404

Takashima M, Sugita T, Toriumi Y, Nakase T (2009) *Cryptococcus tepidarius* sp. nov., a thermotolerant yeast species isolated from a stream from a hotspring area in Japan. Int J Syst Evol Microbiol 59:181–185. doi:10.1099/ijs.0.004515-0

Ueno R, Urano N, Kimura S (2002) Effect of temperature and cell density on ethanol fermentation by a thermotolerant aquatic yeast strain isolated from a hot spring environment. Fish Sci 68:571–578. doi:10.1046/j.1444-2906.2002.00463.x

Voronovsky AY, Rohulya OV, Abbas CA, Sibirny AA (2009) Development of strains of the thermotolerant yeast *Hansenula polymorpha* capable of alcoholic fermentation of starch and xylan. Metab Eng 11:234–242. doi:10.1016/j.ymben.2009.04.001

Watanabe I, Nakamura T, Shima J (2010) Strategy for simultaneous saccharification and fermentation using a respiratory-deficient mutant of *Candida glabrata* for bioethanol production. J Biosci Bioeng 110:176–179. doi:10.1016/j.jbiosc.2010.02.003

Wen F, Sun J, Zhao H (2010) Yeast surface display of trifunctional minicellulosomes for simultaneous saccharification and fermentation of cellulose to ethanol. Appl Environ Microbiol 76:1251–1260. doi:10.1128/AEM.01687-09

Yanase S, Hasunuma T, Yamada R, Tanaka T, Ogino C, Fukuda H, Kondo A (2010) Direct ethanol production from cellulosic materials at high temperature using the thermotolerant yeast *Kluyveromyces marxianus* displaying cellulolytic enzymes. Appl Microbiol Biotechnol 88:381–388. doi:10.1007/s00253-010-2784-z

Ylitervo P, Franzén CJ, Taherzadeh MJ (2011) Ethanol production at elevated temperatures using encapsulation of yeast. J Biotechnol 156:22–29. doi:10.1016/j.jbiotec.2011.07.018

Yuangsaard N, Yongmanitchai W, Yamada M, Limtong S (2013) Selection and characterization of a newly isolated thermotolerant *Pichia kudriavzevii* strain for ethanol production at high temperature from cassava starch hydrolysate. Antonie van Leeuwenhoek Int J Gen Mol Microbiol 103:577–588. doi:10.1007/s10482-012-9842-8

Zhang Q, Fu Y, Wang Y, Han J, Lv J, Wang S (2011) Improved ethanol production of a newly isolated thermotolerant *Saccharomyces cerevisiae* strain after high-energy-pulse-electron beam. J Appl Microbiol 112:280–288. doi:10.1111/j.1365-2672.2011.05209.x

Zhang J, Lynd LR (2010) Ethanol production from paper sludge by simultaneous saccharification and co-fermentation using recombinant xylose-fermenting microorganisms. Biotechnol Bioeng 107:235–244. doi:10.1002/bit.22811

Construction of Baker's Yeast Strains with Enhanced Tolerance to Baking-Associated Stresses

3

Hiroshi Takagi

Abstract

In dough fermentation and baker's yeast production, yeast cells are exposed to numerous stresses, including freeze-thaw, high-sucrose, and air-drying, collectively referred to as baking-associated stresses. These conditions can also induce oxidative stress in yeast, leading to increases in reactive oxygen species levels probably due to damage to the mitochondrial membrane and respiratory chain components and denaturation of antioxidant enzymes. To prevent lethal damage, baker's yeast employs a number of stress tolerance mechanisms, such as the expression of stress proteins, accumulation of stress protectants and compatible solutes, changing the composition of the membrane, and repression of translation via stress-associated signal transduction pathways to regulate the expression of particular genes. As proline and trehalose play important roles in stress tolerance in baker's yeast, altering their metabolism via genetic engineering is a promising approach for the development of more stress-tolerant strains. So-called "omics" approaches, such as comprehensive phenomics and functional genomics, can be used to identify novel genes that affect stress tolerance. Further improvements in the fermentation capability and production efficiency of yeast strains, however, will require elucidation of the mechanisms underlying the stress response, adaptation, and tolerance. We believe that the engineering of not only baker's yeasts but also other important industrial yeasts exhibiting greater stress tolerance would enhance the production of yeast-based products such as bread doughs and alcoholic beverages and facilitate breakthroughs in bioethanol production.

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Contents

3.1 Introduction

Baker's yeast, which generally is comprised of strains of *Saccharomyces cerevisiae*, is an essential ingredient in baked food and beverage products produced by fermentation (Linko et al. 1997; Randez-Gil et al. 1999). Approximately two million tons of baker's yeasts are produced annually worldwide, based on 30% dry weight (Attfield 1997; Evans 1990). In bread making, baker's yeast serves to (1) increase the dough volume by gas generation during fermentation, (2) impart structure and texture to the dough, and (3) add distinctive flavors to the dough (Burrows 1970). Baker's yeast is produced in one of three forms: cream (an aqueous suspension that consists of approximately 20% cells by dry weight), compressed, or dried. Compressed yeast production involves partial dehydration, and the product contains approximately 30% cells (dry weight). Most baker's yeasts produced in Japan are in the cream or compressed forms. However, dried yeast (consisting of <5% water) is imported from other countries and used in home baking and bakeries because it is convenient to use and easy to store.

Flavor is an important factor that determines bread quality. Consumers prefer breads that have a characteristic delicious flavor. The flavor of bread is affected by a variety of compounds produced during the fermentation and baking processes, including alcohols, diacetyl compounds, esters, organic acids, and carbonyl compounds (Pence and Kohler 1961; Wick et al. 1964). Both isobutyl alcohol (i-BuOH) and isoamyl alcohol (i-AmOH) also affect the flavor of baked goods and thus could be utilized in making new types of bread. In Japan, 4-aza-DL-leucine-resistant mutant baker's yeast, which produces large amounts of i-BuOH, is used to enhance the flavor of breads, but high levels of i-AmOH are considered undesirable (Watanabe et al. 1990). The i-BuOH- or i-AmOH-overproducing mutants have been released from inhibition of the key branched-chain amino acid synthesis enzymes acetohydroxy acid synthase and α-isopropylmalate synthase, respectively.

Other molecules targeted in the breeding of baker's yeast are enzymes involved in the utilization of melibiose (a disaccharide converted from raffinose in molasses and used in the production of baker's yeast) and maltose (a free sugar found in flour) (Dequin 2001). Because they lack the enzyme α-galactosidase (melibiase), which

catalyzes the hydrolysis of melibiose into the fermentable sugars galactose and glucose, baker's yeast cannot utilize melibiose. Baker's yeast expressing the α-galactosidase-encoding gene *MEL1* from bottom-fermenting brewing yeast was shown to utilize all of the available melibiose in a beet molasses medium, however, resulting in higher yeast yield. (Liljeström et al. 1991; Liljeström-Suominen et al. 1988). Fermentation continues due to the release of maltose from starch by the activity of amylases present in the dough. The utilization of maltose, which requires the glucose-repressible enzymes maltose permease and maltase (Needleman 1991), results in a lag in carbon dioxide production. To avoid this problem, enzymes involved in maltose utilization can be derepressed by replacing the native maltase and maltose permease promoters with constitutive promoters (Osinga et al. 1988).

Baker's yeast cells are exposed to a numerous stresses during the bread-making process, including freeze-thaw cycles, high sucrose concentrations, and air-drying treatment. These conditions induce oxidative stress in the cells, resulting in the generation of reactive oxygen species (ROS) and potential mitochondrial damage. This chapter examines the mechanisms through which yeast cells respond to and tolerate baking-associated stresses. In addition, the chapter discusses the construction of stress-tolerant baker's yeast strains for use in commercial applications.

3.2 Baking-Associated Stresses

Although the history of bread making dates back many centuries, modern consumer demands have necessitated the development of new baking technologies. An important focus in this regard is the development of baker's yeast strains that exhibit high fermentation capabilities in addition to durability under harsh baking conditions. For example, dried yeast strains are widely used because of their longer storage shelf life and lower transport costs compared with compressed yeast. Sweet dough (high-sugar dough) contains up to 40% sucrose by flour weight. The development of frozen dough technology has greatly expanded consumer access to oven-fresh bakery products. As discussed above, baker's yeast cells are exposed to a variety of stresses during production and the fermentation of dough (baking-associated stresses) (Attfield 1997; Shima and Takagi 2009) (Fig. 3.1). These stresses can occur together or in a sequential manner (e.g., exposure to freeze-thaw cycling plus high-sucrose levels) (Attfield 1997). Exposure to freeze-thaw, high- sucrose, and air-drying treatments is thought to induce oxidative stress in yeast cells. (Ando et al. 2007; Attfield 1997; Landolfo et al. 2008; Sasano et al. 2010, 2012a; Shima et al. 2008). This in turn results in the generation of ROS due to the severe damage that might occur to the mitochondrial membrane and/or respiratory chain components and denaturation of proteins such as antioxidant enzymes.

Most microorganisms are capable of adapting to environmental stress to some degree. Yeast cells used in baking applications must have a variety of stress adaptation mechanisms (e.g., induction of stress protein expression, accumulation of stress protectants or compatible solutes, changes in membrane composition, and repression of translation). These mechanisms involve regulation of the

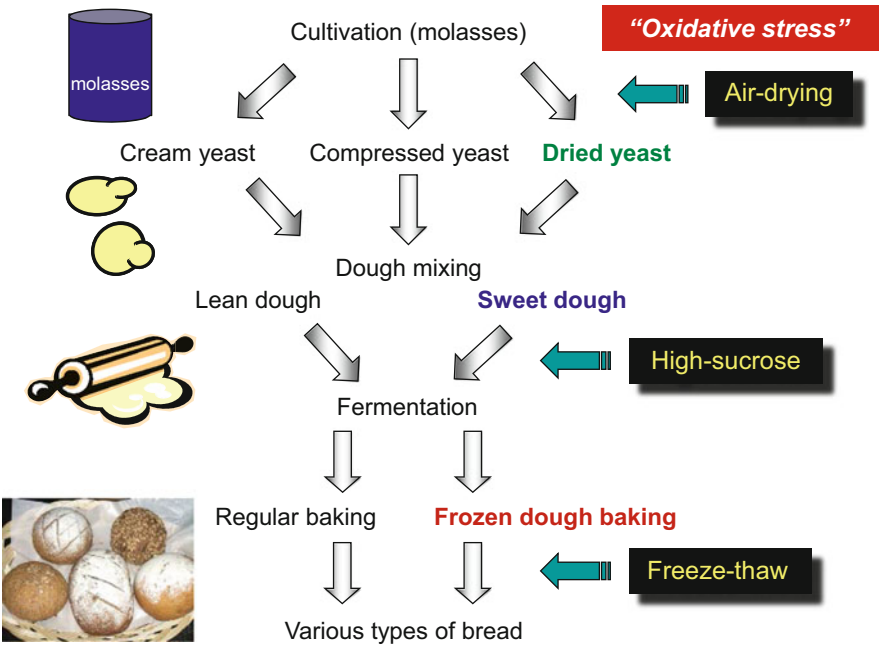

Fig. 3.1 Schematic view of the processes for baker's yeast production and bread making. During these processes, baker's yeast cells are exposed to baking-associated stresses, such as air-drying, high-sucrose, and freeze-thaw. These treatments also induce oxidative stress in yeast cells

corresponding gene expression networks via stress-triggered signal transduction pathways. Under conditions of severe stress that induce protein denaturation and ROS generation with subsequent growth inhibition or cell death, the fermentation capability of yeast can be drastically inhibited. The most important characteristic of yeast used in industrial applications is high stress tolerance. The construction of yeast strains exhibiting high tolerance to a variety of stresses is thus critical in the development of economically viable commercial processes for fermentation and baker's yeast production.

3.2.1 Freeze-Thaw Stress

Baking with frozen dough is less labor intensive and increases the access of consumers to oven-fresh bakery products. However, freezing and subsequent thawing can severely damage yeast cells, and this damage can decrease the fermentation capability of the yeast cells and lead to their death. Freeze-thaw cycling also induces oxidative stress in yeast cells, particularly the generation of free radicals and ROS, which cause oxidative damage to cellular components (Park et al. 1997, 1998). As such, baker's yeast strains tolerant to freeze-thaw stress are highly desired in the baking industry. Though some freeze-thaw-tolerant yeast strains

have been isolated from natural sources (Hahn and Kawai 1990; Hino et al. 1987) and culture collections (Oda et al. 1986), others have been constructed using conventional mutation or hybridization technologies (Nakagawa and Ouchi 1994a). One possible way to minimize freeze-thaw damage to cells is to subject fermented doughs to heat treatment before freezing (Nakagawa and Ouchi 1994b).

Compared with yeast strains intolerant to freezing, strains of *Saccharomyces* used in frozen dough accumulate more trehalose in the cells (Hino et al. 1990; Oda et al. 1986). The synthesis of trehalose, which functions as a stress protectant, can be induced under a variety of stress conditions (Kaino and Takagi 2008; Van Dijck et al. 1995). Levels of trehalose within the cell are controlled metabolically by regulation of the balance between synthesis and degradation. Neutral trehalase, Nth1, is a trehalose-degradation enzyme; disruption of the *NTH1* gene in baker's yeast leads to increased levels of intracellular trehalose and an increase in freeze-thaw tolerance (Shima et al. 1999).

In many plants and bacteria, proline accumulates in response to osmotic stress and functions as an osmoprotectant (i.e., a compatible solute) (Csonka 1981; Verbruggen and Hermans 2008). Glycerol or trehalose synthesis can be induced under stress conditions, but this does not lead to increases in the intracellular level of proline (Kaino and Takagi 2008). Extracellular proline also exhibits cryoprotective activity, to a degree nearly equal to that of glycerol or trehalose (Takagi et al. 1997). Proline plays a number of roles in vitro, including stabilizing the proteins and membranes during freezing and dehydration, decreasing the melting temperature of DNA during salinity stress, and scavenging of hydroxyl radicals under oxidative stress conditions (Takagi 2008). In plants, elevations in the level of proline in response to osmotic stress have been correlated with lower levels of free radicals (Hong et al. 2000). Increases in the degree of oxidation following alcohol exposure in wild-type cells are indicative of ROS generation. Notably, proline accumulation following exposure to ethanol significantly reduces ROS levels and increases the survival rate of yeast cells in the stationary phase (Takagi et al. 2016). The extremely high water solubility of proline suggests that it inhibits the formation of intracellular ice crystals and subsequent dehydration by forming strong hydrogen bonds with intracellular free water. However, the mechanisms underlying this phenomenon in vivo are poorly understood. The Takagi laboratory reported the construction of proline-accumulating *S. cerevisiae* strains. These engineered strains exhibited enhanced tolerance to many stresses, including freeze-thaw cycling, desiccation, and exposure to hydrogen peroxide (H_2O_2) and ethanol (Matsuura and Takagi 2005; Morita et al. 2002, 2003; Takagi et al. 1997, 2000a, 2005; Terao et al. 2003).

γ-Glutamyl kinase (encoded by the *PRO1* gene) is the first enzyme of the proline synthesis pathway via glutamate in *S. cerevisiae*, whereas proline oxidase (encoded by *PUT1*) catalyzes the first step of the proline degradation pathway (Fig. 3.2). Pro1 activity is regulated by feedback inhibition by proline, indicating that Pro1 is the rate-limiting enzyme in the regulation of intracellular proline levels (Sekine et al. 2007). Interestingly, Asp154Asn and Ile150Thr variants of Pro1 are less sensitive to

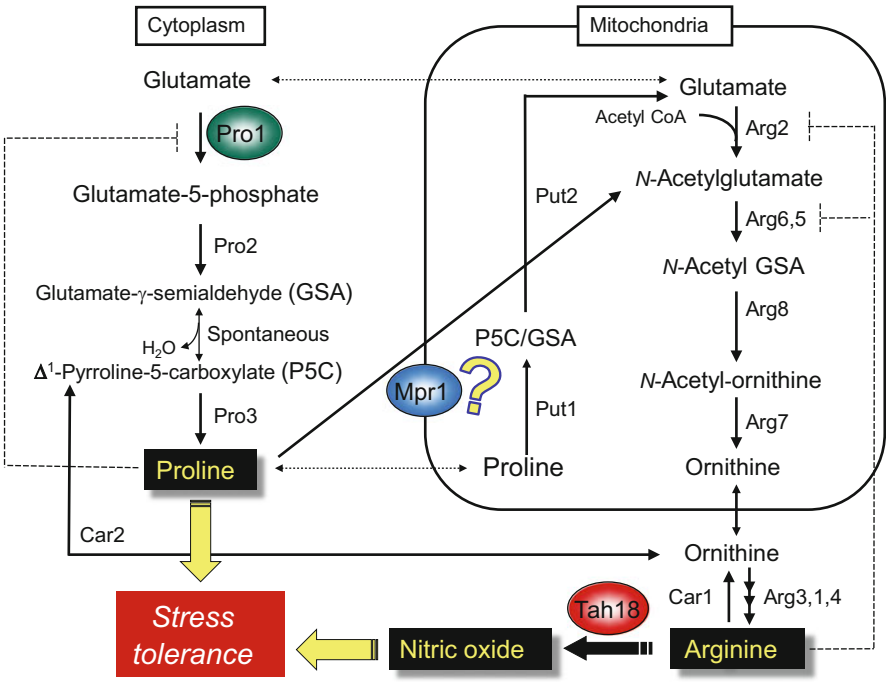

Fig. 3.2 Metabolic pathways of proline and arginine in *Saccharomyces cerevisiae* cells. Normally, both amino acids are synthesized from glutamic acid. In response to oxidative stress, nitric oxide (NO) is produced from the increased arginine through the Mpr1- and Tah18-dependent manner

feedback inhibition and exhibit excessive proline synthesis. Yeast cells expressing these Pro1 variants accumulate proline and exhibit a higher tolerance to freeze-thaw stress (Sekine et al. 2007). Recent research has shown that Pro1-mediated stress tolerance is independent of proline biosynthesis (Tatehashi et al. 2016). *PRO1* interacts with the genes *BRE5* and *UBP3*, which are required for de-ubiquitination activity during ribophagy, the process of selective ribosome degradation. The γ-glutamyl kinase activity of Pro1 plays a role in ribophagy, which is important for ensuring cell survival during nitrogen starvation.

In the industrial yeast strains used in the production of Japanese sake, cells with disruption of the *PUT1* gene or a $PRO1^{D154N}$ mutant allele accumulate proline and exhibit higher tolerance to ethanol stress than control strains (Takagi et al. 2005). The fermentation profiles of proline-accumulating diploid sake yeast strains have also been analyzed (Takagi et al. 2007). With respect to commercial applications of recombinant baker's yeast, consumers might be more accepting of self-cloning (SC) yeast that does not harbor foreign genes or DNA sequences (except for yeast DNA), compared with genetically modified (GM) yeasts. Kaino et al. (2008) constructed SC diploid baker's yeast strains by disrupting *PUT1* and replacing wild-type *PRO1* with the $PRO1^{D154N}$ or $PRO1^{150T}$ allele. In commercial baking

involving frozen dough, pre-freezing fermentation can produce benefits in terms of texture and taste (Teunissen et al. 2002). Yeast cells activated during pre-freezing fermentation produce metabolites such as alcohols and organic acids that can affect the taste and flavor of the bread. Why this process results in a loss of gas production remains unclear; however, it is possible that prolonged pre-freezing fermentation seriously damages the membranes of the yeast cells in the dough (Kline and Sugihara 1968). To overcome this problem, dough was fermented for 120 min at 30 °C before freezing and then kept frozen for 9 days. Gas production by wild-type cells declined dramatically, to 40% of the pre-freezing level (Fig. 3.3a). It is noteworthy that the fermentation capability of proline-accumulating cells was approximately 50% greater than that of wild-type cells (Fig. 3.3a), suggesting that proline-accumulating baker's yeast is more tolerant of freeze-thaw stress and thus suitable for use in frozen-dough baking.

A diploid baker's yeast strain that simultaneously accumulates proline and trehalose was constructed in order to further enhance tolerance to freeze-thaw stress (Sasano et al. 2012c). The mutant strain exhibited greater tolerance to freeze-thaw stress and fermentation capability in frozen dough than strains that accumulate only proline or trehalose. Breads exhibiting enhanced post-freezing swelling, reduced freezing periods, and lower manufacturing costs can be produced using the diploid baker's yeast strain. Engineering novel yeast strains that simultaneously accumulate proline and trehalose could be very useful in frozen-dough baking.

In response to oxidative stress, heat shock, and high ethanol concentrations, the transcriptional activator Msn2 induces the expression of approximately 180 genes (Causton et al. 2001; Estruch 2000). Under the abovementioned stress conditions, Msn2 (which typically forms a heterodimer with Msn4) is translocated into the nucleus, where it binds to the stress-responsive element within the promoter region and activates the transcription of numerous genes encoding stress-related proteins (Marchler et al. 1993; Martinez-Pastor et al. 1996). A number of cellular functions depend upon Msn2-regulated genes, including the oxidative stress response (*CTT1*, *SOD2*), molecular chaperones (*HSP12*, *HSP104*), and trehalose synthesis (*TPS1*, *TPS2*) (Boy-Marcotte et al. 1998). Msn2-overexpressing yeast strains can tolerate oxidative stress due to the high level of transcription of genes associated with antioxidant functions (Cardona et al. 2007; Sasano et al. 2012e; Watanabe et al. 2009; Zuzuarregui and del Olmo 2004). Sasano et al. (2012b) recently constructed an Msn2-overexpressing SC diploid strain of baker's yeast. This strain exhibited enhanced tolerance to freeze-thaw stress and higher intracellular trehalose levels than the wild-type strain. In addition, the fermentation capability in frozen dough of the Msn2-overexpressing strain was approximately 2.5-fold greater than that of the wild-type strain. These data suggest that Msn2-overexpressing baker's yeast strains could also be useful in frozen-dough baking.

The *POG1* gene encodes a transcription factor involved in cell cycle regulation (Leza and Elion 1999) and functions as a multi-copy suppressor of the mutant *S. cerevisiae* E3 ubiquitin ligase Rsp5 (Demae et al. 2007). The *pog1* mutant is stress sensitive, suggesting that *POG1* is involved in stress tolerance. Interestingly, deletion of *POG1* results in a drastic increase (55–70%) in the fermentation

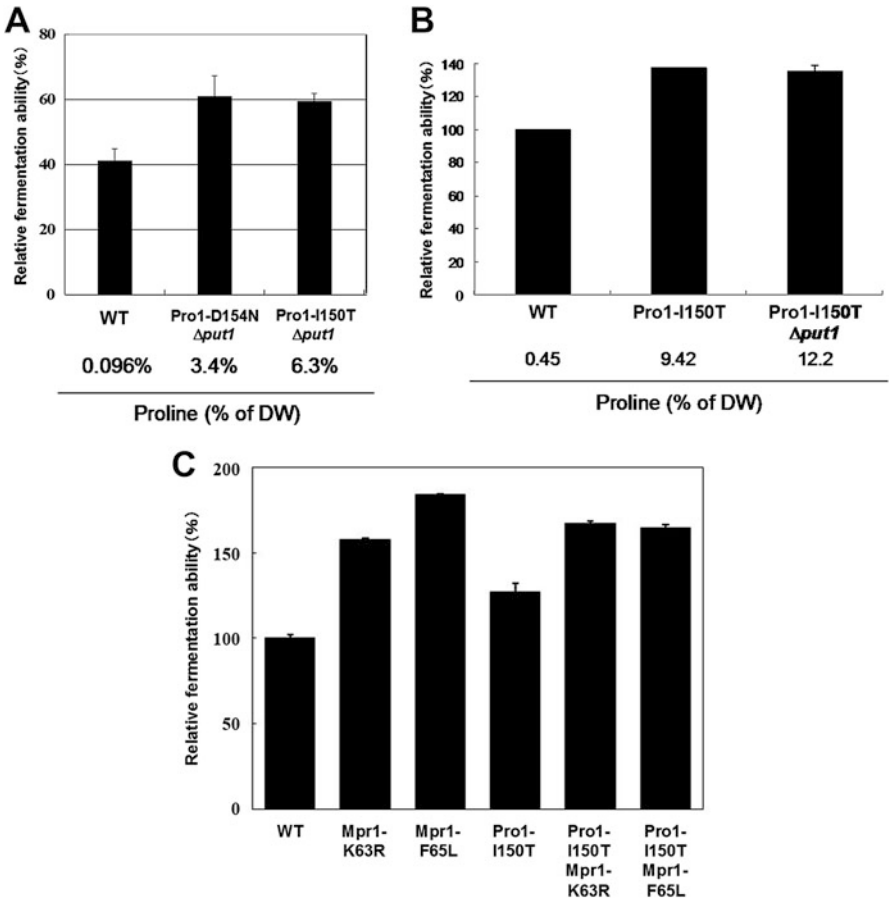

Fig. 3.3 Fermentation abilities of baker's yeast strains under baking-associated stresses. (**a**) The doughs were pre-fermented for 120 min at 30 °C and then frozen for 9 days at −20 °C. The frozen dough was thawed for 30 min at 30 °C, and the remaining CO_2 gas production was measured. The gassing power before freezing was defined as 100% (Kaino et al. 2008). (**b**) Fermentation ability in sweet dough (30% sucrose per weight of flour) was monitored by CO_2 gas production. The total amounts of CO_2 production after 2 h were measured. The gassing power of wild-type strain (WT) was defined as 100% (Sasano et al. 2012d). (**c**) Compressed yeast was treated with air-drying stress for 4 h at 37 °C. The dough containing the stress-treated yeasts was fermented for 3 h, and the remaining CO_2 gas production was measured. The amount of CO_2 production of WT after air-drying stress treatment was defined as 100% (Sasano et al. 2010)

capability in bread dough after freeze-thaw stress, whereas overexpression of this gene enhances the fermentation capability in dough containing high sucrose concentrations (Sasano et al. 2013). Thus, engineering yeast strains with the objective of controlling the expression of *POG1* represents a potentially useful approach for breeding yeast strains applicable to baking.

3.2.2 High-Sucrose Stress

During the dough fermentation process, baker's yeast cells encounter differences in sucrose concentration to which they must adapt (Tanaka et al. 2006). Doughs can be classified as either lean or sweet based on the sugar concentration. Lean dough contains no sugar (sugar-free dough) or small amounts of sugar (<5% per weight of flour). Sweet dough (high-sugar dough) generally can contain up to 40% sucrose per weight of flour. Such high sucrose concentrations exert severe osmotic stress upon yeast, and this stress can seriously damage a variety of cellular components (Verstrepen et al. 2004) and reduce their fermentation capability. Baker's yeast exhibiting osmotolerance would be less susceptible to potentially lethal injury, but development of such yeast will require elucidation of the mechanism underlying high-sucrose (HS)-stress tolerance. The mechanism could involve the induction of stress-related proteins, the accumulation of stress protectants or compatible solutes, and changes in membrane composition (Shima and Takagi 2009).

Upon sensing high osmotic pressure, *S. cerevisiae* cells accumulate glycerol and trehalose (Cronwright et al. 2002; De Virgilio et al. 1994; Hino et al. 1990; Hirasawa et al. 2006; Shima et al. 1999). Microarray and genome-wide screening analyses of a collection of deletion mutant strains revealed that metabolism of the osmoprotectants glycerol and trehalose plays an important role in tolerance to high sucrose concentrations (Ando et al. 2006; Tanaka-Tsuno et al. 2007). Many bacteria and plant cells accumulate proline as an osmoprotectant in response to osmotic stress (Csonka 1981; Verbruggen and Hermans 2008). Under conditions of high osmotic pressure (1 M NaCl), a proline oxidase-deficient strain that accumulated high levels of proline was markedly more osmotolerant than other strains (Takagi et al. 1997). Proline-accumulating baker's yeast exhibits greater fermentation capability in frozen dough than wild-type yeast (Kaino et al. 2008).

These observations suggest that proline confers tolerance to HS stress. Sasano et al. (2012d) constructed proline-accumulating SC diploid baker's yeast strains and determined their viability after inoculating cells into HS liquid fermentation medium in order to examine the effect of proline accumulation on HS-stress tolerance. The proline-accumulating strains were more viable than wild-type cells under these conditions, providing additional data suggesting that proline accumulation confers tolerance to HS stress.

In a study involving wine yeast, Landolfo et al. (2008) reported that during fermentation of medium containing high concentrations of sugar, ROS accumulation caused oxidative damage to the cells, probably as a result of denaturation of antioxidant proteins or damage leading to mitochondrial membrane dysfunction. Inoculation of baker's yeast cells into the HS liquid fermentation medium produced similar results, with an approximately twofold increase in ROS levels in all of the strains tested. Exposure to high sucrose concentrations leads to ROS accumulation in baker's yeast. Proline accumulation appears to confer tolerance to HS stress by inducing a reduction in ROS levels. Compared with wild-type yeast, the specific activity of superoxide dismutase is reportedly significantly higher (approximately

1.7-fold) in proline-accumulating strains. These data indicate that intracellular proline protects antioxidant enzymes from high osmotic pressure.

The tolerance of proline-accumulating strains to high sucrose concentrations was also assayed in sweet dough containing 30% sucrose per weight of flour. Stationary-phase cells were cultivated in cane molasses medium for 48 h and used for sweet-dough fermentation. Interestingly, the gassing power of the proline-accumulating strains was approximately 40% greater than that of the wild-type strain, indicating that proline accumulation enhances the leavening effect of baker's yeast in sweet dough (Fig. 3.3b). It has also been reported that the stress-protective effect requires an appropriate intracellular proline level (approximately 9%) (Fig. 3.3b). These data demonstrate convincingly that proline-accumulating baker's yeast strains are suitable for use in the production of sweet breads. The use of such yeast enables the production of breads exhibiting greater swelling with a shorter fermentation period and at less manufacturing cost.

3.2.3 Air-Drying Stress

The dried yeast manufacturing process exposes the cells to air-drying stress, which can have a number of harmful effects, including the accumulation of misfolded proteins, mitochondrial dysfunction, and vacuolar acidification (Nakamura et al. 2008; Shima et al. 2008), all of which lead to a decrease in fermentation capability. Thus, air-drying stress tolerance is a necessary characteristic of baker's yeast strains in dried yeast preparations. The flow of hot air during the drying process increases the temperature of the cells to around 37 °C. As such, air-drying stress involves the stresses of both high temperature and dehydration, which reportedly induces the intracellular accumulation of ROS (Franca et al. 2007; Nomura and Takagi 2004). A variety of antioxidant enzymes scavenge the ROS produced as by-products during normal respiratory metabolism in all aerobic organisms, including yeast. However, transient heat shock and loss of water can lead to dysfunction in ROS-detoxifying enzymes. The resulting increased ROS levels can damage cellular components and negatively impact fermentation capability and even induce cell death.

Mpr1 is an *N*-acetyltransferase that detoxifies the proline analogue L-azetidine-2-carboxylate (AZC) in the *S. cerevisiae* strain Σ1278b (Nasuno et al. 2013; Shichiri et al. 2001; Takagi et al. 2000b). The Σ1278b background strain has two *MPR* isogenes: *MPR1* on chromosome XIV and *MPR2* on chromosome X (sigma 1278b gene for proline-analogue resistance). The products of these genes (Mpr1 and Mpr2) play similar roles in AZC resistance (Takagi et al. 2000b). Genes homologous to *MPR1* have been identified in the genomes of a number of yeasts and fungi, and AZC acetyltransferase activity has been detected in numerous yeast strains, suggesting that the Mpr1 homologues are widely conserved in both yeasts and fungi (Kimura et al. 2002; Wada et al. 2008). Mpr1 confers tolerance to oxidative stresses such as heat shock, exposure to H_2O_2, freeze-thaw cycling, and ethanol exposure by mediating a reduction in intracellular ROS levels (Du and

Takagi 2005, 2007; Ishchuk et al. 2010; Nomura and Takagi 2004). Nishimura et al. (2010) proposed a mechanism for the antioxidant effect mediated by Mpr1 that involves a novel arginine synthesis pathway. Two Mpr1 variants (Lys63Arg and Phe65Leu) exhibiting enhanced enzymatic activity were isolated via PCR random mutagenesis of *MPR1* (Iinoya et al. 2009). Compared with wild-type Mpr1, overexpression of the K63R variant led to a decreased intracellular ROS levels and enhanced cell viability under oxidative stress conditions. In addition, the F65 L variant exhibited greatly enhanced thermal stability. Recently, two novel thermostable variants, Asn203Lys and Asn203Arg, were constructed using a structure-based molecular design approach. It was hypothesized that overexpression of the stable Mpr1 variants leads to increased arginine synthesis in yeast (Nasuno et al. 2013, 2016). It was hypothesized that overexpression of the stable Mpr1 variants leads to increased arginine synthesis in yeast. Based on the observation that the addition of arginine to the medium increases the fermentation rate of yeast (Gutiérrez et al. 2012), the development of thermostable Mpr1 variants could be useful in constructing industrial yeast strains capable of enhanced arginine synthesis and fermentation capability (Nasuno et al. 2016).

Interestingly, among yeast strains used industrially, Japanese baker's yeast strains harbor one copy of the *MPR2* gene on chromosome X (Sasano et al. 2010). In order to examine the role of *MPR2* in air-drying stress, the viability and intracellular ROS levels of diploid industrial baker's yeast strains were examined (Sasano et al. 2010). After exposure to air-drying stress, wild-type cells exhibited a significant increase in ROS levels. The *Δmpr2* strain was more sensitive to air-drying stress than the wild-type strain. Interestingly, the ROS levels in the *Δmpr2* strain were approximately 40% higher compared with the wild-type strain, suggesting that *MPR2* protects baker's yeast from air-drying stress by mediating a reduction in intracellular ROS levels.

The fermentation capability of the *Δmpr2* strain in dough following exposure to air-drying stress was also examined. No significant differences in gassing power were observed between the wild-type and *Δmpr2* strains prior to exposure to air-drying stress. The fermentation capability of the air-drying-stressed *Δmpr2* strain was only approximately 60% of that of the wild-type strain, however, indicating that *MPR2* affects the dough fermentation performance of air-drying-stressed baker's yeast. Interestingly, after air-drying stress, the fermentation capability of cells expressing the K63R and F65 L Mpr1 variants was greater than that of cells expressing wild-type Mpr1 (Fig. 3.3c). In particular, the gassing power of the F65 L-expressing strain was approximately 1.8-fold greater than that of the strain expressing wild-type Mpr1, most likely due to the greater thermal stability of the F65 L variant (Fig. 3.3c).

The effect of proline accumulation on tolerance to air-drying stress in baker's yeast has also been examined. Proline accumulation was found to significantly enhance the fermentation capability of baker's yeast after air-drying stress (Fig. 3.3c). Furthermore, Mpr1 variant-expressing yeast exhibited an approximately 40% increase in fermentation capability after air-drying stress as compared with cells expressing wild-type Mpr1 (Fig. 3.3c). Hence, the antioxidant enzymes

Mpr1/2 appear to be promising targets in the breeding of novel yeast strains exhibiting enhanced tolerance to air-drying stress.

3.3 Novel Approach and Mechanism for Baking-Associated Stress Tolerance

3.3.1 Omics Approach to Identify the Genes Required for Stress Tolerance

To identify as yet uncharacterized genes that play roles in stress tolerance in yeast, both comprehensive phenomics and functional genomics analyses have been carried out under various stress conditions simulating the commercial baking process. The results of these analyses indicate that numerous genes mediate stress tolerance in baker's yeast.

To identify the genes that mediate freeze-thaw tolerance in yeast, genome-wide screening was performed using a complete deletion strain collection of diploid *S. cerevisiae* (Ando et al. 2007). A total of 58 gene deletions that confer freeze-thaw sensitivity were identified in this screening, and these genes were then classified according to cellular function and localization of the gene product. The results of these analyses revealed that many of the genes involved in freeze-thaw tolerance are associated with vacuole functions and cell wall biogenesis. The greatest number of gene products was found to be components of vacuolar H^+-ATPase. The cross-sensitivity of freeze-thaw-sensitive mutants to oxidative and cell wall stress (both of which are closely related to freeze-thaw stress) was also examined. Ando et al. (2007) reported that vacuolar H^+-ATPase dysfunction confers sensitivity to both oxidative and cell wall stress. By contrast, defects in the products of genes involved in cell wall assembly confer sensitivity to cell wall stress but not oxidative stress. Collectively, these results suggest that at least two different mechanisms mediate freezing injury in yeast: (1) oxidative stress generated during the freeze-thaw process, and (2) defects in cell wall assembly.

The HS-tolerant and maltose-utilizing (LS) yeasts used in modern industrial baking were developed using breeding techniques and are now used commercially. Sugar utilization and HS tolerance differ significantly between HS-tolerant and LS yeasts. Tanaka-Tsuno et al. (2007) characterized the gene expression profiles of HS-tolerant and LS yeasts in media of different sucrose concentrations. The overall gene expression patterns were obtained using two-way hierarchical clustering. These analyses demonstrated that HS-tolerant and LS yeasts exhibit different gene expression patterns. Clusters of genes that are upregulated (cluster 1) or downregulated (cluster 2) under HS conditions were identified using quality threshold clustering analysis. Clusters 1 and 2 contained a number of genes that play roles in carbon and nitrogen metabolism, respectively. Genes involved in metabolism of the osmoprotectants glycerol and trehalose were expressed at higher levels in LS yeast than in HS-tolerant yeast at sucrose concentrations of 5–40%. No clear

correlation between the expression levels of genes involved in osmoprotectant biosynthesis and the intracellular levels of osmoprotectants was observed.

Nakamura et al. (2008) examined how gene expression changes in baker's yeast exposed to the type of air-drying stress encountered during dried yeast production. They reported that intracellular accumulation of trehalose contributes to dehydration tolerance (Gadd et al. 1987). However, the expression of genes involved in trehalose synthesis did not increase during the air-drying process, which could account for the high concentration of intracellular trehalose in the compressed yeast. By contrast, the expression of genes related to molecular chaperone functions, proteasome, and autophagy was transiently upregulated in the early stage of the process, suggesting that proper folding of cellular proteins and the removal of denatured proteins play important roles in the adaptation of cells to air-drying stress. The expression of genes involved in β-oxidation was constitutively upregulated during the air-drying process. One hypothesis holds that yeast cells acquire the energy needed for adaptation to air-drying stress through the degradation of intracellular fatty acids. This hypothesis is supported by the observation that yeast cells are unable to take up nutrients during the air-drying process. These gene expression data could aid efforts to both improve dried yeast production and breed strains with enhanced tolerance to air-drying stress.

The results of the abovementioned studies suggest that vacuolar H^+-ATPase plays an important role in yeast cells subjected to baking-associated stress, particularly air-drying stress (Shima et al. 2008). The results of gene expression and phenotypic analyses suggest that copper ion homeostasis is critical for freeze-thaw stress tolerance in yeast (Takahashi et al. 2009). Moreover, the endoplasmic reticulum membrane protein Eos1, originally identified as being necessary for tolerance to oxidative stress, could be involved in zinc homeostasis (Nakamura et al. 2007, 2010).

In order to gain a more comprehensive understanding of the mechanism underlying oxidative stress tolerance, essential genes were screened in a collection of heterozygous deletion mutants (Okada et al. 2014). Classification of the genes according to function and localization suggested that nuclear functions, such as RNA synthesis, play critical roles in the oxidative stress response.

3.3.2 Nitric Oxide-Mediated Stress-Tolerant Mechanism Found in Yeast

Nitric oxide (NO) is a ubiquitous signaling molecule in pathways that regulate numerous cellular functions in microorganisms including bacteria, yeast, and fungi as well as in higher eukaryotes such as plants and mammals (Astuti et al. 2016). In mammals, NO confers tolerance to oxidative stress by enhancing the intracellular antioxidant status, primarily through cGMP-mediated signaling pathways by activating soluble guanylate cyclase. NO also functions through posttranslational activation of proteins via *S*-nitrosylation. In unicellular eukaryote yeasts, low NO levels have been linked with various stress response pathways, including responses

to high hydrostatic pressure, copper metabolism, and H_2O_2-induced apoptosis (Almeida et al. 2007; Domitrovic et al. 2003; Shinyashiki et al. 2000). However, the mechanism of NO synthesis and its physiologic role in *S. cerevisiae* remain unclear, primarily due to the absence of mammalian NO synthase (NOS) orthologues in the genome. In several recent studies involving a laboratory yeast strain, Nishimura et al. (2010, 2013) described a novel antioxidant mechanism involved in stress-induced NO synthesis mediated by proline and arginine metabolism via Mpr1 (Fig. 3.2).

Expression of the Pro1 I150T variant, which is less sensitive to proline feedback inhibition, and the thermostable Mpr1 F65 L variant was shown to result in enhanced fermentation capability in baker's yeast exposed to the stress of freeze-thaw cycling and air-drying, respectively (Kaino et al. 2008; Sasano et al. 2010). Sasano et al. (2012a) constructed a SC diploid baker's yeast strain exhibiting enhanced proline and NO synthesis due to co-expression of the Pro1 I150T and Mpr1 F65 L variants. Higher intracellular NO levels in response to air-drying stress were observed in the engineered strain, which was tolerant of not only oxidative stress but also air-drying and freeze-thaw stress due to lower intracellular ROS levels (Fig. 3.4a). Compared with the wild-type strain, the engineered strain also exhibited higher leavening activity in bread dough after air-drying and freeze-thaw stress (Fig. 3.4b). These data suggest that baker's yeast synthesizes NO in response to types of oxidative stress that induce ROS generation and that the increase in NO synthesis plays an important role in the mechanism of baking-associated stress tolerance. Engineering yeast strains with enhanced proline and NO synthesis capabilities is thus a potentially promising approach in the breeding of novel baker's yeast strains.

Nishimura et al. (2013) reported a novel NO-mediated antioxidant mechanism in yeast, although the details of the underlying mechanism were not elucidated. The Takagi group recently identified a downstream NO pathway involved in stress tolerance in yeast (Nasuno et al. 2014). The results of microarray and real-time quantitative PCR analyses revealed that exogenous NO treatment induces the expression of copper metabolism genes under control of the transcription factor Mac1. These genes include *CTR1*, which encodes a high-affinity copper transporter. Under conditions of high-temperature stress, NO induced increased transcription of *CTR1*, leading to Mac1-dependent increases in the intracellular copper concentration, activity of the Cu/Zn-superoxide dismutase Sod1, and cell viability. NO had no effect on expression of the *MAC1* gene, indicating that NO activates Mac1 via posttranslational modification. These results led to the proposal of a novel NO-mediated antioxidant mechanism involving activation of Mac1.

The flavoprotein Tah18 transfers electrons to the iron-sulfur cluster protein Dre2 and exhibits NOS-like activity in *S. cerevisiae* (Nishimura et al. 2013). Tah18 also reportedly promotes apoptosis of yeast cells exposed to H_2O_2. Yoshikawa et al. (2016) demonstrated that Tah18-dependent NOS-like activity induces the apoptosis of cells treated with H_2O_2. Tah18-dependent NO production and subsequent cell death are suppressed in cells exhibiting enhanced interaction between Tah18 and its molecular partner, Dre2. These data suggest that in response to deleterious

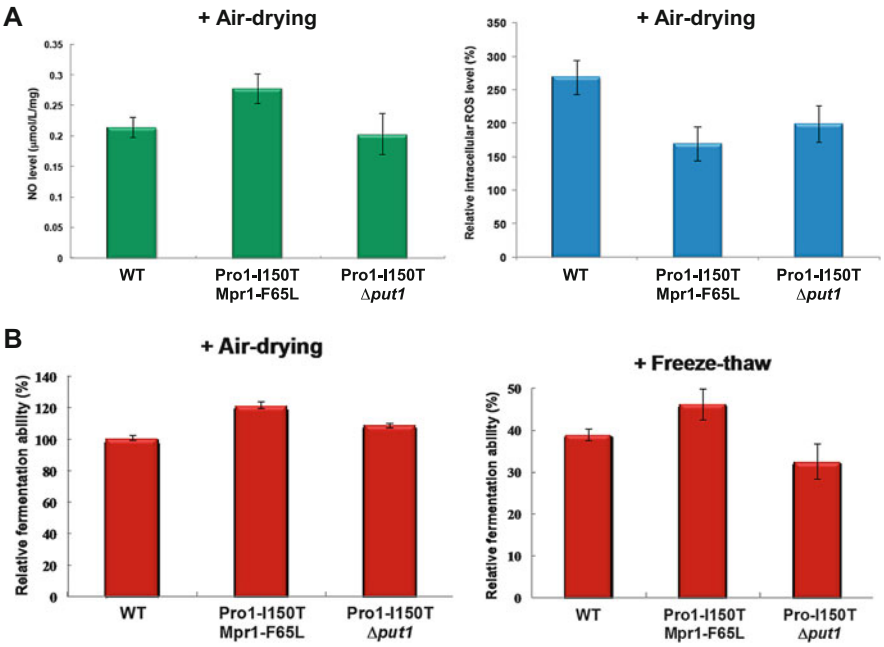

Fig. 3.4 Intracellular NO and ROS levels and fermentation abilities of baker's yeast strains under baking-associated stresses (Sasano et al. 2012a). (**a**) Intracellular NO (*left*) and ROS (*right*) levels after air-drying stress (at 42 °C for 90 min) were measured. For ROS level, the fluorescence intensity before stress treatment of each strain was defined as 100%. (**b**) After exposure to air-drying stress, baker's yeast strains were mixed with dough and fermented. The remaining CO_2 gas production after 2 h was measured. The gassing power of the wild-type strain (WT) is relatively taken as 100% (*left*). The doughs were pre-fermented for 2 h at 30 °C and then frozen at −20 °C for 3 weeks. The frozen dough was thawed for 30 min at 30 °C, and the remaining CO_2 gas production after 2 h was measured. The gassing power before freezing of each strain was defined as 100% (*right*)

environmental changes, the Tah18-Dre2 complex induces cell death by acting as a molecular switch via Tah18-dependent NOS-like activity. NO acts as a figurative double-edged sword due to its highly diffusible and reactive nature, exerting opposing effects on cells (Pacher et al. 2007). The apoptosis of *S. cerevisiae* cells following exposure to high levels of H_2O_2 is dependent on an NOS-like activity requiring Tah18. By contrast, yeast cells are protected from high-temperature stress by NO produced via NOS-like activity (Liu et al. 2015; Nasuno et al. 2014). These observations raise the possibility that the Tah18-Dre2 complex regulates many more biological functions than previously thought (including cell survival and death) by acting as a NO-dependent molecular switch that responds to environmental changes.

The observation that NO plays an important role in the stress response of industrially important yeast strains suggests that targeting NO production at both the physiologic and genetic levels could aid in the development of additional useful

yeast strains. Modulating NO biosynthesis as a means of enhancing the fermentation capability of baker's yeast has been reported (Sasano et al. 2012a, b, c, d, e). As such, producing industrially useful yeast strains tolerant of a wide variety of environmental stresses during fermentation that simultaneously exhibit enhanced fermentation rates will require the development of more advanced engineering strategies (Astuti et al. 2016).

3.4 Conclusions and Future Perspective

As described in this chapter, baker's yeasts used in industrial and commercial baking applications are exposed to multiple environmental stresses, including freeze-thaw cycling, high sucrose concentrations, and air-drying. Yeast cells employ a wide variety of strategies to cope with these potentially deleterious environmental conditions. However, fermentation rates can decline significantly if the stress level increases beyond that to which the yeast cells are capable of adapting. When discussing applications of yeast strains, stress "tolerance" is the key characteristic. For example, proline and trehalose play important roles in mediating the stress tolerance of baker's yeast. In fact, targeting proline and trehalose metabolism is a promising approach in the development of more stress-tolerant baker's yeast strains. To further improve the fermentation capability and production efficiency of yeast, however, the details of the mechanisms underlying the response, adaptation, and tolerance to a variety of stresses must be fully elucidated at the molecular, metabolic, and cellular levels. We believe that baker's yeast used in bread baking as well as other important industrial yeasts [such as those used in the brewing of alcoholic beverages (e.g., beer, wine, sake, and shochu) and bioethanol production] that exhibit enhanced tolerance to a variety of stresses could greatly expand these endeavors.

A number of issues aside from scientific and technologic problems need to be addressed to expand the potential practical uses of engineered baker's yeasts. First, breeding industrial baker's yeast strains with a higher stress tolerance than laboratory strains can be difficult. Approaches involving the exogenous addition of stress protectants or antioxidant compounds to cultures or dough are too laborious for practical application. However, the use of Pro1 variant-expressing baker's yeast strains that accumulate proline or strains that express the Mpr1 variant and exhibit improved antioxidant activity could negate this problem. Second, Japanese government guidelines specify that it is not necessary to treat SC yeast as GM because SC processes are considered equivalent to naturally occurring gene conversion events such as recombination, deletion, and transposition (Fig. 3.5) (Hino 2002). However, neither the baking industry as a whole nor consumers have accepted this technology. We therefore propose an alternative method for consumers to construct baker's yeast strains exhibiting the same stress tolerance mechanisms (Fig. 3.6). The toxic proline analogue AZC is taken up by cells via proline permease and causes misfolding of the proteins into which it is incorporated, competitively with proline, thereby inhibiting cell growth. By introducing favorable mutations in the *PRO1* or

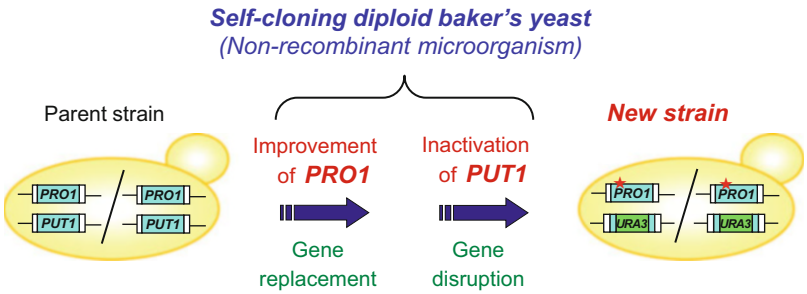

Fig. 3.5 Construction of self-cloning diploid baker's yeast strain with proline accumulation

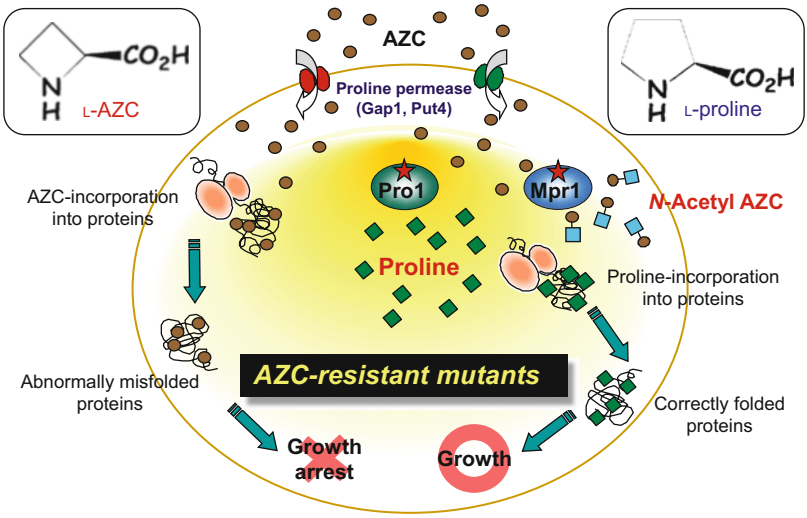

Fig. 3.6 Construction of commercial baker's yeast strains. By introducing a favorable mutation in the *PRO1* or *MPR1* gene, yeast cells that accumulate large quantities of proline or acetylate L-azetidine-2-carboxylate (AZC) efficiently are tolerant to AZC, respectively

MPR1 genes, cells capable of efficiently accumulating large quantities of proline or acetylate AZC would be rendered tolerant to AZC due to the incorporation of proline into the proteins. After treatment with a conventional mutagen, desirable strains derived from AZC-resistant mutants could be isolated.

Tsolmonbaatar et al. (2016) recently reported the isolation of AZC-resistant mutants derived from diploid *S. cerevisiae*. Of those mutants that accumulated more intracellular proline, five were more viable than the parent wild-type strain under freeze-thaw or HS-stress conditions. Two of the strains carried novel mutations in the *PRO1* gene (encoding the Pro247Ser and Glu415Lys variants of Pro1 [γ-glutamyl kinase]). Interestingly, these mutations were found to impart AZC resistance in the yeast cells and desensitize the cells to proline feedback inhibition of Pro1, leading to intracellular proline accumulation. Moreover, baker's yeast

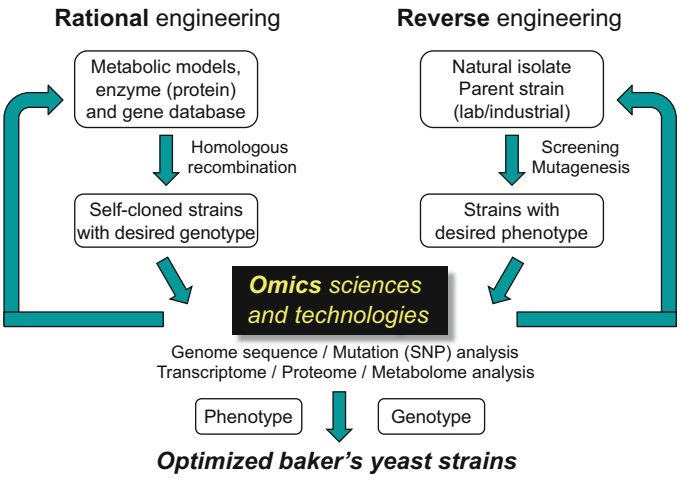

Fig. 3.7 Schematic view of omics-based baker's yeast breeding

strains expressing the *PRO1*^{P247S} and *PRO1*^{E415K} genes were more tolerant to freeze-thaw stress than strains expressing wild-type *PRO1*. The approach described here thus represents a practical method for breeding proline-accumulating baker's yeast strains exhibiting enhanced tolerance to baking-associated stresses.

In summary, in this chapter we have proposed novel sophisticated "omics" strategies using both rational and reverse engineering approaches for constructing baker's yeast strains exhibiting desirable properties (Fig. 3.7).

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References

Almeida B, Buttner S, Ohlmeier S, Silva A, Mesquita A, Sampaio-Marques B, Osorio NS, Kollau A, Mayer B, Leao C, Laranjinha J, Rodrigues F, Madeo F, Ludovico P (2007) NO-mediated apoptosis in yeast. J Cell Sci 120:3279–3288

Ando A, Tanaka F, Murata Y, Takagi H, Shima J (2006) Identification and classification of genes required for tolerance to high-sucrose stress revealed by genome-wide screening of *Saccharomyces cerevisiae*. FEMS Yeast Res 6:249–267

Ando A, Nakamura T, Murata Y, Takagi H, Shima J (2007) Identification and classification of genes required for tolerance to freeze-thaw stress revealed by genome-wide screening of *Saccharomyces cerevisiae* deletion strains. FEMS Yeast Res 7:244–253

Astuti RI, Nasuno R, Takagi H (2016) Nitric oxide signaling in yeast. Appl Microbiol Biotechnol 100:9483–9497

Attfield PV (1997) Stress tolerance: the key to effective strains of industrial baker's yeast. Nat Biotechnol 15:1351–1357

Boy-Marcotte E, Perrot M, Bussereau F, Boucherie H, Jacquet M (1998) Msn2p and Msn4p control a large number of genes induced at the diauxic transition which are repressed by cyclic AMP in *Saccharomyces cerevisiae*. J Bacteriol 180:1044–1052

Burrows S (1970) In: Rose AH, Harrison JS (eds) The yeast volume 3 Yeast technology. Academic, London, pp 349–420

Cardona F, Carrasco P, Perez-Ortin JE, del Olmo M, Aranda A (2007) A novel approach for the improvement of stress resistance in wine yeasts. Int J Food Microbiol 114:83–91

Causton HC, Ren B, Koh SS, Harbison CT, Kanin E, Jennings EG, Lee TI, True HL, Lander ES, Young RA (2001) Remodeling of yeast genome expression in response to environmental changes. Mol Biol Cell 12:323–337

Cronwright GR, Rohwer JM, Prior BA (2002) Metabolic control analysis of glycerol synthesis in *Saccharomyces cerevisiae*. Appl Environ Microbiol 68:4448–4456

Csonka LN (1981) Proline over-production results in enhanced osmotolerance in Salmonella typhimurium. Mol Gen Genet 182:82–86

De Virgilio C, Hottiger T, Dominguez J, Boller T, Wiemken A (1994) The role of trehalose synthesis for the acquisition of thermotolerance in yeast. I. Genetic evidence that trehalose is a thermoprotectant. Eur J Biochem 219:179–186

Demae M, Murata Y, Hisano M, Haitani Y, Shima J, Takagi H (2007) Overexpression of two transcriptional factors, Kin28 and Pog1, suppresses the stress sensitivity caused by the *rsp5* mutation in *Saccharomyces cerevisiae*. FEMS Microbiol Lett 277:70–78

Dequin S (2001) The potential of genetic engineering for improving brewing, wine-making and bread making. Appl Microbiol Biotechnol 56:577–588

Domitrovic T, Palhano FL, Barja-Fidalgo C, DeFreitas M, Orlando MT, Fernandes PM (2003) Role of nitric oxide in the response of *Saccharomyces cerevisiae* cells to heat shock and high hydrostatic pressure. FEMS Yeast Res 3:341–346

Du X, Takagi H (2005) *N*-acetyltransferase Mpr1 confers freeze tolerance on *Saccharomyces cerevisiae* by reducing reactive oxygen species. J Biochem 138:391–397

Du X, Takagi H (2007) *N*-Acetyltransferase Mpr1 confers ethanol tolerance on *Saccharomyces cerevisiae* by reducing reactive oxygen species. Appl Microbiol Biotechnol 75:1343–1351

Estruch F (2000) Stress-controlled transcription factors, stress-induced genes and stress tolerance in budding yeast. FEMS Microbiol Rev 24:469–486

Evans IH (1990) In: Spencer JFT, Spencer DM (eds) Yeast Technology. Springer, Berlin, pp 13–45

Franca MB, Panek AD, Eleutherio EC (2007) Oxidative stress and its effects during dehydration. Comp Biochem Physiol A Mol Integr Physiol 146:621–631

Gadd GM, Chalmers K, Reed RH (1987) The role of trehalose in dehydration resistance of *Saccharomyces cerevisiae*. FEMS Microbiol Lett 48:249–254

Gutiérrez A, Chiva R, Sancho M, Beltran G, Arroyo-López FN, Guillamon JM (2012) Nitrogen requirements of commercial wine yeast strains during fermentation of a synthetic grape must. Food Microbiol 31:25–32

Hahn YS, Kawai H (1990) Isolation and characterization of freeze- tolerant yeasts from nature available for the frozen-dough method. Agric Biol Chem 54:829–831

Hino A (2002) Safety assessment and public concerns for genetically modified food products: the Japanese experience. Toxicol Pathol 30:126–128

Hino A, Takano H, Tanaka Y (1987) New freeze-tolerant yeast for frozen dough preparations. Cereal Chem 64:269–275

Hino A, Mihara K, Nakashima K, Takano H (1990) Trehalose levels and survival ratio of freeze-tolerant versus freeze-sensitive yeasts. Appl Environ Microbiol 56:1386–1391

Hirasawa T, Nakakura Y, Yoshikawa K, Ashitani K, Nagahisa K, Furusawa C, Katakura Y, Shimizu H, Shioya S (2006) Comparative analysis of transcriptional responses to saline stress

in the laboratory and brewing strains of *Saccharomyces cerevisiae* with DNA microarray. Appl Microbiol Biotechnol 70:346–357

Hong Z, Lakkineni K, Zhang Z, Verma DPS (2000) Removal of feedback inhibition of Δ^1-pyrroline-5-carboxylate synthetase results in increased proline accumulation and protection of plants from osmotic stress. Plant Physiol 122:1129–1136

Iinoya K, Kotani T, Sasano Y, Takagi H (2009) Engineering of the yeast antioxidant enzyme Mpr1 for enhanced activity and stability. Biotechnol Bioeng 103:341–352

Ishchuk OP, Abbas CA, Sibirny AA (2010) Heterologous expression of *Saccharomyces cerevisiae MPR1* gene confers tolerance to ethanol and L-azetidine-2-carboxylic acid in *Hansenula polymorpha*. J Ind Microbiol Biotechnol 37:213–218

Kaino T, Takagi H (2008) Gene expression profiles and intracellular contents of stress protectants in *Saccharomyces cerevisiae* under ethanol and sorbitol stresses. Appl Microbiol Biotechnol 79:273–283

Kaino T, Tateiwa T, Mizukami-Murata S, Shima J, Takagi H (2008) Self-cloning baker's yeasts that accumulate proline enhance freeze tolerance in doughs. Appl Environ Microbiol 74:5845–5849

Kimura Y, Nakamori S, Takagi H (2002) Polymorphism of the *MPR1* gene required for toxic proline analogue resistance in the *Saccharomyces cerevisiae* complex species. Yeast 19:1437–1445

Kline L, Sugihara TG (1968) Factors affecting the stability of frozen bread doughs. I. Prepared by the straight dough method. Bakers Dig 42:44–69

Landolfo S, Politi H, Angelozzi D, Mannazzu I (2008) ROS accumulation and oxidative damage to cell structures in *Saccharomyces cerevisiae* wine strains during fermentation of high-sugar-containing medium. Biochim Biophys Acta 1780:892–898

Leza MA, Elion EA (1999) *POG1*, a novel yeast gene, promotes recovery from pheromone arrest via the G1 cyclin *CLN2*. Genetics 151:531–543

Liljeström PL, Tubb, RS, Korhola MP (1991) Construction of new α-galactosidase producing yeast strains and the industrial application of these strains. US Patent 5,055,401

Liljeström-Suominen PL, Joutsjoki V, Korhola MP (1988) Construction of a stable α-galactosidase producing baker's yeast. Appl Environ Microbiol 54:245–249

Linko Y, Javanainen P, Linko S (1997) Biotechnology of bread baking. Trends Food Sci Technol 8:339–344

Liu WC, Yuan HM, Li YH, Lu YT (2015) *CKA2* functions in H_2O_2-induced apoptosis and high-temperature stress tolerance by regulating NO accumulation in yeast. FEMS Yeast Res 15:fov051. doi:10.1093/femsyr/fov051

Marchler G, Schuller C, Adam G, Ruis H (1993) A *Saccharomyces cerevisiae* UAS element controlled by protein kinase A activates transcription in response to a variety of stress conditions. EMBO J 12:1997–2003

Martinez-Pastor MT, Marchler G, Schuller C, Marchler-Bauer A, Ruis H, Estruch F (1996) The *Saccharomyces cerevisiae* zinc finger proteins. Msn2p and Msn4p are required for transcriptional induction through the stress-response element. (STRE). EMBO J 15:2227–2235

Matsuura K, Takagi H (2005) Vacuolar functions are involved in stress-protective effect of intracellular proline in *Saccharomyces cerevisiae*. J Biosci Bioeng 100:538–544

Morita Y, Nakamori S, Takagi H (2002) Effect of proline and arginine metabolism on freezing stress of *Saccharomyces cerevisiae*. J Biosci Bioeng 94:390–394

Morita Y, Nakamori S, Takagi H (2003) L-proline accumulation and freeze tolerance of *Saccharomyces cerevisiae* are caused by a mutation in the *PRO1* gene encoding γ-glutamyl kinase. Appl Environ Microbiol 69:212–219

Nakagawa S, Ouchi K (1994a) Construction from a single parent of baker's yeast strains with high freeze tolerance and fermentative activity in both lean and sweet doughs. Appl Environ Microbiol 60:3499–3502

Nakagawa S, Ouchi K (1994b) Improvement of freeze tolerance of commercial baker's yeasts in dough by heat treatment before freezing. Biosci Biotechnol Biochem 58:2077–2079

Nakamura T, Ando A, Takagi H, Shima J (2007) Eos1, whose deletion confers sensitivity to oxidative stress, is involved in *N*-glycosylation in *Saccharomyces cerevisiae*. Biochem Biophys Res Commun 353:293–298

Nakamura T, Mizukami-Murata S, Ando A, Murata Y, Takagi H, Shima J (2008) Changes in gene expression of commercial baker's yeast during an air-drying process that simulates dried yeast production. J Biosci Bioeng 106:405–408

Nakamura T, Takahashi S, Takagi H, Shima J (2010) Multicopy suppression of oxidant-sensitive *eos1* mutation by *IZH2* in *Saccharomyces cerevisiae* and the involvement of Eos1 in zinc homeostasis. FEMS Yeast Res 10:259–269

Nasuno R, Hirano Y, Itoh T, Hakoshima T, Hibi T, Takagi H (2013) Structural and functional analysis of the yeast *N*-acetyltransferase Mpr1 involved in oxidative stress tolerance via proline metabolism. Proc Natl Acad Sci U S A 110:11821–11826

Nasuno R, Aitoku M, Manago Y, Nishimura A, Sasano Y, Takagi H (2014) Nitric oxide-mediated antioxidative mechanism in yeast through the activation of the transcription factor Mac1. PLoS One 9:e113788. doi:10.1371/journal.pone.0113788

Nasuno R, Hirase S, Norifune S, Watanabe D, Takagi H (2016) Structure-based molecular design for thermostabilization of *N*-acetyltransferase Mpr1 involved in a novel pathway of L-arginine synthesis in yeast. J Biochem 159:271–277

Needleman R (1991) Control of maltase synthesis in yeast. Mol Microbiol 5:2079–2084

Nishimura A, Kotani T, Sasano Y, Takagi H (2010) An antioxidative mechanism mediated by the yeast *N*-acetyltransferase Mpr1: oxidative stress-induced arginine synthesis and its physiological role. FEMS Yeast Res 10:687–698

Nishimura A, Kawahara N, Takagi H (2013) The flavoprotein Tah18-dependent NO synthesis confers high-temperature stress tolerance on yeast cells. Biochem Biophys Res Commun 430:137–143

Nomura M, Takagi H (2004) Role of the yeast acetyltransferase Mpr1 in oxidative stress: regulation of oxygen reactive species caused by a toxic proline catabolism intermediate. Proc Natl Acad Sci U S A 101:12616–12621

Oda Y, Uno K, Ohta S (1986) Selection of yeasts for breadmaking by the frozen-dough method. Appl Environ Microbiol 52:941–943

Okada N, Ogawa J, Shima J (2014) Comprehensive analysis of genes involved in the oxidative stress tolerance using yeast heterozygous deletion collection. FEMS Yeast Res 14:425–434

Osinga KA, Beudeker RF, van der Plaat JB, de Hollander JA (1988) New yeast strains providing for an enhanced rate of the fermentation of sugars, a process to obtain such strains and the use of these strains. Eur Patent 0306107A2

Pacher P, Beckman J, Liaudet L (2007) Nitric oxide and peroxynitrite in health and disease. Physiol Rev 87:315–424

Park JI, Grant CM, Attfield PA, Dawes IW (1997) The freeze-thaw stress response of the yeast *Saccharomyces cerevisiae* is growth phase specific and is controlled by nutritional state via the *RAS*-cyclic AMP signal transduction pathway. Appl Environ Microbiol 63:3818–3824

Park JI, Grant CM, Davies MJ, Dawes IW (1998) The cytoplasmic Cu, Zn superoxide dismutase of *Saccharomyces cerevisiae* is required for resistance to freeze-thaw stress. J Biol Chem 273:22921–22928

Pence JW, Kohler GO (1961) Investigations of bread flavor in the U.S. Department of Agriculture. Brot Gebaeck 15:129–134

Randez-Gil F, Sanz P, Pietro JA (1999) Engineering baker's yeast: room for improvement. Trends Biotechnol 17:237–244

Sasano Y, Takahashi S, Shima J, Takagi H (2010) Antioxidant *N*-acetyltransferase Mpr1/2 of industrial baker's yeast enhances fermentation ability after air-drying stress in bread dough. Int J Food Microbiol 138:181–185

Sasano Y, Haitani Y, Hashida K, Ohtsu I, Shima J, Takagi H (2012a) Enhancement of the proline and nitric oxide synthetic pathway improves fermentation ability under multiple baking-

associated stress conditions in industrial baker's yeast. Microb Cell Fact 11:40. doi:10.1186/1475-2859-11-40

Sasano Y, Haitani Y, Hashida K, Ohtsu I, Shima J, Takagi H (2012b) Overexpression of the transcription activator Msn2 enhances fermentation ability of industrial baker's yeast in frozen dough. Biosci Biotech Biochem 76:624–627

Sasano Y, Haitani Y, Hashida K, Ohtsu I, Shima J, Takagi H (2012c) Simultaneous accumulation of proline and trehalose in industrial baker's yeast enhances fermentation ability in frozen dough. J Biosci Bioeng 113:592–595

Sasano Y, Haitani Y, Ohtsu I, Shima J, Takagi H (2012d) Proline accumulation in baker's yeast enhances high-sucrose stress tolerance and fermentation ability in sweet dough. Int J Food Microbiol 152:40–43

Sasano Y, Watanabe D, Ukibe K, Inai T, Ohtsu I, Shimoi H, Takagi H (2012e) Overexpression of the yeast transcription activator Msn2 confers furfural resistance and increases the initial fermentation rate in ethanol production. J Biosci Bioeng 113:451–455

Sasano Y, Haitani Y, Hashida K, Oshiro S, Shima J, Takagi H (2013) Improvement of fermentation ability under baking-associated stress conditions by altering the POG1 gene expression in baker's yeast. Int J Food Microbiol 165:241–245

Sekine T, Kawaguchi A, Hamano Y, Takagi H (2007) Desensitization of feedback inhibition of the *Saccharomyces cerevisiae* gamma-glutamyl kinase enhances proline accumulation and freezing tolerance. Appl Environ Microbiol 73:4011–4019

Shichiri M, Hoshikawa C, Nakamori S, Takagi H (2001) A novel acetyltransferase found in *Saccharomyces cerevisiae* Sigma1278b that detoxifies a proline analogue, azetidine-2-carboxylic acid. J Biol Chem 276:41998–42002

Shima J, Takagi H (2009) Stress-tolerance of baker's-yeast (*Saccharomyces cerevisiae*) cells: stress-protective molecules and genes involved in stress tolerance. Biotechnol Appl Biochem 53:155–164

Shima J, Hino A, Yamada-Iyo C, Suzuki Y, Nakajima R, Watanabe H, Mori K, Takano H (1999) Stress tolerance in doughs of *Saccharomyces cerevisiae* trehalase mutants derived from commercial Baker's yeast. Appl Environ Microbiol 65:2841–2846

Shima J, Ando A, Takagi H (2008) Possible roles of vacuolar H^+-ATPase and mitochondrial function in tolerance to air-drying stress revealed by genome-wide screening of *Saccharomyces cerevisiae* deletion strains. Yeast 25:179–190

Shinyashiki M, Chiang KT, Switzer CH, Gralla EB, Valentine JS, Thiele DJ, Fukuto JM (2000) The interaction of nitric oxide (NO) with the yeast transcription factor Ace1: A model system for NO-protein thiol interactions with implications to metal metabolism. Proc Natl Acad Sci U S A 97:2491–2496

Takagi H (2008) Proline as a stress protectant in yeast: physiological functions, metabolic regulations, and biotechnological applications. Appl Microbiol Biotechnol 81:211–223

Takagi H, Iwamoto F, Nakamori S (1997) Isolation of freeze-tolerant laboratory strains of *Saccharomyces cerevisiae* from proline-analogue-resistant mutants. Appl Microbiol Biotechnol 47:405–411

Takagi H, Sakai K, Morida K, Nakamori S (2000a) Proline accumulation by mutation or disruption of the proline oxidase gene improves resistance to freezing and desiccation stresses in *Saccharomyces cerevisiae*. FEMS Microbiol Lett 184:103–108

Takagi H, Shichiri M, Takemura M, Mohri M, Nakamori S (2000b) *Saccharomyces cerevisiae* sigma 1278b has novel genes of the *N*-acetyltransferase gene superfamily required for L-proline analogue resistance. J Bacteriol 182:4249–4256

Takagi H, Takaoka M, Kawaguchi A, Kubo Y (2005) Effect of L-proline on sake brewing and ethanol stress in *Saccharomyces cerevisiae*. Appl Environ Microbiol 71:8656–8662

Takagi H, Matsui F, Kawaguchi A, Wu H, Shimoi H, Kubo Y (2007) Construction and analysis of self-cloning sake yeasts that accumulate proline. J Biosci Bioeng 103:377–380

Takagi H, Taguchi J, Kaino T (2016) Proline accumulation protects *Saccharomyces cerevisiae* cells in the stationary phase from ethanol stress by reducing reactive oxygen species levels. Yeast 33:355–363

Takahashi S, Ando A, Takagi H, Shima J (2009) Insufficiency of copper ion homeostasis causes freeze-thaw injury of yeast cells revealed by indirect gene expression analysis. Appl Environ Microbiol 75:6706–6711

Tanaka F, Ando A, Nakamura T, Takagi H, Shima J (2006) Functional genomic analysis of commercial baker's yeast during initial stages of model dough-fermentation. Food Microbiol 23:717–728

Tanaka-Tsuno F, Mizukami-Murata S, Murata Y, Nakamura T, Ando A, Takagi H, Shima J (2007) Functional genomics of commercial baker's yeasts that have different abilities for sugar utilization and high-sucrose tolerance under different sugar conditions. Yeast 24:901–911

Tatehashi Y, Watanabe D, Takagi H (2016) γ-Glutamyl kinase is involved in selective autophagy of ribosomes in *Saccharomyces cerevisiae*. FEBS Lett 590:2906–2914

Terao Y, Nakamori S, Takagi H (2003) Gene dosage effect of L-proline biosynthetic enzymes on L-proline accumulation and freeze tolerance in *Saccharomyces cerevisiae*. Appl Environ Microbiol 69:6527–6532

Teunissen A, Dumortier F, Gorwa MF, Bauer J, Tanghe A, Loïez A, Smet P, Van Dijck P, Thevelein JM (2002) Isolation and characterization of a freeze-tolerant diploid derivative of an industrial baker's yeast strain and its use in frozen doughs. Appl Environ Microbiol 68:4780–4787

Tsolmonbaatar A, Hashida K, Sugimoto Y, Watanabe D, Furukawa S, Takagi H (2016) Isolation of baker's yeast mutants with proline accumulation that showed enhanced tolerance to baking-associated stresses. Int J Food Microbiol 238:233–240

Van Dijck P, Colavizza D, Smet P, Thevelein JM (1995) Differential importance of trehalose in stress resistance in fermenting and nonfermenting *Saccharomyces cerevisiae* cells. Appl Environ Microbiol 61:109–115

Verbruggen N, Hermans C (2008) Proline accumulation in plants: a review. Amino Acids 35:753–759

Verstrepen KJ, Iserentant D, Malcorps P, Derdelinckx G, Van Dijck P, Winderickx J, Pretorius IS, Thevelein JM, Delvaux FR (2004) Glucose and sucrose: hazardous fast-food for industrial yeast? Trends Biotechnol 22:531–537

Wada M, Okabe K, Kataoka M, Shimizu S, Yokota H, Takagi H (2008) Distribution of L-azetidine-2-carboxylate *N*-acetyltransferase in yeast. Biosci Biotechnol Biochem 72:582–586

Watanabe M, Fukuda K, Asano K, Ohta S (1990) Mutants of baker's yeasts producing a large amount of isobutyl alcohol or isoamyl alcohol, flavor components of bread. Appl Microbiol Biotechnol 34:154–159

Watanabe M, Watanabe D, Akao T, Shimoi H (2009) Overexpression of *MSN2* in a sake yeast strain promotes ethanol tolerance and increases ethanol production in sake brewing. J Biosci Bioeng 107:516–518

Wick EL, De Figueiredo M, Wallace DH (1964) The volatile components of white bread prepared by a preferent method. Cereal Chem 41:300–315

Yoshikawa Y, Nasuno R, Kawahara N, Nishimura A, Watanabe D, Takagi H (2016) Regulatory mechanism of the flavoprotein Tah18-dependent nitric oxide synthesis and cell death in yeast. Nitric Oxide 57:85–91

Zuzuarregui A, del Olmo ML (2004) Expression of stress response genes in wine strains with different fermentative behavior. FEMS Yeast Res 4:699–710

Anhydrobiosis and Dehydration of Yeasts

4

Alexander Rapoport

Abstract

Anhydrobiosis is a unique phenomenon of nature which gives possibility to various live organisms to survive during very hot and dry seasons of the year. It is the state in which all processes of metabolism are temporarily reversibly suspended as the result of strong dehydration of the cells. The state of anhydrobiosis is used for industrial production of active dry yeasts. Results of detailed studies of mechanisms of anhydrobiosis and of changes in the cells which take place during their drying by different methods help to improve the dehydration resistance of yeast strains necessary in traditional and nonconventional biotechnologies. The progress which has been made in these researches within the last decades has led to a formulation of the major factors influencing the maintenance of yeast viability during their drying. It was revealed that all intracellular organelles are subjected to definite changes during dehydration and rehydration procedures. A number of earlier unknown intracellular protective reactions which can be artificially activated for the improvement of cells' resistance have been found. These results are used also at the construction of new biotechnologically efficient yeast strains which are very resistant to dehydration process.

Contents

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4.1 Introduction

Since the last century, more and more popular in various branches of biotechnology is the use of active dry yeast. Active dry yeast is yeast in the state of anhydrobiosis in which their metabolism is temporarily reversibly suspended (with the maintenance of yeast cells' viability) as the result of their strong dehydration. The increased interest of industry to this state use for practical goals is linked with many reasons, and main of them is economical one. It is much cheaper and more convenient to transport the dry yeast containing only 5–8% of water than fresh ones in which the content of water can reach 75–80%. On the one hand, in this case the same amount of dry yeasts takes considerably smaller volume, but on the other hand, refrigeration is not necessary for dry yeast transportation. The use of active dry yeasts is much more convenient also for customers because refrigerators are not necessary for their storage, and they can be stored for very long periods of time which can reach 2–3 years if the quality of active dry yeast is good enough. To achieve such high quality of active dry yeast, first of all clarification of the major factors promoting preservation of their viability at dehydration is necessary. It means that we have to understand what happens with yeast cells at their dehydration and subsequent rehydration/reactivation as well as to reveal the effects of various factors of different industrial dehydration methods. Then it is necessary to find the possibilities to improve the resistance of biotechnologically important yeast strains to dehydration–rehydration treatments and to diminish the negative effects of damaging factors of industrial drying procedures. Our current knowledge of these problems is the main topic of this review.

The history of research of anhydrobiosis starts at the beginning of the eighteenth century in 1701 when great Dutch naturalist Antonie van Leewenhoek discovered this state using his own very simple handmade microscopes. He studied the sand taken from the roof gutter of his house after very hot summer and added a drop of water to the sample of this sand. Unexpectedly, he found in this preparation a number of moving organisms which he called as "animalcules." He was very surprised that there were live organisms in such dry sample. He repeated this

procedure a lot of times, and every time he has seen the restoration of active life of small organisms when the water was added. Now we know that these organisms were rotifers. Only in 1743 appeared a new paper of T. Needham which revealed similar effect in other conditions for other organisms—nematodes. Then a number of well-known researchers of the eighteenth century including H. Backer, L. Spallanzani, and others continued the experiments in this direction. In the twentieth century, results of experiments of W. Preyer were published which once more evidenced the maintenance of viability by dry rotifers, tardigrades, and nematodes and gave the term for this phenomenon—"anabiosis." From this time the studies of anabiosis were actively continued in different countries. Another term, synonym of anabiosis—"cryptobiosis"—was proposed by Keilin (1959). D. Keilin has written that it is "the state of an organism when it shows no visible signs of life and when its metabolic activity becomes hardly measurable or comes reversibly to a standstill" (Keilin 1959). New stage of the studies of anabiosis (cryptobiosis) started at the second half of the twentieth century because of the development of powerful techniques for cytological, biophysical, biochemical research, and accumulation of new more-less modern knowledge. This new stage in the studies of anabiosis (cryptobiosis) was started mainly by the experiments of John Crowe in the USA, Folkert Hoekstra in the Netherlands, and some research groups in Russia and Latvia.

Today following the proposal of Keilin (1959), we distinguish some kinds of anabiosis (cryptobiosis), which correspond to the various ways how it can be reached by live organisms. These forms are the following:

- Anhydrobiosis—the metabolism is suspended by the water evaporation from the cells.
- Osmobiosis—caused by extraction of water by solutions with high osmotic pressure.
- Cryobiosis—when liquid water, the medium for metabolic reactions is eliminated by freezing.
- Anoxybiosis—caused by the decrease of oxygen concentration below critical limits.

Now, on the basis of the results of active studies of G. El-Registan's group, it is possible to discuss one more form which conditionally can be called as "autochemoanabiosis." It can be reached under the influence of chemical factors produced by the cells themselves and which are the autoinducers of anabiosis—alkylhydroxybenzenes (Mulyukin et al. 1996, 2001; Bespalov et al. 2000; Stepanenko et al. 2004; El-Registan et al. 2005; Nikolaev et al. 2006 etc.) .

There is a number of live organisms belonging to completely different groups which are able for the transition into the state of anhydrobiosis at the unfavorable conditions of the environment. They are seeds of many plants, mosses, cysts of crustaceans, and microscopic animals such as rotifers, tardigrades, and nematodes, as well as the majority of microorganisms.

There are a number of different factors which may determine microorganisms' viability at their dehydration. One of the attempts to summarize these factors was made by Morgan et al. (2006). In accordance with this classification the main factors are:

- Resistance of microorganisms to dehydration treatment
- Growth media taken for the cultivation of microorganisms
- Growth phase of microbial culture
- Concentration of the cells taken for drying
- Protective substances which are used at dehydration stage
- Methods of dehydration which are used
- Conditions at rehydration/reactivation stage

At the same time, all these factors are rather conditional ones, and here are some data which may evidence such postulate. For example, if we are thinking about resistance of microorganisms to dehydration, usually it is supposed that yeasts are more resistant to dehydration treatments comparing with bacteria. Nevertheless, the comparison of genetically closely related yeasts *Saccharomyces cerevisiae*, *Saccharomyces mikatae*, and *Naumovia castellii* showed that they very significantly differ in their resistance to dehydration treatment. The viability of the cells after dehydration–rehydration was 84, 20, and 40% correspondingly, but in the case these yeasts were dehydrated in the presence of trehalose, their viability increased approximately for 20% for the case of each of these three species (Lopez-Martinez et al. 2014). Also the resistance to drying of various strains of the same species can be rather different. And what is more, the resistance of the same strain differs rather considerably depending on the conditions of cultivation, rehydration, and reactivation. For example, the viability of yeast strain *Saccharomyces cerevisiae* 14 grown in molasses containing medium after dehydration–rehydration may reach 95% and higher, whereas after growth in the ethanol-containing medium, this figure reaches only 30% (Beker and Rapoport 1987). At the same time, viability of *Lactobacillus reuteri* dehydrated after their growth at pH 5 was at the level of 90% whereas after their growth at pH 6—about 65% (Palmfeldt and Hahn-Hagerdal 2000). Another example may be linked with the influence of microbial culture growth phase upon the resistance to dehydration–rehydration. Usually microbial cells (both bacteria and yeasts) are the most resistant to dehydration in stationary growth phase, but in exponential growth phase, their viability after dehydration–rehydration only in the best cases can reach 30% (Beker and Rapoport 1987). Some recent studies showed that this postulate also is not common rule. Very high resistance to dehydration–rehydration treatment was shown for yeast *Debaryomyces hansenii* in exponential growth phase too (Khroustalyova et al. 2001). High resistance to dehydration in exponential growth phase was found for *S. cerevisiae* thermoresistant strain (Rapoport et al. 2014). If to think about optimum cell concentration for the highest viability at dehydration–rehydration, it depends from the used protective medium. When taking optimum protective medium, optimum cell concentration depends from the concentration of protective medium (Morgan et al. 2006). The conclusion

which was made on the basis of analysis of all these factors was that the maintenance of high viability of microorganisms at their drying "appears to be still a science based on empirical testing rather than facts and tested theories. Major problem to overcome seems to be the lack of generic theories" (Morgan et al. 2006). Current review is devoted to the attempt to make an additional step toward generation of such theories. It is linked only with one kind of anabiosis (cryptobiosis), the form which was discovered by Antonie van Leeuwenhoek—to anhydrobiosis and will discuss the information received only for one group of microorganisms—for yeasts.

4.2 Influence of Dehydration Treatment upon Yeast Cells

Main studies of structural-and-functional changes of yeast cells during their dehydration were performed in 1970–1987 and were summarized in the review published 30 years ago (Beker and Rapoport 1987). Therefore, in this review they will be described rather briefly, but the main attention will be paid to the results of later studies in this direction.

4.2.1 Cell Wall

Studies of this surface structure response to dehydration treatment which have been performed using different methodological approaches have shown that in these conditions, there are changes both in its main components—mannoproteins and glucans. In the case of mannoproteins, its changes were revealed using scanning electron microscopy; there were fibrils on the surface of the cells instead of the smooth cell wall in native cells (Rapoport et al. 1983; Ventina et al. 1984). These changes were accompanied with the increase of negative and positive charges of cell wall (Rapoport and Beker 1985; Rapoport and Muter 1995). It can be explained by disruption of certain connections between the molecules. As a result "free ends" of mannoprotein molecules with negative and positive charges may be formed and appear on the cell wall surfaces. The conclusion on the changes of glucan component of the cell wall was made on the basis of revealed changes of the shape of the cell (Rapoport et al. 1986a) which mainly depends from the structure and stability of glucans. It is important to mention that all these changes of the cell walls were revealed for the viable dehydrated and rehydrated after dehydration yeast cells. So, the conclusion was made that these changes are reversible and do not influence cell viability. More recent studies of mannoprotein importance for the stability of yeast cell at its dehydration showed that it definitely was underestimated. It was shown recently that the absence of some mannoproteins (GPI-anchored cell wall protein Ccw12) in the cell wall unexpectedly may have very positive effects upon the higher resistance of yeast to dehydration. At the same time, mutants lacking proteins belonging to two structurally and functionally unrelated groups (proteins non-covalently attached to the cell wall and Pir proteins) possessed significantly

lower cell resistance to dehydration-rehydration than the mother wild-type strain (Borovikova et al. 2016). On the basis of these results, the conclusion can be made that the cell wall structure and mannoprotein composition are characteristics which may be very important for the resistance of yeasts to drying procedure. It simultaneously means that possible damages of cell wall proteins during yeast dehydration can be an unfavorable factor for cell resistance and viability. Besides that, the hypothesis was proposed that increased amounts of chitin may have positive effects for the stabilization of yeast cell walls in the conditions of dehydration–rehydration (Borovikova et al. 2016). Recently additional rather interesting indirect information was received specifying the definite changes of glucan component in the cell walls during dehydration of the cells. It appeared that β-glucans isolated from dry yeast cells possess higher immunogenic activity comparing it with β-glucans isolated from native yeast biomass (Liepins et al. 2015). The reasons of this effect of yeast cells' dehydration procedure, of course, have to be found in the future studies. As one of the working hypothesis, the idea that dehydration treatment leads to essential weakening of chemical bonds between mannoproteins and glucans in yeast cell walls can be proposed. As a result it facilitates to the easier isolation of β-glucans from dry yeast biomass, and obtained preparations of β-glucans have higher level of purity.

4.2.2 Plasma Membrane

Essential changes were found also for plasma membrane of yeast cells at their dehydration–rehydration. Because of significant decrease of cells sizes during this treatment to maintain its integrity, plasma membrane becomes strongly folded (Fig. 4.1) (Biryusova and Rapoport 1978). These structural changes are accompanied with essential increase of its permeability characteristics at the rehydration stage (Beker et al. 1984; Novichkova and Rapoport 1984). It is supposed that the changes of molecular organization of plasma membrane which lead to the increase of membrane permeability and significant leakage from the cells of various intracellular compounds at their rehydration may be one of the main reasons of their losses of viability (Beker and Rapoport 1987). A model which describes the changes in membranes at the dehydration and subsequent rehydration of the cells was developed (Crowe et al. 1989). In the accordance with this model, dehydration leads to the phase transitions of membrane lipids from liquid crystalline phase to gel phase. If back phase transition at dry cell rehydration takes place in the water medium, it can be rather dangerous for the state of membrane and accompanied at least by its high permeability. This model postulates that to decrease such damage, it would be necessary to increase the rehydration temperature above the phase transition temperature or to reach this phase transition in waterless conditions, for example, in the vapors of water. The correctness of these postulates was confirmed also for dehydrated yeast cells (Leslie et al. 1994; Rapoport et al. 1995, 2009). It is already known for many years that to have the resistance to dehydration, yeast cells have to accumulate rather significant amounts of trehalose (Panek 1985, 1995;

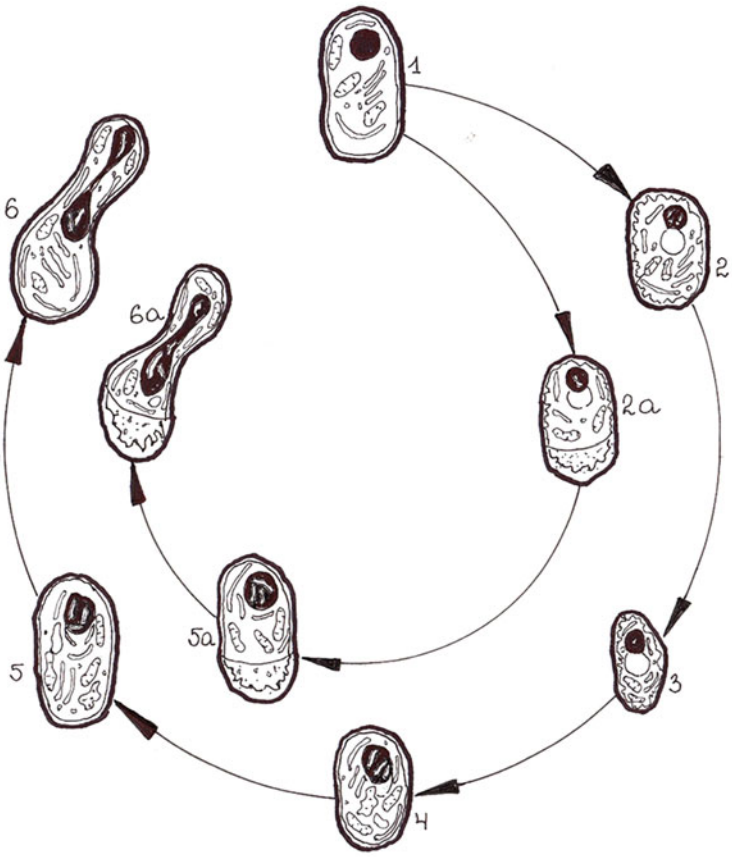

Fig. 4.1 A scheme of several structural transformations in yeast cell during its dehydration (1–3) and subsequent rehydration/reactivation (4–6). (1) Initial cell; (2–3) formation of plasma membrane invaginations (folds) during dehydration, chromatin condensation, its localization at one of the poles of the nucleus, and separation of chromosome containing part of the nucleus; (2a) formation of new intracellular plasma membrane during yeast dehydration which separates irreversibly damaged part of the cell from the viable one; (4–6) rehydration and reactivation of dry cell, further degradation of the part of the nucleus to be separated and its elimination by phagosomes during autophagy; (5a) autolysis in the separated irreversibly damaged part of the cell; (6, 6a) division of a reactivated cell (Beker and Rapoport 1987, modified)

Eleutherio et al. 2015; etc). The water replacement hypothesis was proposed to explain its role in the protection of the cells (Crowe 2008; Crowe et al. 1992). In accordance with the developments of this hypothesis, trehalose hydrogen bonds to polar residues in dry phospholipids (mainly the phosphate of the headgroup) and maintains their lateral spacing similar to substituted water thereby preserving membrane native structures (Crowe 2008). It is supposed also that besides interaction with lipids, trehalose can form also glassy matrix with high glass transition

temperature (Crowe et al. 1998) which prevents phase separation in membranes during drying of live organisms and maintains existing phases or rafts (Ricker et al. 2003). A number of the papers appeared during last years which show that yeast cells can survive desiccation treatment also in the absence of trehalose (Ratnakumar and Tunnacliffe 2006; Petitjean et al. 2015; etc.). It is possible that in this case the trehalose for the membrane and macromolecules protection may be substituted by other compounds, for example, with sugar alcohols (Rapoport et al. 1988: Krallish et al. 1997). Recently water replacement hypothesis has been additionally developed on the basis of the results of molecular dynamic simulation. Up to these new ideas, trehalose interaction with membrane phospholipids at dehydration of the cells results in increased spacing between lipids and disordering of the hydrocarbon chains in concentration-dependent manner (Golovina et al. 2009, 2010). It was shown recently that the kinetics of both processes—dehydration and rehydration—may very significantly influence the level of plasma membrane possible damages. The lowering of both processes facilitates to more safe reorganization of its lipid components (Dupont et al. 2010; Lemetais et al. 2012). It was also shown that essential importance in the maintenance of plasma membrane integrity during yeast cell dehydration has ergosterol (Dupont et al. 2011, 2012). It provides the mechanical and structural stability to membrane and also protects yeast cells from oxidation damages (Dupont et al. 2011, 2012). Another new direction in the studies of plasma membrane changes is directed to the understanding of the role of membrane proteins in the maintenance of cell viability at dehydration–rehydration treatments. First experiments have been performed using a collection of deletion mutants, the strains lacking definite membrane proteins—transporters of potassium and glycerol. Their results clearly showed the importance of potassium transporter Trk2 which is responsible for the accumulation by cells of relatively high intracellular potassium content and that it is crucial for the maintenance of yeast cell viability during their transfer into the state of anhydrobiosis and subsequent reactivation to active life (Borovikova et al. 2014c). Similar results were received also regarding glycerol transporters Stl1p (glycerol importer) and Fps1p (glycerol exporter). It appears that both of them contribute to the cell ability to survive during the transfer into the state of anhydrobiosis (Duskova et al. 2015). It means that damages of at least these membrane proteins at the dehydration–rehydration of the cells may be very dangerous for their viability. These results are the first indication that not only lipid components of plasma membrane but also its protein components play important role at yeast transfer into the state of anhydrobiosis and subsequent restoration and return to active life.

4.2.3 Mitochondria

In spite of the importance of mitochondria for the life of any eukaryotic organism, there is still too small information regarding these organelles' role and possible changes at the cells' transition into the state of anhydrobiosis and subsequent restoration of their normal physiological activities. At the same time, it is necessary

to keep in mind that besides production of energy necessary for reactivation of cells and repair processes during oxidative phosphorylation, there appear also reactive oxygen species (ROS) as the by-products. ROS can be rather dangerous for the cells and especially when they are in such weakened state after anhydrobiosis. It is well known that oxidative stress processes are one of the reasons of cell death during dehydration–rehydration treatments (Espindola et al. 2003; Franca et al. 2005; Dupont et al. 2014). It means that any information about these organelles is not only interesting but is also important for the further understanding of the ways for the increase of yeast resistance to dehydration. Nevertheless, our knowledge about their changes is at the moment too scarce. It is known that mitochondria are the organelles which restoration from inactive state in anhydrobiosis may be revealed earlier than of any other organelles. Their multiplication by dividing was found by electron microscopy at the very early stages of yeast reactivation from the state of anhydrobiosis (Rapoport and Kostrikina 1973). Studies of possible mitochondrial DNA changes as the result of cells' dehydration have not revealed them till the moment. There was not revealed any increase of petite mutants occurrence after dehydration treatment as well as mtDNA copy number changes, sequence rearrangements, and resistance to mutagen challenge (Finn and Stewart 2002; Jenkins et al. 2010). At the same time, it was shown that yeast cell viability after dehydration–rehydration correlates with mtDNA resistance (Picazo et al. 2015). This fact was revealed by comparing the viability of hybrid strain clones which contained mtDNA of *S. cerevisiae* or of *S. uvarum*. In the first case, viability of yeast after dehydration was significantly higher (Picazo et al. 2015). Electron microscopy studies showed also that during yeast dehydration the condensation of mitochondrial genome takes place (Rapoport and Kostrikina 1973). It definitely specifies on the attempts of the cells to protect mtDNA from possible damages during this treatment and transfer into the state of anhydrobiosis. Additional studies in this direction are undoubtedly necessary.

4.2.4 Nucleus

Studies of the changes in yeast nucleus during yeast transition into the state of anhydrobiosis first of all revealed the condensation of chromatin which makes it inactive and more resistant to extreme factors of the environment (Rapoport et al. 1973). Now it is supposed as the main protective reaction in the cells, and without it cells cannot maintain their viability during dehydration–rehydration processes. Electron microscopy investigations showed also the changes of nucleus shape as well the existence of opened areas of nucleus which are not covered by nuclear membrane (Rapoport et al. 1973). Such pictures are interpreted now as the evidences of the considerable changes of lipid components of nuclear membrane which are the targets for fixing agents used at the preparation of the samples for electron microscopy studies (Dupont et al. 2014). Usually in these areas are located mitochondria and lipid droplets which most probably are necessary as the sources of energy and lipids for the restoration of changed areas of nuclear membranes

during reactivation of cells from the anhydrobiosis (Rapoport et al. 1973). Further studies have not revealed any changes of the size and numbers of chromosomes as well as sequence changes in the DNA of brewer's yeast at their dehydration–rehydration (Jenkins et al. 2010). It was revealed that all the genes needed for desiccation tolerance are already transcribed and translated prior to desiccation (Calahan et al. 2011). Studies of gene expression after rehydration of dry yeast revealed activation of some genes linked with the fermentation pathway and with the nonoxidative branch of the pentose phosphate pathway. The induction of genes linked with ribosomal biogenesis and protein synthesis was shown too (Novo et al. 2007). Observations made at transcriptomic and proteomic levels showed that after rehydration of dry yeast cells very quickly there can be revealed the start of protein synthesis and expression of various genes including those which are responsible for the assimilation of various nutrient compounds (Singh et al. 2005; Rossignol et al. 2006; Novo et al. 2007; Vaudano et al. 2009, 2010; Noti et al. 2015). All these results lead to the conclusion that if the transition of yeast cells into the state of anhydrobiosis takes place in adequate conditions, there are no serious damages of the cellular genome and nucleus.

4.2.5 Other Intracellular Organelles

Rather limited amount of information was accumulated till the moment regarding reactions of other intracellular organelles during dehydration–rehydration as well as on their changes during these processes in spite that some of them have very important roles in the maintenance of yeast cell physiological activities. One of such important organelles is *vacuole* which has a lot of functions including the regulation of cellular homeostasis. Besides, it is a storage organelle, and what can be very important for the cases of cell transfer into the state of anhydrobiosis is it is linked with degradation, turnover of various intracellular molecules, and processes of autophagy. It was revealed that at dehydration–rehydration, yeast cell vacuoles can diminish in their sizes as well as fragment. There takes place also redistribution of protein molecules at least at some limited areas of vacuolar membrane that specifies changes in molecular organization of membrane (Rapoport et al. 1986b). It is clear that such changes can be rather dangerous for the cell if extrapolating our knowledge about plasma membrane changes and strong increase of plasma membrane permeability. Vacuoles contain a lot of lytic enzymes which leakage from these organelles can lead to the death of the cells. The increase of the permeability of vacuolar membranes has been already shown at least for the Mg^{2+} and Ca^{2+} ions (Rapoport et al. 1982). Electron microscopy studies revealed also the activation of autophagy during rehydration and reactivation of yeast cells from the state of anhydrobiosis (Beker and Rapoport 1987). It definitely can be supposed that autophagy is an important protective reaction of the cells at these processes because it gives the possibility to isolate from actively functioning organelles in the rest of the cell and subject to degradation (and further recycling) irreversibly damaged molecules and structures which can be dangerous for the cell.

The only information which we have at the moment about changes of *peroxisomes* at yeast cell dehydration–rehydration was obtained in the experiments with yeast *Candida boidinii*. Their cells contain the packets of peroxisomes. Electron microscopy studies showed obvious changes of lipid components of their membranes. Such conclusion was made because in dehydrated-rehydrated cells, it was not possible to visualize peroxisomal membranes inside the packet of peroxisomes. Similar observations were made also at the studies of changes of membranes of nuclei (they have been discussed above). Such effects revealed in electron microscopy investigations are linked with bad chemical fixation of these membranes at the preparation of the samples for the studies by standard methods used also for the native (not subjected to dehydration treatment) yeast samples. Because fixation procedure is linked on the chemical interaction of the fixing agent with membrane lipids, the differences in the fixation results may be explained only by the changes of lipid components of membranes during dehydration of yeast cells (Rapoport et al. unpublished).

One more interesting organelles are *lipid droplets* (Klug and Daum 2014). Lipids which are located in lipid droplets are linked with energy production via β-oxidation, intracellular membrane synthesis, protein modification, signaling, secretion within lipoproteins, and cell reactions upon stress treatments (Klug and Daum 2014; Barbosa et al. 2015; Wang 2016). In spite of the recently essentially increased attention of researchers to these organelles, we still have too scarce information regarding their changes at yeast dehydration–rehydration. Electron microscopy studies revealed that dehydration treatment may result in the merging of lipid droplets in baker's yeast cells. At the reactivation stage, sometimes there can be found the existence of the direct contacts between lipid droplets and vacuoles as well as intrusion of merged lipid droplets into the vacuole in the yeast cells (Ventina et al. 1986). These observations give the possibility to think about active participation of lipid droplets in the processes of repair and restoration of membrane structure. Experiments performed with yeast species which genetically are closely related to *S. cerevisiae*—with *S. paradoxus* and *S. bayanus*—revealed the correlation between higher amount of phosphatidylcholine accumulated by cells in lipid droplets as the result of special cultivation conditions and increased resistance of these cells at the dehydration treatment (Rodrıguez-Porrata et al. 2011). It was shown also that lipid droplets may be linked with the storage or inactivation of misfolded or aggregated proteins which can appear during dehydration–rehydration (Wang 2016).

Unfortunately, we still have no information about possible changes of *endoplasmic reticulum* membranes as well as about *Golgi complex*.

4.2.6 Intracellular Protective Reactions

To survive in the constantly changing conditions of the environment, under the influence of its various unfavorable physical and chemical factors, all live organisms including yeasts have worked out during their long evolution period a

lot of special protective reactions. In principle, the suspension of metabolism and transfer into the state of anhydrobiosis also is the protective reaction which helps live organisms to maintain their viability during very dry periods of the year. Sometimes it happens that it is rather difficult to separate the protective reactions and just the changes of organisms or cells at the influence of extreme conditions of the environment. Such example is the changes of plasma membrane which becomes strongly folded at the dehydration of the cells (Fig. 4.1). Taking into account that cells during dehydration process considerably diminish their volumes, to maintain its integrity plasma membrane has to become strongly folded, and it is reached by the formation of folded net from the small folds which existed in the membranes of native cells. So, it can be supposed that such changes also are one of protective reactions. Similar situation is with already abovementioned autophagy. It also can be supposed as protective reaction because its role is definitely linked with the necessity to eliminate damaged molecules and structures, such as proteins, membranes, or in some cases also organelles which can be toxic to actively functioning cells (Ratnakumar et al. 2011). Besides that, electron microscopy studies revealed some other protective reactions. The main of them most probably is the condensation of chromatin in the nucleus and of mtDNA in mitochondria which makes DNA molecules inactive and protect them from the damages. For the nucleus there was found the possibility to be diminished significantly in its sizes with the formation of new nuclear membrane covering condensed chromatin-containing part of the nucleus (Fig. 4.1) (Rapoport et al. 1973). In some cases, the possibility of the formation of new intracellular plasma membrane which separates two parts of the cell was discovered—dead and viable (Fig. 4.1) (Rapoport 1973). A number of chemical compounds have protective functions. The most well known of them is trehalose which can protect the membranes and macromolecules by substitution of water which is necessary for the maintenance of their structure and conformation (Panek 1995; Eleutherio et al. 2015). In spite a number of researchers have showed the possibility of mutant yeast cells to maintain their viability also in the case of trehalose absence, most probably it evidences only that cells have the alternatives how to survive in these conditions. It was found that one of alternatives to trehalose can be the group of sugar alcohols (Rapoport et al. 1988, 2009; Krallish et al. 1997). A number of substances including catalase, glutathione, and proline protect cells from the oxidative stress during dehydration–rehydration (Takagi et al. 2000, 2016; Takagi 2008; Espindola et al. 2003; Franca et al. 2005). The information on the protective function of proline is discussed in detail by H. Takagi in this book chapter “Construction of baker’s yeast strains with enhanced tolerance to baking-associated stresses.” It was revealed that higher amounts of a thioredoxin enhance oxidative stress resistance in wine yeast strains. The thioredoxin and also the glutathione/glutaredoxin system constitute the most important defense against oxidation. Both these antioxidant systems are very important at various stages of active dry yeast production starting from biomass propagation (Grant 2001; Herrero et al. 2008; Gómez-Pastor et al. 2010). Proteins, such as thioredoxins and glutaredoxins, participate also in the protection of protein activity against oxidative damage by repairing chemically modified proteins or by

modulating the redox state of protein sulfhydryl groups (Grant 2001). In accordance to these effects of the protection mechanisms against oxidative stress, lower levels of molecular damage were revealed, and both lipid peroxidation and protein carbonylation were diminished at yeast dehydration if its cells contained higher amounts of these protective proteins (Gómez-Pastor et al. 2010, 2012). Some researchers showed the protective role of heat shock proteins and especially of Hsp70 (Guzhova et al. 2008; Dupont et al. 2014; Capece et al. 2016) which can be important in the facilitation of endoplasmic reticulum-associated degradation of damaged proteins. Recently it was shown that extremely important compound is ergosterol which as it is believed can increase the mechanical resistance of plasma membrane (Dupont et al. 2011, 2012, 2014). The most recent discovery in this direction is the function of one more important protein trehalose-6P synthase which in addition to its catalytic function in trehalose synthesis is a sensing/signaling intermediate with regulatory function(s), at least in energy homeostasis. Such function in preventing energy depletion definitely may be very essential to withstand adverse conditions (Petitjean et al. 2015). This short list of different intracellular protective reaction is not complete, of course, but it definitely shows how complex and various reactions of the cells are in the unfavorable conditions of the environment. Such conclusion can be supported by recent research results which showed that mutants defective in trehalose biosynthesis, hydrophilins, responses to hyperosmolarity, and hypersalinity, reactive oxygen species (ROS) scavenging, and DNA damage repair nevertheless retain these strain desiccation tolerance (Calahan et al. 2011). It means that there are a lot of various alternatives which give the possibility to substitute one protective reaction by the other. It is also clear that further studies of mechanisms of anhydrobiosis in yeasts will lead to new discoveries of earlier unknown for us intracellular protective reactions. Some of them may appear very important not only for basic biology and industrial biotechnology but also for the goals of medicine. It can be expected that this new knowledge will give the possibilities for the development of new approaches for the protection of human organisms and artificial increase of their resistance which can be necessary, for example, in the cases of complicated medical procedures and treatments.

4.3 Some Aspects of Industrial Drying and Use of Dry Yeasts

Different methods of dehydration are used for the obtaining of active dry yeast preparations as well as for the production of dry starter cultures for various biotechnological processes. There are freeze-drying (Lodato et al. 1999; Abadias et al. 2001; Ale et al. 2015), drum dryers, fluidized bed drying (Bayrock and Ingledew 1997), spray-drying (Luna-Solano et al. 2005; Isono et al. 1995), convective dehydration, or air-blast processes between these approaches. All these approaches have their advantages and disadvantages. For example, disadvantage of freeze-drying is linked with rather high expenses necessary for this method application. Fluidized bed drying and spray-drying sometimes can give rather low

viability of yeast cells in the preparations obtained after dehydration (Lee et al. 2016). Therefore, a lot of studies during the last years were devoted to the attempts to improve and optimize these drying methods to get the possibility to use them rather efficiently. Just as the examples below will be discussed, several results are obtained during the last 15–20 years using some drying approaches—fluidized bed drying, spray-drying, and air-drying.

4.3.1 Fluidized Bed Drying

Fluidized bed technology has been used in industry already for many years. Between different products which were dehydrated with this technology, there are various wet solid products including also yeasts (Turker et al. 2006). The main advantages of this technology are linked with large contact surface area between solids and gas, good degree of solids mixing, and rapid transfer of heat and moisture between solids and gas that shortens drying time as well as the level of damaging of heat-sensitive biological materials. At the same time, the main problem of industrial fluidized bed dryers is scaling up of the processes developed in the laboratory conditions. The most important issues linked with the application of this technology and constructions of various possible fluidized bed dryer models are discussed in detail by Daud (2008). It is reported that multistage fluidized bed drying process can be used as a commercial effective method for yeast drying (Akbari et al. 2012).

Some researchers suppose that fluidized bed technologies have significant advantages comparing them with other ways of industrial yeast dehydration (Grabowski et al. 1997; Bayrock and Ingledew 1997). Bayrock and Ingledew (1997) showed that the viability of yeasts in the fluidized bed dryer was not significantly affected by the drying temperature used during the warming-up and constant-rate drying periods (when moisture contents were greater than 15%). On the basis of obtained results, these researchers stated that the mechanisms of death for fluidized bed drying are very different from those for spray-drying. It is suggested that factors other than thermal destruction influence the viability seen during fluidized bed drying.

Studies directed to the understanding of possible role of oxidative stress in the case of fluidized bed drying of yeasts showed that it is a critical one (Grant 2001; Herrero et al. 2008; Gómez-Pastor et al. 2010). It was shown in these studies that one of the factors which provide yeast strains resistance at their dehydration in fluidized bed dryer is the accumulation of glutathione. In the experiments with the simulation of industrial growth conditions, yeast cells accumulated more than 1% of glutathione, and this percentage increased during the first period of drying, reaching approximately 2%. Such results led to the low level of lipid peroxidation in dry biomass (Garre et al. 2010), and it was five- to tenfold lower than in other experiments with laboratory dehydration conditions (Espindola et al. 2003).

Mathematical modeling has been used for the analysis of drying processes, and a number of more or less complex empirical or theoretical models are available (see:

Debaste et al. 2008). The operating conditions such as temperature, loading rate of compressed yeast granules, and hot air humidity had direct effects on both yeast activity and viability. The most important factors that affected the quality of the product were loading rate and the operational temperature in each zone of the bed. Compared with the normal operating conditions at the plant, the optimization resulted in more than 12% and 27% improvement in the yeast activity and viability, respectively (Akbari et al. 2012). A model has been developed for the drying of granular baker's yeast in fluidized bed dryer. The model was applied to the drying of granular baker's yeast with different granular dimensions. Good correspondence was found between model predictions and experimental measurements for small granule sizes. As a result, up to the opinion of this model authors, it may be successfully used in the technologies linked with the drying of relatively small particles where diffusion is not the limiting step (Turker et al. 2006). Current models take into account several simultaneous mechanisms of heat, liquid, and vapor diffusion (Turker et al. 2006). Comparatively recent modeling of yeast drying in a fluidized bed led to the construction of mathematical model in which a deep physical meaning was developed, using Fick's law to model molecular diffusion of vapor inside the tortuous porous medium (Debaste et al. 2008).

The important aspects in the control and optimization of drying processes are the energy cost, drying time, the product quality, and the moisture content in material at the end of the process (Koni et al. 2009). The optimal control algorithm is proposed to improve the drying process of baker's yeast in batch fluidized bed. The developed model helps to reach smaller energy consumption and quality loss at the end of the drying process using proposed optimization procedure (Koni et al. 2009).

4.3.2 Spray-Drying

Spray-drying is another process for the production of active dry microbial preparations including yeasts at industrial scale (Luna-Solano et al. 2005; Aponte et al. 2016). This approach gives the possibilities of high production rates with low operating costs. It is commonly used for the production of various stable dry food products (Silva et al. 2011; Aponte et al. 2016). The following advantages of this process are usually mentioned: (1) heat-sensitive materials can be dried under atmospheric pressure and at low temperatures, (2) it allows pilot-scale and large-scale productions in continuous operation with relatively simple equipment, (3) spray-dried products are usually homogeneous, and (4) different types of carriers and protective compounds can be used to enhance the microbial survival (Fu and Chen 2011; Aponte et al. 2016).

Similar to other industrial processes directed to the dehydration of yeast, also spray-drying may lead to rather serious changes and damages in DNA, RNA, cell wall, plasma membrane, and other organelles (Labuza et al. 1970; Santivarangkna et al. 2008; Aponte et al. 2016).

In a number of studies, there were investigated optimal conditions of spray-drying process for several kinds of yeast. For example, for the obtaining of dry

brewer's yeast with rather high viability, it was recommended to use the addition of 10% of maltodextrins and the following temperatures: inlet, 145 °C and outlet, 60 °C (Luna-Solano et al. (2000, 2003, 2005). Another study resulted in the obtaining of high quality of dry yeast when a soluble starch was used as a protective compound, but inlet temperature was lowered to 120 °C with the same outlet temperature (60 °C). Further decrease of inlet/outlet temperatures to 110/45 °C also gave rather high viability of dry preparations, but the level of remaining water in these powders was higher than is usually recommended, and as the result it can be unfavorable for long-term storage of such dry preparations (Gomez-Pastor et al. 2011).

Some researchers separate thermal and dehydration inactivation at their studies of main factors affecting yeast damages during drying procedure. It was shown by them that yeast fermentative activity was lost very sharply if drying temperature was over 40 °C, but when it was at the level of 30 °C, thermal inactivation was negligible. At the same time, dehydration inactivation occurred also at such low temperature. It was concluded also that in spite it is not easy to control drying rate for spray-drying; low dehydration rates result in higher activities of dried yeast preparations (Fujii et al. 2011).

The possibilities for the optimization of spray-drying process are discussed in a number of papers (Luna-Solano et al. 2000, 2003, 2005). A combination of simulation with experimental treatments of spray-drying process was developed in order to minimize the energy resources required to obtain a production of spray-dried brewer's yeast at given viability and moisture. Cost function, viability, output moisture, and production were related with process variables, both empirically and by simulation with a nonlinear space state. This developed methodology is not specific for the definite technical construction of the dryers but is rather general for yeast spray-drying (Luna-Solano et al. 2005). An approach to determine the kinetics of degradation of the substances, as a function of the current state of the material (temperature, residual moisture content, and rates of their changes) during the spray-drying process of yeasts, is described by Gordienko et al. (2015). The obtained kinetic equations were used to predict the quality of yeast biomass dried in a pilot-scale spray equipment (Gordienko et al. 2015).

Studies of efficient storage of dry yeast preparations produced using spay drying method showed that there can be the losses of viability from 10 to 25% per year at 20 °C, but at the same time, there was no revealed significant changes in viability of dry yeast preparations during 3 years if they have been stored at 4 °C in a dry atmosphere (Gomez-Pastor et al. 2011).

Spray-drying is used for the microencapsulation process in the food industry. Microencapsulation is necessary to cover bioactive compounds with protective materials which can protect bioactive core from various environmental stress factors (Huq et al. 2013). In the case of yeast *Saccharomyces boulardii*, this process gives the possibility for their safe passing through gastrointestinal system of humans and animals and for this yeast use as an efficient probiotic. It can be used also as preventative and therapeutic agent for the curing of diarrhea (Holzapfel and Schillinger 2002; Kuhle et al. 2005; Czerucka et al. 2007; McFarland 2010;

Rajkowska et al. 2012). *S. boulardii* was microencapsulated by spray-drying in a number of studies (Joshi and Thorat 2011; Duongthingoc et al. 2013a, b). Gelatin and gum arabic are recommended as promising protective "wall" materials for *S. boulardii* microencapsulation. An inlet temperature of 125 °C should be preferred in this spray-drying microencapsulation process (Arslan et al. 2015).

4.3.3 Air-Drying of Yeast

Some researchers prefer to use air-blast drying which up to their data can lower the cost fivefold, decrease cell damage during drying procedure, as well as provide easier control of moisture of yeast preparations comparing with other drying methods (Santivarangkna et al. 2007; Lee et al. 2016). In spite of these advantages, rather small amount of studies related to the air-blast dried yeast have been performed. One of them was directed to the optimization of production of *Saccharomyces cerevisiae* and non-*Saccharomyces* (*Hanseniaspora uvarum* and *Issatchenkia orientalis*) yeast starters at the industrial level using this method (Lee et al. 2016). The tasks of this work included also the selection of efficient excipients and protective compounds for the dehydration treatment which are very important because intracellular accumulation of the appropriate solutes can significantly improve microbial cells survival during and after drying procedure (Kets et al. 1996; Champagne et al. 2012; Lee et al. 2016 etc.). Seven types of sugars were studied in this work with the goal to improve the viability of air-dried yeast cells. The best results were obtained at the use of 10% trehalose, 10% sucrose, and 10% glucose. The viability of these dehydrated yeasts was at the level of 80–97%. Also after 3 months of storage, *S. cerevisiae* and *H. uvarum* still had good survival rates, whereas the viability of *I. orientalis* decreased rather significantly. Dried *S. cerevisiae* and *H. uvarum* also after their storage at 4 °C showed metabolic activities similar to those of non-dried yeast cells (Lee et al. 2016).

Air-drying or convective drying was used at the development of novel approach for yeast cell immobilization (Rapoport et al. 2011). As the result of this technology stable in technological processes, biotechnologically active and economically efficient immobilized yeast preparations were obtained. It was shown that their use might also be more efficient than the use of free microbial preparations, for example, in such biotechnological processes as the purification of wastewaters from heavy metals (Rapoport et al. 2011). Yeast preparations immobilized by this method with drying procedure on hydroxylapatite or chamotte were tested also in bioethanol production. In these experiments it was shown that immobilized preparations produced the same amount of ethanol as free cells after 36 h of fermentation. At the same time during the early stages of fermentation, immobilized yeast cells produced ethanol at a higher rate than free cells. Yeast preparations immobilized on both supports (hydroxylapatite and chamotte) were successfully used at least in six sequential batch fermentations without any loss of biotechnological activity (Borovikova et al. 2014a). Special study was performed with the goal of revealing if the dehydration procedure used in this new

immobilization method noticeably decreases the viability of yeast cells in immobilized preparations. Various yeasts were used in this research: *S. cerevisiae* cells that were rather sensitive to dehydration and had been aerobically grown in an ethanol-containing medium, a recombinant strain of *S. cerevisiae* grown in aerobic conditions which was completely nonresistant to dehydration, and an anaerobically grown bakers' yeast strain *S. cerevisiae*, as well as a fairly resistant *Pichia pastoris* strain. Performed experiments showed that immobilization of all these strains essentially increased their resistance to a dehydration-rehydration treatment. The increase of cells' viability (compared with control cells dehydrated in similar conditions) was from 30 to 60%. It is concluded that a new immobilization method, which includes a dehydration stage, does not lead to an essential loss of yeast cell viability. Correspondingly, there is no risk of losing the biotechnological activities of immobilized preparations. At the same time, this research showed new interesting possibility of producing dry, active yeast preparations also for those strains that are very sensitive to dehydration and which can be used in biotechnology in an immobilized form. Finally, the immobilization with drying approach can be used also for the development of efficient methods for the long-term storage of biotechnologically active recombinant yeast strains (Borovikova et al. 2014b).

Similar approach of yeasts immobilization with dehydration stage was recently used in other studies, and it was found that five yeast strains representing *S. cerevisiae*, *Hanseniaspora uvarum*, and *Issatchenkia orientalis* entrapped in 2% air-blast-dried Ca-alginate beads with protective agents had rather high survival rates after storage at 4 °C for 3 months. The protective agents efficiently used in this research were 10% skim milk containing 10% sucrose or 10% raffinose or 10% trehalose or 10% glucose. In all these cases, the initial viability of immobilized dried yeasts was at the level of at least 87% and higher. After storage during 3 months at 4 °C, survival rate of these yeast preparations were maintained at the level of at least 51% (Kim et al. 2017).

4.3.4 Rehydration Stage

It is well known that rehydration process is one of the most important stages of reactivation of dehydrated organisms which can help to restore their active state as well as in non-adequate conditions increase their cells' damages which can lead also to their death. Therefore, it is important to find the ways how to promote the repair of the conformation of macromolecules, state of membrane lipids and proteins, molecular organization of membranes which in its turn will lead to the decrease of membrane permeability, and losses of intracellular compounds during this rehydration stage (Crowe et al. 1984, 1989, 1992; Beker and Rapoport 1987). Such studies were performed in laboratory conditions also with dehydrated yeast cells (Beker and Rapoport 1987; Beker et al. 1984; Rapoport et al. 1995). To understand what should be recommended for the industrial use of active dry yeast preparations at the production of wine, the best yeast rehydration conditions for ensuring good cell viability and vitality before their inoculation into the must were

revealed. In this study the biochemical and biophysical behaviors of dehydrated yeast after their rehydration in the various media under oenological conditions (incubation at 37 °C for 30 min) were investigated. It was shown that the addition of magnesium into rehydration medium provided the highest vitality rate as well as significantly diminished the period necessary for the reactivation of the cells and beginning of their biotechnological activity (approximately for 60%) (Rodríguez-Porrata et al. 2008). The magnesium rehydrating conditions revealed in this study definitely may have a broad industrial application.

There are also some other additives to the rehydration media which can influence the restoration of high viability and of vitality which up to some recent data (Vaudano et al. 2014) better corresponds to the fermentation activities of active dry yeast preparations. They include ergosterol (Soubeyrand et al. 2005), addition of inactive dry yeast which contains a lot of active compounds (Vaudano et al. 2014), ammonium (Vaudano et al. 2014), and ascorbic acid which most probably is efficient due to its antioxidant activity (Díaz-Hellín et al. 2013).

It is known that there are a lot of factors which can influence the viability of the cells at the rehydration stage. They include duration of the rehydration period, rehydration temperature (Poirier et al. 1999), and pH of the medium (Zikmanis et al. 1984). These factors probably affect the structure of the yeast membrane by altering the permeability and flow of molecules and ions, resulting in higher or lower vitality/viability of the rehydrated cells (Attfield 1997; Poirier et al. 1999). Up to some results, mechanical stirring of dry yeast preparations during rehydration may lead to the decrease of the number of vital cells by 5–7% and the consumption of sugars in fermentation by 18–26%. Other studies revealed the possibility of weak positive effect of a slight stirring during rehydration (reviewed by Ferrarini et al. 2007), but the experiments of Attfield et al. (2000) showed that an increase in the number of viable cells may be reached without pre-soaking and stirring. The detailed study performed by Ferrarini et al. (2007) showed that dispersion procedures play an important role in the recovery of dry yeast preparations. The authors of this research suppose that the stirring is necessary for breaking the clusters of yeast cells that in its turn would facilitate an easier water absorption, positively influence the decrease of osmotic pressure in cells cytoplasm, and avoid leakage of cell material. The other proposal or recommendation of these researchers is linked with the elongation of traditional duration of rehydration procedure (till 30 min) to 120 min at 38 °C. At the same time, it is necessary to keep in mind that these conditions may be suitable just for the strains used in this study, and therefore most probably such conclusions should be confirmed also in other conditions and with other strains of dry wine yeast preparations.

Some researchers suppose that additional procedure of “propagation” or “conditioning” of active dry yeast may reduce lag times of fermentation and correspondingly the overall fermentation duration. This issue was studied by Bellissimi and Ingledew (2005). These researchers introduced also a new term—metabolic acclimatization—to describe the events which took place during the procedure of conditioning. At the same time, their research showed that metabolic acclimatization was not necessary at least prior to alcoholic fermentation. Lag times were reduced, but overall fermentation times were unaffected (Bellissimi and Ingledew 2005).

4.3.5 Construction of New Strains

Another direction of the studies linked with the improvement of the quality of industrially produced active dry yeast preparations is the construction of new biotechnologically efficient yeast strains which are very resistant to dehydration process. One of the successful examples of this approach is recent development of new strain for the production of bioethanol (Zheng et al. 2013). In this work researchers applied the method of whole genome shuffling in which gene recombination is achieved at different positions throughout the whole genome by recursive protoplast fusion or crossing (Gong et al. 2009; Zheng et al. 2013). In this process, genetic diversity is generated, and resultant mutants with the desired phenotypes can be selected using specific screening methods (Patnaik et al. 2002; Zhang et al. 2002; Zheng et al. 2013). Application of this approach gave possibility to this work authors to obtain novel yeast strain with high resistance to dehydration treatment. During the following analysis of this novel strain and its differences from the initial strain, there were a lot of revealed proteomics and physiological changes in this new strain. In total 479 genes were with different expression levels. The further studies showed that 299 upregulated genes in new strain are linked with ribosome biosynthesis, proteasome, glycolysis/gluconeogenesis, and secondary metabolite biosynthesis. At the same time, 180 downregulated genes were enriched in pathways for oxidative phosphorylation, endocytosis, RNA transport, and vitamin B6 metabolism (Zheng et al. 2013). Besides that it was revealed also that this new strain accumulates higher amounts of trehalose which can protect macromolecules conformation and molecular organization of various intracellular membranes including also plasma membrane. As it was shown earlier in this review, it is one of the main factors which may determine the stability of the cells at dehydration–rehydration. Very interesting features of the new strain were revealed at the comparison of fatty acid content in initial and new strains. It appeared that in normal conditions there were no distinct differences in the four main fatty acid ratios between these two strains. At the same time, after the treatment there was found an increased content of unsaturated fatty acids (palmitoleic acid, oleic acid, and linoleic acid) in both strains, resulting in 17.6% and 24.6% increases in the unsaturation ratios of the initial and new strains, respectively. New strain had a higher proportion of C16:1 and a lower proportion of C18:1. It is supposed that higher amount of palmitoleic acid (C16:1) contents may be important for the maintenance of the membrane fluidity (Zheng et al. 2013). New strain accumulated also 14.6% more ergosterol (Zheng et al. 2013) which as it was shown earlier stabilizes the molecular organization of plasma membrane at the dehydration–rehydration of yeast cells (Dupont et al. 2011).

One more interesting attempt to construct new efficient and dehydration-resistant strain was performed by Kotarska with colleagues (Kotarska et al. 2011). These researchers using electrofusion between *S. cerevisiae* and *S. diastaticus* constructed new strain with rather high amylolytic activities for the industrial use in the ethanol production. They checked the technological efficiency and stability of biotechnological application of this strain after its dehydration.

Very good results were obtained in these experiments. This dry distillery yeast showed high fermentation activity and yield of ethanol production. It was concluded that application of this new strain in the form of active dry yeast in distillery practice significantly lowers the costs of spirit production by saving 30% of glucoamylase preparations used for starch saccharification. Besides that authors of this research confirmed that the use of active dry yeast preparation considerably simplifies the technological process in distillery. The application of this strain in the form of dry preparation makes the starting of the fermentation at optional time easier as well as eliminates the possibility of mash infection which sometimes takes place when conventional yeast culture was applied (Kotarska et al. 2011).

4.3.6 Application of Active Dry Yeasts

The most traditional application of active or instant dry yeasts is linked with classic technologies and biotechnologies. They include the production of bread, beer, ethanol, and wine (Fig. 4.2). The simplicity of transportation and storage and long shelf life are the main reasons why dry yeast becomes more and more popular in these areas of industry. Several reviews describe different issues relating to these classical technologies (Strumillo and Kudra 1986; Adamiec et al. 1995; Beker and Rapoport 1987; Schmidt and Henschke 2015; etc.).

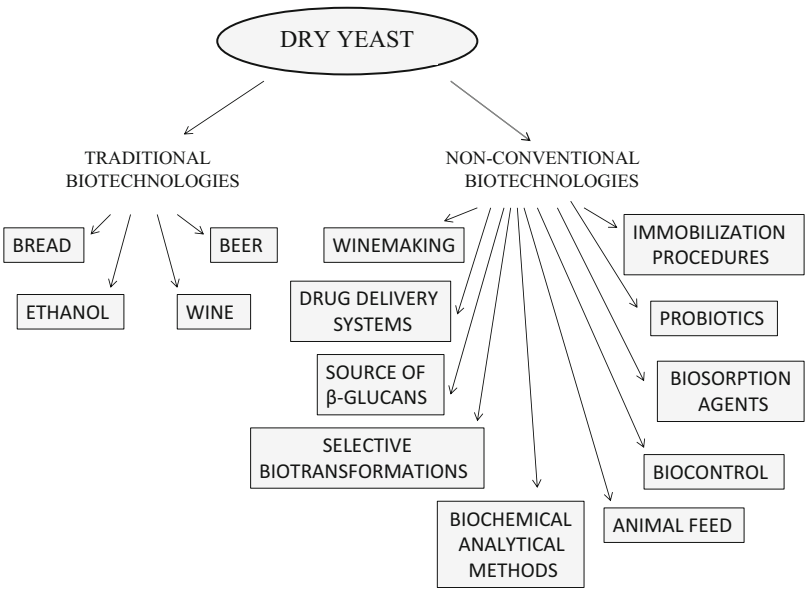

Fig. 4.2 Use of dry yeasts in traditional and non-conventional biotechnologies (Rapoport et al. 2016, modified)

During the last two decades, there appeared a number of new interesting ideas and proposals for the additional, non-conventional use of dry yeast preparations (Rapoport et al. 2016). They are linked with biomedical, pharmaceutical, agricultural, environmental, and chemical possible applications of dry yeasts (Fig. 4.2). One of such biomedical applications was already mentioned above, and it is the use of dry microencapsulated yeast *S. boulardii* as efficient probiotic in medicine (Arslan et al. 2015). Another biomedical application of dry yeast is their use as drug delivery systems. This idea is based on the use of dry recombinant yeasts which can either carry out a bioconversion reaction or produce compounds of interest directly in the digestive environment (Blanquet et al. 2005). One more interesting example is the possibility to use dry yeast as a source of efficient immunomodulators—β-glucans (Liepins et al. 2015). Dry yeasts are already used in animal feed because yeast biomass has a good composition of amino acids; high levels of lysine, threonine, and leucine; and high levels of B-complex vitamins (Rapoport et al. 2016). Because of the changes of cell wall surface characteristics during yeast dehydration, it appears that they become much more active in sorption of various pollutants including such dangerous substances as heavy metals. This makes various dry yeasts a very promising agent for the protection and/or purification of the environment (Muter et al. 2001a, b, 2002; Muter and Rapoport 2014). It was already mentioned above the use of drying procedure for the obtaining of very stable and active immobilized preparation of dry yeasts (Rapoport et al. 2011; Kim et al. 2017). Some yeast possesses biocontrol activities against various diseases of fruits and cereal grains. Dry preparations of these yeasts give the possibility of their long-term storage without losses of activity as well as decrease expenses for their transportation to various countries and regions (Melin et al. 2007, 2011; Rapoport et al. 2016). An interesting application of dry yeasts was recently proposed at the development of new efficient yeast model system for the rapid evaluation of possible efficiency of various chemical compounds (Borovikova et al. 2015). Dry yeasts can be used also in some biochemical analytical methods and in selective biotransformations (Mowshowitz 1976; Buzzini et al. 2012; Tsuda et al. 2012; Forti et al. 2015; etc.). Then, in the winemaking besides the already classical use of dry yeasts as the starter cultures, they can be successfully applied also for other goals. This nonconventional application can improve the sensorial characteristics of wines and may be efficient for the removal of unpleasant smells of volatile phenols. They have effects on wine fermentation, composition, and organoleptic characteristics, as well as on wine safety that depends from various antioxidant compounds produced by the yeast (Pozo-Bayon et al. 2009a, b; Rodriguez-Bencomo et al. 2016).

4.4 Conclusions

The last four decades have given a lot for the beginning of our understanding of general mechanisms of anhydrobiosis and correspondingly of events which take place at the dehydration of yeasts during which they can be transferred into this state. Especially big progress was reached recently, already in the twenty-first

century. An accumulated knowledge helps producers of active dry yeast preparations to find new interesting approaches for the improvement of the quality of their products. In spite of very high quality of baker's active (or instant) dry yeast produced now by leading yeast companies, this problem still is very actual for the case of brewer's, ethanol, and wine yeasts. In addition to the undoubted value of the acquired information for the purposes of practical biotechnology, it is very important also for basic science. We know now much more than earlier about the role of water for the maintenance of biological systems, structures, and macromolecules. Studies of anhydrobiosis considerably developed our knowledge on the molecular organization of biological membranes and on the intracellular protective reactions which sometimes are rather unexpected for us. We understood that there are a lot of various alternatives for the protection of the most important functions of live organisms in the unfavorable conditions and that the losses of some protective compounds are not critical because they may be substituted by the rapid synthesis of other ones which will perform necessary protection. And what is more, theoretical ideas obtained in the studies of anhydrobiosis in yeast (which is the optimal model of any eukaryotic cell) can be extrapolated to the higher organisms including animals and people. They can be used, for example, for medical purposes for the special artificial increase or improvement of organisms' resistance in the case of various diseases or complicated therapeutic treatments and surgical procedures. At the same time, it is necessary to underline once more that we are now still at the early stages of the understanding of the mechanisms of anhydrobiosis and potential of live organisms in the unfavorable conditions of the environment or physical and chemical treatments. John H. Crowe, the most well-known world leader in the studies of anhydrobiosis, in 1971 and in 2015 published two big reviews with similar titles—"Anhydrobiosis: an unsolved problem" —indicating a number of problems which still have to be solved by the researchers of this unique phenomenon of live nature (Crowe 1971, 2015). And it is clear that already in some next years, we have to expect very rapid progress in this direction.

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References

Abadias M, Benabarre A, Teixido N, Usall J, Vinas I (2001) Effect of freeze-drying and protectants on viability of the biocontrol yeast *Candida sake*. Int J Food Microbiol 65:173–182

Adamiec J, Kaminski W, Markowski AS, Strumillo C (1995) Drying of biotechnological products. In: Mujumdar AS (ed) Handbook of industrial drying. Marcel Dekker, New York, pp 775–808

Akbari H, Karimia K, Lundin M, Taherzadeh MJ (2012) Optimization of baker's yeast drying in industrial continuous fluidized bed dryer. Food Bioprod Process 90:52–57

Ale CE, Otero MC, Pasteris SE (2015) Freeze-drying of wine yeasts and *Oenococcus oeni* and selection of the inoculation conditions after storage. J Bioprocess Biotech 5:248

Aponte M, Troianiello GD, Di Capua M, Romano R, Blaiotta G (2016) Impact of different spray-drying conditions on the viability of wine *Saccharomyces cerevisiae* strains. World J Microbiol Biotechnol 32:13

Arslan S, Erbas M, Tontul I, Topuz A (2015) Microencapsulation of probiotic *Saccharomyces cerevisiae* var. *boulardii* with different wall materials by spray drying. LWT – Food Sci Technol 63:685–690

Attfield PV (1997) Stress tolerance: the key to effective strains of industrial baker's yeast. Nat Biotechnol 15:1351–1357

Attfield PV, Kletsas S, Veal DA, Van Rooijen R, Bell PJL (2000) Use of flow cytometry to monitor cell damage and predict fermentation activity of dried yeasts. J Appl Microbiol 89:207–214

Barbosa AD, Savage DB, Siniossoglou S (2015) Lipid droplet–organelle interactions: emerging roles in lipid metabolism. Curr Opin Cell Biol 35:91–97

Bayrock D, Ingledew WM (1997) Fluidized bed drying of baker's yeast: moisture levels, drying rates, and viability changes during drying. Food Res Int 30:407–415

Beker MJ, Blumbergs JE, Ventina EJ, Rapoport AI (1984) Characteristics of cellular membranes at rehydration of dehydrated yeast *Saccharomyces cerevisiae*. Eur J Appl Microbiol Biotechnol 19:347–352

Beker MJ, Rapoport AI (1987) Conservation of yeasts by dehydration. Adv Biochem Eng/Biotechnol 35:127–171

Bellissimi E, Ingledew WM (2005) Metabolic acclimatization: preparing active dry yeast for fuel ethanol production. Process Biochem 40:2205–2213

Bespalov MM, Kolpako AI, Loiko NG, Doroshenko EV, Mulyukin AL, Kozlova AN, Varlamova EA, Kurganov BI, El-Registan GI (2000) The role of microbial dormancy autoinducers in metabolism blockade. Microbiology 69:217–223

Biryusova VI, Rapoport AI (1978) Cryofractographic investigation of the structure of yeast cells in an anabiotic state. Microbiology 47:245–251

Blanquet S, Garrait G, Beyssac E, Perrier C, Denis S, Hebrard G, Alric M (2005) Effects of cryoprotectants on the viability and activity of freeze dried recombinant yeasts as novel oral drug delivery systems assessed by an artificial digestive system. Eur J Pharm Biopharm 61:32–39

Borovikova D, Herynkova P, Rapoport A, Sychrova H (2014c) Potassium uptake system Trk2 is crucial for yeast cell viability during anhydrobiosis. FEMS Microbiol Lett 350:28–33

Borovikova D, Muiznieks I, Rapoport A (2015) New test-system based on the evaluation of yeast cells resistance to dehydration-rehydration stress. Open Biotechnol J 9:49–53

Borovikova D, Rozenfelde L, Pavlovska I, Rapoport A (2014b) Immobilisation increases yeast cells resistance to dehydration-rehydration treatment. J Biotechnol 184:169–171

Borovikova D, Scherbaka R, Patmalnieks A, Rapoport A (2014a) Effects of yeast immobilisation on bioethanol production. Biotechnol Appl Biochem 61:33–39

Borovikova D, Teparic R, Mrsa V, Rapoport A (2016) Anhydrobiosis in yeast: cell wall mannoproteins are important for yeast *Saccharomyces cerevisiae* resistance to dehydration. Yeast 33:247–353

Buzzini P, Goretti M, Ponzoni C, Caselli E, Branda E, Cramarossa MR, Turchetti B, Forti L (2012) Asymmetric reduction of (4S)-(+)-carvone catalyzed by enoate reductases (ERs) expressed by non-conventional yeast (NCY) whole-cells. In: Whittall J, Sutton P (eds) Practical methods for biocatalysis and biotransformations. Wiley, New York, pp 104–108

Calahan D, Dunham M, DeSevo C, Koshland DE (2011) Genetic analysis of desiccation tolerance in *Saccharomyces cerevisiae*. Genetics 189:507–519

Capece A, Votta S, Guaragnella N, Zambuto M, Romaniello R, Romano P (2016) Comparative study of *Saccharomyces cerevisiae* wine strains to identify potential marker genes correlated to desiccation stress tolerance. FEMS Yeast Res 16:fow015

Champagne CP, Raymond Y, Simon JP (2012) Effect of water activity and protective solutes on growth and subsequent survival to air-drying of *Lactobacillus* and *Bifidobacterium* cultures. Appl Microbiol Biotechnol 95:745–756

Crowe JH (1971) Anhydrobiosis: an unsolved problem. Am Nat 105:563–574

Crowe JH (2008) Trehalose as a "chemical chaperone": fact and fantasy. Adv Exp Med Biol 594:143–158
Crowe JH (2015) Anhydrobiosis: an unsolved problem with applications in human welfare. Subcell Biochem 71:263–280
Crowe JH, Carpenter JF, Crowe LM (1998) The role of vitrification in anhydrobiosis. Annu Rev Physiol 60:73–103
Crowe JH, Crowe LM, Chapman D (1984) Preservation of membranes in anhydrobiotic organisms: the role of trehalose. Science 223:701–703
Crowe JH, Hoekstra FA, Crowe LM (1989) Membrane phase transitions are responsible for imbibitional damage in dry pollen. Proc Natl Acad Sci U S A 86:520–523
Crowe JH, Hoekstra FA, Crowe LM (1992) Anhydrobiosis. Annu Rev Physiol 54:579–599
Czerucka D, Piche T, Rampal P (2007) Yeast as probiotics – *Saccharomyces boulardii*. Aliment Pharmacol Ther 26:767–778
Daud WRW (2008) Fluidized bed dryers – recent advances. Adv Powder Technol 19:403–418
Debaste F, Halloin V, Bossart L, Haut B (2008) A new modeling approach for the prediction of yeast drying rates in fluidized beds. J Food Eng 84:335–347
Díaz-Hellín P, Úbeda J, Briones A (2013) Improving alcoholic fermentation by activation of *Saccharomyces* species during the rehydration stage. LWT – Food Sci Technol 50:126–131
Duongthingoc D, George P, Gorczyca E, Kasapis S (2013a) Studies on the viability of *Saccharomyces boulardii* within microcapsules in relation to the thermomechanical properties of whey protein. Food Hydrocoll 42:232–238
Duongthingoc D, George P, Katopo L, Gorczyca E, Kasapis S (2013b) Effect of whey protein agglomeration on spray dried microcapsules containing *Saccharomyces boulardii*. Food Chem 141:1782–1788
Dupont S, Beney L, Ferreira T, Gervais P (2011) Nature of sterols affects plasma membrane behavior and yeast survival during dehydration. Biochim Biophys Acta 1808:1520–1528
Dupont S, Beney L, Ritt JF, Lherminier J, Gervais P (2010) Lateral reorganization of plasma membrane is involved in the yeast resistance to severe dehydration. Biochim Biophys Acta 1798:975–985
Dupont S, Lemetais G, Ferreira T, Cayot P, Gervais P, Beney L (2012) Ergosterol biosynthesis: a fungal pathway for life on land? Evolution 66:2961–2968
Dupont S, Rapoport A, Gervais P, Beney L (2014) The survival kit of *Saccharomyces cerevisiae* for anhydrobiosis. Appl Microbiol Biotechnol 98:8821–8834
Duskova M, Borovikova D, Herynkova P, Rapoport A, Sychrova H (2015) The role of glycerol transporters in yeast cells in various physiological and stress conditions. FEMS Microbiol Lett 362:1–8
Eleutherio E, Panek A, De Mesquita JF, Trevisol D, Magalhães R (2015) Revisiting yeast trehalose metabolism. Curr Genet 61:263–274
El-Registan GI, Mulyukin AL, Nikolaev YA, Stepanenko IY, Kozlova AN, Martirosova EI, Shanenko EF, Strakhovskaya MG, Revina AA (2005) The role of microbial low-molecular-weight autoregulatory factors (alkylhydroxybenzenes) in resistance of microorganisms to radiation and heat shock. J Adv Space Res 36:1718–1728
Espindola AS, Gomes DS, Panek AD, Eleutherio EC (2003) The role of glutathione in yeast dehydration tolerance. Cryobiology 47:236–241
Ferrarini R, Bocca E, Cavazza A (2007) Mechanical dispersion procedures improve the rehydration of active dry yeast. Enzym Microb Technol 40:1251–1255
Finn DA, Stewart GG (2002) Fermentation characteristics of dried brewers yeast: effect of drying on flocculation and fermentation. J Am Soc Brew Chem 60:135–139
Forti L, Di Mauro S, Cramarossa MR, Filippucci S, Turchetti B, Buzzini P (2015) Non-conventional yeasts whole cells as efficient biocatalysts for the production of flavors and fragrances. Molecules 20:10377–10398
Franca MB, Panek AD, Eleutherio ECA (2005) The role of cytoplasmic catalase in dehydration tolerance of *Saccharomyces cerevisiae*. Cell Stress Chaperones 10:167–170

Fu N, Chen XD (2011) Towards a maximal cell survival in convective thermal drying processes. Food Res Int 44:1127–1149
Fujii S, Sakamoto Y, Aso T, Aktas T, Yoshimoto N, Yamamoto S (2011) Drying of yeasts—factors affecting inactivation during drying. Dry Technol 29:1981–1985
Garre E, Raginel F, Palacios A, Julien A, Matallana E (2010) Oxidative stress responses and lipid peroxidation damage are induced during dehydration in the production of dry active wine yeasts. Int J Food Microbiol 136:295–303
Golovina EA, Golovin AV, Hoekstra FA, Faller R (2009) Water replacement hypothesis in atomic detail – factors determining the structure of dehydrated bilayer stacks. Biophys J 97:490–499
Golovina E, Golovin A, Hoekstra F, Faller R (2010) Water replacement hypothesis in atomic details: effect of trehalose on the structure of single dehydrated POPC bilayers. Langmuir 26:11118–11126
Gómez-Pastor R, Pérez-Torrado R, Cabiscol E, Ros J, Matallana E (2010) Reduction of oxidative cellular damage by overexpression of the thioredoxin *TRX2* gene improves yield and quality of wine yeast dry active biomass. Microb Cell Fact 9:9
Gomez-Pastor R, Perez-Torrado R, Cabiscol E, Ros J, Matallana E (2012) Engineered Trx2p industrial yeast strain protects glycolysis and fermentation proteins from oxidative carbonylation during biomass propagation. Microb Cell Fact 11:4
Gomez-Pastor R, Perez-Torrado R, Garre E, Matallana E (2011) Recent advances in yeast biomass production. In: Matovic MD (ed) Biomass-detection, production and usage. InTech, Rijeka
Gong JX, Zheng HJ, Wu ZJ, Chen T, Zhao XM (2009) Genome shuffling: progress and applications for phenotype improvement. Biotechnol Adv 27:996–1005
Gordienko MG, Voynovskiy AA, Menshutina NV (2015) Design of a kinetic model for degradation of substances during spray drying using yeast biosuspension, oil-in-water emulsion, and alumosilicate suspension. Dry Technol 33:24–36
Grabowski S, Mujumdar AS, Ramaswamy HS, Strumillo C (1997) Evaluation of fluidized versus spouted bed drying of baker's yeast. Dry Technol 15:625–634
Grant CM (2001) Role of the glutathione/glutaredoxin and thioredoxin systems in yeast growth and response to stress conditions. Mol Microbiol 39:533–541
Guzhova I, Krallish I, Khroustalyova G, Margulis B, Rapoport A (2008) Dehydration of yeast: changes in the intracellular content of Hsp 70 family proteins. Process Biochem 43:1138–1141
Herrero E, Ros J, Belli G, Cabiscol E (2008) Redox control and oxidative stress in yeast cells. Biochim Biophys Acta 1780:1217–1235
Holzapfel WH, Schillinger U (2002) Introduction to pre- and probiotics. Food Res Int 35:109–116
Huq T, Khan A, Khan RA, Riedl B, Lacroix M (2013) Encapsulation of probiotic bacteria in biopolymeric system. Crit Rev Food Sci Nutr 53:909–916
Isono Y, Araya G, Hoshino A (1995) Immobilization of *Saccharomyces cerevisiae* for ethanol fermentation on γ-alumina particles using a spray-dryer. Process Biochem 30:743–746
Jenkins DM, Powell CD, Smart KA (2010) Dried yeast: impact of dehydration and rehydration on brewing yeast DNA integrity. J Am Soc Brew Chem 68:132–138
Joshi VS, Thorat BN (2011) Formulation and cost-effective drying of probiotic yeast. Dry Technol 29:749–757
Keilin D (1959) The problem of anabiosis or latent life: history and current concept. The Leeuwenhoek lecture. Proc R Soc Lond B150:148–191
Kets EPW, Teunissen PJM, de Bont JAM (1996) Effect of compatible solutes on survival of lactic acid bacteria subjected to drying. Appl Environ Microbiol 62:259–261
Khroustalyova GM, Adler L, Rapoport AI (2001) Exponential growth phase cells of the osmotolerant yeast *Debaryomyces hansenii* are extremely resistant to dehydration stress. Process Biochem 36:1163–1166
Kim D-H, Lee S-B, Park H-D (2017) Effect of air-blast drying and the presence of protectants on the viability of yeast entrapped in calcium alginate beads with an aim to improve the survival rate. Appl Microbiol Biotechnol 101:93–102
Klug L, Daum G (2014) Yeast lipid metabolism at a glance. Yeast FEMS Res 14:369–388

Koni M, Yuzgec U, Turker M, Dincer H (2009) Optimal quality control of baker's yeast drying in large scale batch fluidized bed. Chem Eng Process 48:1361–1370
Kotarska K, Kłosowski G, Czuprynski B (2011) Characterization of technological features of dry yeast (strain I-7-43) preparation, product of electrofusion between *Saccharomyces cerevisiae* and *Saccharomyces diastaticus*, in industrial application. Enzym Microb Technol 49:38–43
Krallish I, Jeppsson H, Rapoport A, Hahn-Hagerdal B (1997) Effect of xylitol and trehalose on dry resistance of yeasts. Appl Microbiol Biotechnol 47:447–451
Kuhle AVDA, Skovgaard K, Jespersen L (2005) In vitro screening of probiotic properties of *Saccharomyces cerevisiae* var. *boulardii* and food-borne *Saccharomyces cerevisiae* strains. Int J Food Microbiol 101:29–39
Labuza TP, Le Roux JP, Fan TS, Tannenbaum SR (1970) Engineering factors in single-cell protein production. Spray drying and cell viability. Biotechnol Bioeng 12:135–140
Lee S-B, Choi W-S, Jo H-J, Yeo S-H, Park H-D (2016) Optimization of air-blast drying process for manufacturing *Saccharomyces cerevisiae* and non-*Saccharomyces* yeast as industrial wine starters. AMB Express 6:105
Lemetais G, Dupont S, Beney L, Gervais P (2012) Air-drying kinetics affect yeast membrane organization and survival. Appl Microbiol Biotechnol 96:471–480
Leslie SB, Teter SA, Crowe LM, Crowe JH (1994) Trehalose lowers membrane phase transitions in dry yeast cells. Biochim Biophys Acta 1192:7–13
Liepins J, Kovacova E, Shvirksts K, Grube M, Rapoport A, Kogan G (2015) Drying enhances immunoactivity of spent brewer's yeast cell wall beta-D-glucans. J Biotechnol 206:12–16
Lodato P, Segovia de Huergo M, Buera MP (1999) Viability and thermal stability of a strain of *Saccharomyces cerevisiae* freeze-dried in different sugar and polymer matrices. Appl Microbiol Biotechnol 52:215–220
Lopez-Martinez G, Borull A, Poblet M, Rozes N, Cordero-Otero R (2014) Metabolomic characterization of yeast cells after dehydration stress. Int Microbiol 17:131–139
Luna-Solano G, Salgado-Cervantes MA, Garcia-Alvarado MA, Rodriguez-Jimenes G (2000) Improved viability of spray dried brewer's yeast by using starch (grits) and maltodextrin as processing aids. J Food Process Eng 23:453–462
Luna-Solano G, Salgado-Cervantes MA, Ramirez-Lepe M, Garcia-Alvarado MA, Rodriguez-Jimenes GC (2003) Effect of drying type and drying conditions over fermentative ability of brewer's yeast. J Food Process Eng 26:135–147
Luna-Solano G, Salgado-Cervantes MA, Rodríguez-Jimenes GC, García-Alvarado MA (2005) Optimization of brewer's yeast spray drying process. J Food Eng 68:9–18
McFarland LV (2010) Systematic review and meta-analysis of *Saccharomyces boulardii* in adult patients. World J Gastroenterol 16:2202–2222
Melin P, Hakansson S, Schnurer J (2007) Optimization of liquid and dry formulations of the biocontrol yeast *Pichia anomala*. Appl Microbiol Biotechnol 73:1008–1016
Melin P, Schnurer J, Hakansson S (2011) Formulation and stabilization of the biocontrol yeast *Pichia anomala*. Antonie van Leeuwenhoek 99:107–112
Morgan CA, Herman N, White PA, Vesey G (2006) Preservation of micro-organisms by drying; A review. J Microbiol Methods 66:183–193
Mowshowitz DB (1976) Permeabilization of yeast for enzyme assays: an extremely simple method for small samples. Anal Biochem 70:94–99
Mulyukin AL, Demkina EV, Kozlova AN, Soina VS, El-Registan GI (2001) Synthesis of anabiosis autoinducers by non-spore-forming bacteria as a mechanism regulating their activity in soil and subsoil sedimentary rocks. Microbiology 70:535–541
Mulyukin AL, Lusta KA, Gryaznova MN, Kozlova AN, Duzha MV, Duda VI, El-Registan GI (1996) Formation of resting cells by Bacillus cereus and Micrococcus luteus. Microbiology 65:683–689
Muter O, Lubinya I, Millers D, Grigorjeva L, Ventinya E, Rapoport A (2002) Cr (VI) sorption by intact and dehydrated *Candida utilis* cells in the presence of other metals. Process Biochem 38:123–131

Muter O, Millers D, Grigorjeva L, Ventina E, Rapoport A (2001a) Cr(VI) sorption by intact and dehydrated *Candida utilis* cells: differences in mechanisms. Process Biochem 37:505–511
Muter O, Patmalnieks A, Rapoport A (2001b) Interrelations of the yeast *Candida utilis* and Cr(VI): metal reduction and its distribution in the cell and medium. Process Biochem 36:963–970
Muter O, Rapoport A (2014) Some physiological aspects of chromium sorption by yeasts. Lambert Academic Publishing, Saarbrucken
Nikolaev YA, Mulyukin AL, Stepanenko IY, El-Registan GI (2006) Autoregulation of stress response in microorganisms. Mikrobiologiya 75:420–426
Noti O, Vaudano E, Pessione E, Garcia-Moruno E (2015) Short-term response of different *Saccharomyces cerevisiae* strains to hyperosmotic stress caused by inoculation in grape must: RT-qPCR study and metabolite analysis. Food Microbiol 52:49–58
Novichkova AT, Rapoport AI (1984) State of the intracellular pool of free amino acids in dehydrated yeast organisms. Microbiology 53:1–4
Novo M, Beltran G, Rozes N, Guillamon J-S, Sokol S, Leberre V, Francois J, Mas A (2007) Early transcriptional response of wine yeast after rehydration: osmotic shock and metabolic activation. FEMS Yeast Res 7:304–316
Palmfeldt J, Hahn-Hagerdal B (2000) Influence of culture pH on survival of *Lactobacillus reuteri* subjected to freeze-drying. Int J Food Microbiol 55:235–238
Panek AD (1985) Trehalose metabolism and its role in *Saccharomyces cerevisiae*. J Biotechnol 3:121–130
Panek AD (1995) Trehalose metabolism – new horizons in technological applications. Braz J Med Biol Res 28:169–181
Patnaik R, Louie S, Gavrilovic V, Perry K, Stemmer WPC, Ryan CM, Del Cardayre S (2002) Genome shuffling of *Lactobacillus* for improved acid tolerance. Nat Biotechnol 20:707–712
Petitjean M, Teste M-A, Francois JM, Parrou J-L (2015) Yeast tolerance to various stresses relies on the trehalose-6P synthase (Tps1) protein, not on trehalose. J Biol Chem 290:16177–16190
Picazo C, Gamero-Sandemetrio E, Orozco H, Albertin W, Marullo P, Matallana E, Aranda A (2015) Mitochondria inheritance is a key factor for tolerance to dehydration in wine yeast production. Lett Appl Microbiol 60:217–222
Poirier I, Maréchal PA, Richard S, Gervais P (1999) *Saccharomyces cerevisiae* viability is strongly dependant on rehydration kinetics and the temperature of dried cells. J Appl Microbiol 86:87–92
Pozo-Bayon MA, Andujar-Ortiz I, Alcaide-Hidalgo JM, Martin-Alvarez PJ, Moreno-Arribas MV (2009b) Characterization of commercial inactive dry yeast reparations for enological use based on their ability to release soluble compounds and their behavior toward aroma compounds in model wines. J Agric Food Chem 57:10784–10792
Pozo-Bayon MA, Andujar-Ortiz I, Moreno-Arribas MV (2009a) Scientific evidences beyond the application of inactive dry yeast preparations in winemaking. Food Res Int 42:754–761
Rajkowska K, Kunicka-Styczynska A, Rygala A (2012) Probiotic activity of *Saccharomyces cerevisiae* var. *boulardii* against human pathogens. Food Technol Biotechnol 50:230–236
Rapoport AI (1973) Rejection of areas of damaged cytoplasm by microorganisms in a state of anabiosis. Microbiology 42:317–318
Rapoport AI, Beker ME (1985) Changes in the surface charge of yeast cells during their dehydration and rehydration. Microbiology 54:362–365
Rapoport AI, Berestennikova ND, Yanovsky KA, Beker MJ (1986a) Changes in the shape and size of yeast cells upon their dehydration followed by reactivation. Microbiologiya 55:881–882 (in Russian)
Rapoport AI, Birjusova VI, Meissel MN (1973) The structure of the yeast cell nucleus in an anabiotic state. Rep Acad Sci USSR 213:708–710 (in Russian)
Rapoport AI, Biryusova VI, Svetlichnaya TP (1986b) Freeze-fracture study of changes in vacuoles during dehydration of *Saccharomyces cerevisiae* cells. Microbiology 55:835–839
Rapoport AI, Biryuzova VI, Beker ME (1983) Effect of dehydration on the surface structure of the yeast cell wall. Microbiology 52:204–207

Rapoport A, Borovikova D, Kokina A, Patmalnieks A, Polyak N, Pavlovska I, Mezinskis G, Dekhtyar Y (2011) Immobilisation of yeast cells on the surface of hydroxyapatite ceramics. Process Biochem 46:665–670

Rapoport AI, Khroustalyova GM, Camanis GJ, Beker MJ (1995) Yeast anhydrobiosis: permeability of the plasma membrane. Mikrobiologiya 64:275–278

Rapoport AI, Khroustalyova GM, Crowe LM, Crowe JH (2009) Anhydrobiosis in yeast: stabilization by exogenous lactose. Microbiology 78:624–629

Rapoport AI, Kostrikina NA (1973) Cytological investigation of an anabiosis state of yeast organisms. Proc Acad Sci USSR Biol 5:770–773 (in Russian)

Rapoport AI, Markovskii AB, Beker ME (1982) Increase in the permeability of intracellular membranes on dehydration and rehydration of *Saccharomyces cerevisiae* yeast. Microbiology 51:707–710

Rapoport A, Muter O (1995) Biosorption of hexavalent chromium by yeasts. Process Biochem 30:145–149

Rapoport AI, Puzyrevskaya OM, Saubenova MG (1988) Polyols and resistance of yeasts to dehydration. Microbiology 57:269–271

Rapoport A, Rusakova A, Khroustalyova G, Walker G (2014) Thermotolerance in *Saccharomyces cerevisiae* is linked to resistance to anhydrobiosis. Process Biochem 49:1889–1892

Rapoport A, Turchetti B, Buzzini P (2016) Application of anhydrobiosis and dehydration of yeasts for non-conventional biotechnological goals. World J Microbiol Biotechnol 32:104

Ratnakumar S, Hesketh A, Gkargkas K, Wilson M, Rash BM, Hayes A, Tunnacliffe A, Oliver SG (2011) Phenomic and transcriptomic analyses reveal that autophagy plays a major role in desiccation tolerance in *Saccharomyces cerevisiae*. Mol BioSyst 7:139–149

Ratnakumar S, Tunnacliffe A (2006) Intracellular trehalose is neither necessary nor sufficient for desiccation tolerance in yeast. FEMS Yeast Res 6:902–913

Ricker JV, Tsvetkova NM, Wolkers WF, Leidy C, Tablin F, Longo M, Crowe JH (2003) Trehalose maintains phase separation in an air-dried binary lipid mixture. Biophys J 84:3045–3051

Rodriguez-Bencomo JJ, Andujar-Ortiz I, Sanchez-Patan F, Moreno-Arribas MV, Pozo-Bayon MA (2016) Fate of the glutathione released from inactive dry yeast preparations during the alcoholic fermentation of white musts. Aust J Grape Wine Res 22:46–51

Rodrıguez-Porrata B, Lopez-Martinez G, Redon M, Sancho M, Mas A, Rozes N, Cordero-Otero R (2011) Enhancing yeast cell viability after dehydration by modification of the lipid profile. World J Microbiol Biotechnol 27:75–83

Rodríguez-Porrata B, Novo M, Guillamon J, Rozes N, Mas A, Cordero-Otero R (2008) Vitality enhancement of the rehydrated active dry wine yeast. Int J Food Microbiol 126:116–122

Rossignol T, Postaire O, Storaı J, Blondin B (2006) Analysis of the genomic response of a wine yeast to rehydration and inoculation. Appl Microbiol Biotechnol 71:699–712

Santivarangkna C, Kulozik U, Foerst P (2007) Alternative drying processes for the industrial preservation of lactic acid starter cultures. Biotechnol Prog 23:302–315

Santivarangkna C, Kulozik U, Foerst P (2008) Inactivation mechanisms of lactic starter cultures preserved by drying processes. J Appl Microbiol 105:1–13

Schmidt SA, Henschke PA (2015) Production, reactivation and nutrient requirements of active dried yeast in winemaking: theory and practice. Aust J Grape Wine Res 21:651–662

Silva J, Freixo R, Gibbs P, Teixeira P (2011) Spray-drying for the production of dried cultures. Int J Dairy Technol 64:321–335

Singh J, Kumar D, Ramakrishnan N, Singhal V, Jervis J, Garst J, Slaughter S, DeSantis A, Potts M, Helm R (2005) Transcriptional response of *Saccharomyces cerevisiae* to desiccation and rehydration. Appl Environ Microbiol 17:8752–8763

Soubeyrand V, Luparia V, Williams P, Doco T, Vernhet A, Ortiz-Julien A, Salmon JM (2005) Formation of micella containing solubilized sterols during rehydration of active dry yeasts improves their fermenting capacity. J Agric Food Chem 53:8025–8032

Stepanenko IY, Strakhovskaya MG, Belenikina NS, Nikolaev YA, Mulyukin AL, Kozlova AN, Revina AA, El-Registan GI (2004) Protection of *Saccharomyces cerevisiae* against oxidative and radiation-caused damage by alkylhydroxybenzenes. Microbiology 73:163–169

Strumillo C, Kudra T (1986) Drying: principles applications and design. Gordon and Breach Science Publishers, New York

Takagi H (2008) Proline as a stress protectant in yeast: physiological functions, metabolic regulations, and biotechnological applications. Appl Microbiol Biotechnol 81:211–223

Takagi H, Sakai K, Morida K, Nakamori S (2000) Proline accumulation by mutation or disruption of the proline oxidase gene improves resistance to freezing and desiccation stresses in *Saccharomyces cerevisiae*. FEMS Microbiol Lett 184:103–108

Takagi H, Taguchi J, Kaino T (2016) Proline accumulation protects *Saccharomyces cerevisiae* cells in stationary phase from ethanol stress by reducing reactive oxygen species levels. Yeast 33:355–363

Tsuda H, Matsumoto T, Ishimi Y (2012) Time reduction of vitamin B6 and inositol assay by using lyophilized *Saccharomyces cerevisiae* ATCC 9080. J Nutr Sci Vitaminol 58:149–151

Turker M, Kanarya A, Yuzgec U, Kapucu H, Senalp Z (2006) Drying of baker's yeast in batch fluidized bed. Chem Eng Process 45:1019–1028

Vaudano E, Costantini A, Cersosimo M, Del Prete V, Garcia-Moruno E (2009) Application of real-time RT–PCR to study gene expression in active dry yeast (ADY) during the rehydration phase. Int J Food Microbiol 129:30–36

Vaudano E, Costantini A, Noti O, Garcia-Moruno E (2010) An RT-qPCR approach to study the expression of genes responsible for sugar assimilation during rehydration of active dry yeast. Food Microbiol 27:802–808

Vaudano E, Noti O, Costantini A, Garcia-Moruno E (2014) Effect of additives on the rehydration of *Saccharomyces cerevisiae* wine strains in active dry form: influence on viability and performance in the early fermentation phase. J Inst Brew 120:71–76

Ventina EJ, Saulite LA, Rapoport AI, Beker ME (1984) Electron-microscopic study of yeasts in a state of anabiosis and reactivated from this state. Microbiology 53:536–541

Ventina EJ, Saulite LA, Rapoport AI, Beker ME (1986) Lipid inclusions in cells and changes in them during dehydration and reactivation of yeasts. Microbiology 55:99–104

Wang C-W (2016) Lipid droplets, lipophagy and beyond. Biochim Biophys Acta 1861:793–805

Zhang YX, Perry K, Vinci VA, Powell K, Stemmer WPC, Del Cardayre SB (2002) Genome shuffling leads to rapid phenotypic improvement in bacteria. Nature 415:644–646

Zheng D, Zhang K, Gao K, Liu Z, Zhang X, Li O, Sun J, Zhang X, Du F, Sun P, Qu A, Wu X (2013) Construction of novel *Saccharomyces cerevisiae* strains for bioethanol active dry yeast (ADY) production. PLoS One 8:e85022

Zikmanis PB, Auzan SI, Kruche RV, Zikmane MA, Auzinia LP (1984) Rehydration conditions for dehydrated *Saccharomyces cerevisiae* yeasts and cell viability. Mikrobiologiia 53:208–212

Biotechnology of Glycerol Production and Conversion in Yeasts

5

Marta Semkiv, Kostyantyn Dmytruk, Charles Abbas, and Andriy Sibirny

Abstract

Glycerol is widely used in the cosmetic, paint, automotive, food, and pharmaceutical industries and for the production of explosives. Currently glycerol is available in commercial quantities as a by-product of biodiesel production, but its purification is cost prohibitive. The industrial production of glycerol from glucose by fermentation using *Candida* sp. and *Saccharomyces cerevisiae* yeasts has been described. This review covers in depth the biosynthesis of glycerol by yeast as well as glycerol bioconversion to different useful chemical compounds.

Contents

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5.1 Introduction

Glycerol or propane-1,2,3-triol (also known as glycerine or glycerin) is a simple polyol that is colorless, odorless, highly viscous, sweet tasting, and a nontoxic liquid. Glycerol is a constituent of neutral fats (triacylglycerides). Glycerol is widely used in the food industry as a sweetener and humectant, in pharmaceutical industry, and for production of cosmetics, toothpastes, paints, drugs, paper, textiles, leather, and explosives (Wang et al. 2001). It was discovered in 1779 by the Swiss pharmacist, K. W. Scheele, who was the first to isolate this compound by heating a mixture of litharge (PbO) with olive oil (Quispe et al. 2013). Glycerol could be produced chemically from oils (triacylglycerides) or from propylene or by microbial synthesis in large concentration by some osmophilic yeast. Most recently glycerol has been readily available commercially in low purity as a by-product from the biodiesel industry. This glycerol is contaminated by methanol, salts, and other impurities, so its purification is quite expensive. In this review we describe pathways of glycerol biosynthesis and catabolism in different yeast species and the construction of yeast strains capable of aerobic and anaerobic glycerol oversynthesis as well as strains which effectively convert glycerol to valuable compounds.

5.2 Glycerol Metabolism in Yeast Cells

Glycerol is very important for living cells as it forms the backbone of phospholipids (common constituents of cell membranes) and triglycerides (widespread storage lipids). The free form of glycerol is both utilized and produced by many living organisms, in particular, yeasts. Glycerol metabolism is most comprehensively described for laboratory strains of the conventional yeast *Saccharomyces cerevisiae* (Fig. 5.1), but for other yeasts available information is rather fragmentary.

5.2.1 Glycerol Synthesis

In the yeast *S. cerevisiae*, glycerol is synthesized from the glycolysis intermediate dihydroxyacetone phosphate (DHAP) in two consecutive reactions catalyzed by the cytosolic enzymes glycerol-3-phosphate dehydrogenase and glycerol-3-phosphate

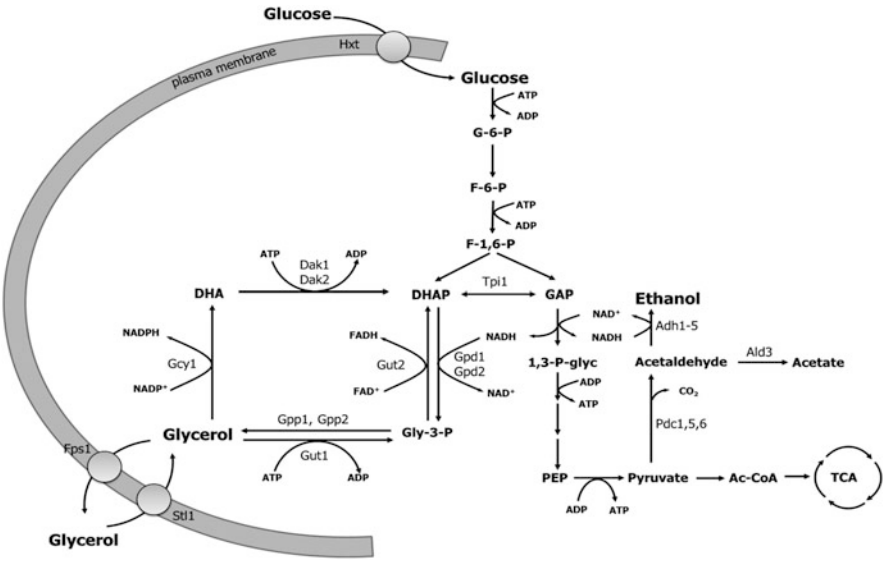

Fig. 5.1 Glycerol metabolism in *S. cerevisiae*. *G-6-P* glucose-6-phosphate; *F-6-P* fructose-6-phosphate; *F-1,6-P* fructose-1,6-bisphosphate; *DHAP* dihydroxyacetone phosphate; *DHA* dihydroxyacetone; *GAP* glyceraldehyde-3-phosphate; *G-3-P* glycerol-3-phosphate; *1,3-P-glyc* 1,3-bisphosphoglycerate; *PEP* phosphoenolpyruvate; *Ac-CoA* acetyl coenzyme A; *TCA* tricarboxylic acid cycle; *Tpi1* triose phosphate isomerase; *Adh1-5* alcohol dehydrogenases; *Pdc1,5,6* pyruvate decarboxylases; *Ald3* aldehyde dehydrogenase; *Gpd1, Gpd2* cytosolic glycerol-3-phosphate dehydrogenases; *Gpp1*, *Gpp2* glycerol-3-phosphate phosphatases; *Gut1* glycerol kinase; *Gut2* glycerol-3-phosphate dehydrogenase; *Gcy1* glycerol dehydrogenase; *Dak1*, *Dak2* dihydroxyacetone kinase; *Hxt*, *Stl1*, *Fps1* membrane transporters

phosphatase. DHAP originates from the split of fructose-1,6-bisphosphate into DHAP and glyceraldehyde-3-phosphate (GAP). These two compounds are the starting point of two metabolic pathways, which lead to the final products: ethanol from GAP and glycerol from DHAP. DHAP can be converted into GAP and vice versa by the action of the enzyme triose phosphate isomerase. Cytosolic NAD^+-dependent glycerol-3-phosphate dehydrogenase (GPDH) catalyzes the reduction of DHAP to glycerol-3-phosphate with simultaneous oxidation of NADH to NAD^+. This is the "rate-limiting" step in glycerol production (Remize et al. 2001). In *S. cerevisiae* this enzyme is encoded by the two isogenes *GPD1* and *GPD2*. The *GPD1* gene was first discovered by Larsson et al. (1993) and Albertyn et al. (1994); *GPD2* gene was discovered later by Eriksson et al. (1995) and Ansell et al. (1997). The amino acid sequences of Gpd1 and Gpd2 are 69% identical (Eriksson et al. 1995), and the kinetic properties of the two isoenzymes are similar. Despite the similar physical and catalytic properties of their gene products, the *GPD1* and *GPD2* genes are regulated differently at the transcriptional level. Also, the mutant phenotypes indicate that Gpd1 and Gpd2 fulfill different roles in metabolism. The growth of a *gpd1Δ* mutants was clearly inhibited at high osmolarity (in the presence

of 0.7 M NaCl), whereas a *gpd2Δ* mutant exhibited a growth rate similar to the wild type under these conditions. The transcription of *GPD1* gene is strongly induced by MAPK Hog1, which plays a central role in the cell response to osmotic stress (Albertyn et al. 1994). However, glycerol production following osmotic stress increases almost instantly, before the transcriptional regulation can influence enzyme production (Klipp et al. 2005). This fact led to the suggestion that the activation of the existing Gpd1 pool may be the mechanism by which rapid glycerol production is achieved. Indeed, there is some recent evidence that indicates the activity of the Gpd1 enzyme is regulated at the posttranslational level by phosphorylation (Lee et al. 2012; Oliveira et al. 2012). The two TORC2-dependent protein kinases Ypk1 and Ypk2 phosphorylate Gpd1 at the four adjacent serine residues. Change of all these four sites to alanine results in GPDH that is twofold more active than the original wild type (Oliveira et al. 2012). Following hyperosmotic shock, Ypk1 and Ypk2 are apparently no longer stimulated by TORC2, and Gpd1 becomes dephosphorylated by an unknown protein phosphatase, leading to its activation (Lee et al. 2012).

The transcription of the *GPD2* gene has been shown not to be affected by changes in external osmolarity; however, *gpd2Δ* mutants showed poor growth without oxygen supply, because Gpd2 activity is important for maintenance of cellular redox balance under anaerobic conditions. The mRNA level of *GPD2*, in contrast to *GPD1*, was ninefold upregulated after a shift of *S. cerevisiae* from aerobic to anaerobic conditions (Ansell et al. 1997). During prolonged anaerobic growth, expression of *GPD2* decreased, and in steady-state anaerobic chemostat cultures, the mRNA concentration stabilized at 1.5 times the aerobic level (ter Linde et al. 1999). A *gpd1Δ gpd2Δ* double mutant was totally unable to produce glycerol and did not grow at all under anaerobic conditions, unless an NADH-oxidizing agent, such as acetaldehyde or acetoin, was added (Bjorkqvist et al. 1997). At high osmolarity, the double mutant does not grow either (Ansell et al. 1997).

Genes encoding NAD^+-dependent GPDH have also been characterized in other yeast species such as *Candida magnoliae* (Lee et al. 2008), *Candida glycerinogenes* (Chen et al. 2008), and *Debaryomyces hansenii* (Thome 2004) and in the filamentous fungus, *Aspergillus nidulans* (Fillinger et al. 2001). In addition, $NADP^+$-dependent GPDH activity has been discovered in other yeast species, such as *Candida versatilis* (Watanabe et al. 2008).

The second step in the glycerol synthesis is the irreversible hydrolysis of the phosphate group from glycerol-3-phosphate, a reaction catalyzed by glycerol-3-phosphatase (G3P) (Norbeck et al. 1996). Similarly as for the dehydrogenase, two structural genes encoding isoenzymes of the phosphatase have been identified: *GPP1* and *GPP2*. The sequences of these corresponding proteins are 95% identical (Norbeck et al. 1996). Both isoenzymes have a high specificity for DL-glycerol-3-phosphate and have an optimum pH of 6.5. The expression of *GPP1* and *GPP2* genes is regulated differently as is the case for *GPD* genes. Mutants lacking Gpp1 activity showed poor growth under anaerobic conditions and *GPP1* expression seemed to be induced upon switch to anaerobic conditions (Pahlman et al. 2001).

The expression of *GPP2* is strongly activated through the high osmolarity glycerol (HOG) pathway in the presence of hyperosmotic stress (Norbeck et al. 1996; Pahlman et al. 2001). Single mutant *gpp1Δ* or *gpp2Δ* did not reveal growth impairment during osmotic stress, indicating that the two paralogs can substitute well for each other (Pahlman et al. 2001). However, the deletion of both genes in the *gpp1*Δ *gpp2*Δ mutant caused a growth inhibition under anaerobic conditions and increased sensitivity to osmotic stress. The *gpp1*Δ *gpp2*Δ mutant accumulated increased amounts of glycerol-3-phosphate and produced only minor amounts of glycerol (Pahlman et al. 2001).

In filamentous fungi and in the fission yeast *Schizosaccharomyces pombe*, another possible pathway for glycerol synthesis through dihydroxyacetone (DHA) has been proposed. The first step in this pathway is assumed to be catalyzed by an uncharacterized enzyme that dephosphorylates DHAP to DHA. The second step is catalyzed by an $NADP^+$-dependent glycerol dehydrogenase. This pathway has been suggested based on the fact that $NADP^+$-dependent glycerol dehydrogenase activities converting DHA to glycerol have been identified in *S. pombe* (Marshall et al. 1989) and species of filamentous fungi belonging to the *Aspergillus* genus (Redkar et al. 1995; Schuurink et al. 1990; Ruijter et al. 2004). More precisely this pathway was studied in *A. nidulans* (Fillinger et al. 2001; de Vries et al. 2003). Mutants with disruption of the gene *gldB* encoding NADP+-dependent glycerol dehydrogenase had a significant reduction in glycerol amounts and showed a severe growth defect under hyperosmotic conditions (de Vries et al. 2003), while the deletion of *gfdA* encoding cytosolic GPDH did not abolish glycerol biosynthesis (Fillinger et al. 2001). Expression of the *A. nidulans* paralog *gldB* gene was strongly induced under conditions of hyperosmotic shock. These studies suggested that glycerol formation through DHA is the major pathway responsible for glycerol accumulation under osmotic stress in *A. nidulans*, and the pathway through glycerol-3-phosphate does not play a significant role in this organism. But in order to clearly establish this alternative pathway for glycerol production, the "DHAPase" activity needs to be allocated to a certain enzyme, which so far has not been demonstrated.

5.2.2 Glycerol Utilization

As glycerol is relatively abundant in nature, there is no wonder that many microorganisms can use it as a source of carbon and energy. Similarly as glycerol production, glycerol utilization can occur through glycetol-3-phosphate or through dihydroxyacetone. The dihydroxyacetone phosphate metabolically produced can either enter into central catabolism via the transformation to glyceraldehyde-3-phosphate by a triose phosphate isomerase or can serve as a substrate for lipid synthesis. The pathway through glycetol-3-phosphate involves a glycerol kinase and a FAD^+-dependent glycerol-3-phosphate dehydrogenase that is located at the outer surface of the inner mitochondrial membrane. The latter enzyme directly transfers the electrons via $FADH_2$ to the respiratory chain. In *S. cerevisiae*, this

pathway represents the main route for glycerol utilization. In *S. cerevisiae*, glycerol kinase is encoded by the *GUT1* gene (Sprague and Cronan 1977; Pavlik et al. 1993) and glycerol-3-phosphate dehydrogenase is encoded by gene *GUT2* (Ronnow and Kielland-Brandt 1993). *S. cerevisiae* mutants that are defective in *GUT1* or *GUT2* are unable to utilize glycerol as a sole carbon source (Sprague and Cronan 1977). Mitochondrial Gut2 glycerol-3-phosphate dehydrogenase together with the cytosolic glycerol-3-phosphate dehydrogenases Gpd1 and Gpd2 also contributes to the "glycerol-3-phosphate shuttle" of yeast, which serves for the conversion of mitochondrial NADH into NAD^+ during anaerobic growth of *S. cerevisiae* (Larsson et al. 1998). The route of glycerol dissimilation through glycerol-3-phosphate intermediate has also been observed in a number of other yeasts, e.g., *D. hansenii* (Adler et al. 1985), *Zygosaccharomyces rouxii* (van Zyl et al. 1991), and *C. glycerinogenes* (Wang et al. 2000). A number of yeasts are thought to dissimilate glycerol via the second pathway with dihydroxyacetone as an intermediate. The first step in this pathway is the oxidation of glycerol to dihydroxyacetone by glycerol dehydrogenase followed by the phosphorylation of dihydroxyacetone by dihydroxyacetone kinase. These enzymes are encoded by the genes *GCY1*, *DAK1*, and *DAK2*. In *S. cerevisiae*, the existence of this pathway was proposed by Norbeck and Blomberg (1997). Jung and coworkers recently confirmed that Gcy1 is the $NADP^+$-dependent glycerol dehydrogenase (Jung et al. 2012). The function of this pathway in *S. cerevisiae* is unclear but most probably does not involve utilization of glycerol as a carbon source, because it cannot substitute nonfunctioning glycerol-3-phosphate catabolic route in *gut1Δ* or *gut2Δ* mutants. The dihydroxyacetone pathway in this yeast might play a role in the regulation of the glycerol concentration during hyperosmotic stress (Blomberg 2000) or for redox regulation (Costenoble et al. 2000). However, in other yeasts, this pathway may be more significant. Overall, the information about the role of one or another route for glycerol utilization in certain yeast species is not well understood. Based on the presence of glycerol kinase or NAD^+-dependent glycerol dehydrogenase activity in cell-free extracts, Tani and Yamada (1987) divided the yeast species they investigated into three groups: (1) the first group being assumed to exclusively use the glycerol-3-phosphate pathway (e.g., *Candida boidinii*); (2) the second group that used only the dihydroxyacetone pathway (e.g., *Hansenula ofunaensis*); and (3) the third group where both pathways were considered to be functional (e.g., *Candida valida*). However, the evidence they provided for the presence of activities of the enzymes attributed to the specific pathways is not sufficient to establish the functionality of these metabolic pathways. More explicit evidence is needed that demonstrates that the deletion of the gene that encodes a key enzyme in a particular catabolic pathway leads to the reduction or total elimination of yeast growth on glycerol, as was shown for *gut1Δ* or *gut2Δ* mutants of *S. cerevisiae* (Sprague and Cronan 1977). This type of strong evidence was provided for the dihydroxyacetone pathway for glycerol utilization in the yeast *Schizosaccharomyces pombe* (Matsuzawa et al. 2010). An *S. pombe* strain with deletion of the gene *gld1* had reduced NAD^+-dependent glycerol dehydrogenase activity and was not able to grow in media containing glycerol as the sole carbon source.

Different yeast species and even different strains of the same species show high diversity with regard to ability for growth on glycerol as the sole source of carbon (Kurtzman et al. 2011). Most strains of the conventional yeast *S. cerevisiae* have low growth on a medium with glycerol as the sole carbon source, but their growth can be improved by the addition of amino acids, nucleotide bases, or complex supplements to the cultivation medium (Merico et al. 2011; Swinnen et al. 2013). Among 52 *S. cerevisiae* strains that were compared, a strain with the fastest biomass accumulation on glycerol was chosen and compared to common laboratory strain in respect to its genetic features (Swinnen et al. 2013). It turned out that better biomass accumulation was caused by three genetic determinants—specific alleles of genes *UBR2*, *SSK1*, and *GUT1* (Swinnen et al. 2016). The role of the *GUT1* gene in glycerol utilization is obvious, but the role of the genes *UBR2* and *SSK1* is uncertain, and they encode, respectively, a ubiquitin ligase (Finley et al. 2012) and a component of HOG pathway (Maeda et al. 1994). Two independent studies have reported that wild-type *S. cerevisiae* strains that were not able to grow in a synthetic glycerol medium can be relatively easily evolved by repeated batch cultivations in a glycerol medium to reach better growth levels under these conditions (Ochoa-Estopier et al. 2011; Merico et al. 2011). These results may imply that *S. cerevisiae* ancestors were able to utilize glycerol more efficiently, but this feature was lost during evolution. Such "loss of function" might occur due to the fact that *S. cerevisiae* has evolved to overgrow other competing microorganisms through fast sugar utilization and rapid ethanol production caused by the strong Crabtree effect. In this way, potential competitors for the consumption of the fermentation products ethanol and glycerol during the post-diauxic growth phase have already been outcompeted during the sugar-consuming phase.

Some other yeast species show much better ability to grow on glycerol than *S. cerevisiae*. For example, when the ability of 42 different yeast species to grow on glycerol was tested, the yeasts *Pichia jadinii* and *P. anomala* showed the highest growth rate, which was approximately three times higher than for *S. cerevisiae* (Lages et al. 1999). Current industrial applications employing glycerol-based cultivation media use such yeasts as *Yarrowia (Candida) lipolytica, Komagataella (Pichia) pastoris*, and *Pachysolen tannophilus*. When their growth performance on glycerol was tested, these species showed 1.3–3.3 higher growth rates than that of the best glycerol-utilizing *S. cerevisiae* strain (Klein et al. 2016).

5.2.3 Glycerol Transport Across Plasma Membrane

In order to be used as a carbon source, glycerol must be transported into the cell. By comparison the excessive amounts of glycerol that are produced from NAD^+ re-oxidation must be exported from the cell. The molecular details of the glycerol flux through a plasma membrane have been best studied in *S. cerevisiae*; in other yeasts and in filamentous fungi, the available information is fragmentary. In *S. cerevisiae* the process of identification of the glycerol transport mechanisms

was not straightforward, so all possible hypotheses have been considered which include passive and facilitated diffusion and active transport.

For example, previously glycerol was generally regarded as a molecule that is able to permeate a plasma membrane by passive diffusion that is driven by a concentration gradient (Gancedo et al. 1968; Heredia et al. 1968; Romano 1986). But Oliveira et al. (2003) based on the comparison of olive oil/water partitioning coefficient with that of other molecules, speculated that glycerol is unlikely to significantly diffuse through biological membranes, although they indicated that some passive diffusion across the lipid bilayer cannot be completely excluded. Since plasma membrane is mostly impermeable to glycerol, then any detectable passive glycerol diffusion into the cell must be mediated via a channel-like facilitator protein. Several experimental studies demonstrated a saturable glycerol uptake component in *S. cerevisiae*, which was believed to be connected with the protein Fps1, a member of the major intrinsic protein (MIP) family of channel proteins (Luyten et al. 1995; Sutherland et al. 1997). This protein shows a high sequence homology to the glycerol facilitator GlpF from *E. coli* (Heller et al. 1980), the only transporter responsible for glycerol uptake in this bacterium. Subsequent experiments showed that the previously detected saturable glycerol uptake kinetics was an artifact caused by considerable Gut1 activity that interfered with the experimental setup (Oliveira et al. 2003). The deletion of *GUT1* gene eliminated such effect. It has also been shown that *S. cerevisiae fps1Δ* mutants were able to grow on glycerol as sole carbon source (Luyten et al. 1995). Further studies confirmed that Fps1 in *S. cerevisiae* is involved rather in glycerol export during osmoregulation than in glycerol uptake (Tamas et al. 1999).

The existence of a glycerol active transport system in *S. cerevisiae* was predicted based on the observation of simultaneous uptake of glycerol and protons accompanied by intracellular glycerol accumulation (Sutherland et al. 1997). *GUP1* and its paralog *GUP2* had been suggested to encode the proteins responsible for glycerol active transport (Holst et al. 2000). These authors showed that the Gup1 protein is essential for the alleviation of the osmotic stress-induced cell death of *gpd1Δ gpd2Δ* mutants: deletion of *GUP1* in a *gpd1Δ gpd2Δ* double deletion mutant suppresses the rescuing effect of glycerol supplementation during hyperosmotic shock (Holst et al. 2000). However, further studies did not provide support for this theory. It has been shown that glycerol uptake activity was enhanced after switch to a medium with glycerol or with high salt concentration, whereas *GUP1* and *GUP2* expression was found to be constitutive (Oliveira and Lucas 2004). Also, a double deletion mutant *gup1Δ gup2Δ* still showed active glycerol uptake under salt stress (Neves et al. 2004). Therefore, proteins Gup1 and Gup2 probably are involved in glycerol uptake only indirectly.

Ferreira et al. (2005) revealed that a crucial role for glycerol uptake is played by the glycerol/H^+-symporter Stl1. The deletion of *STL1* gene completely abolished active glycerol transport and prevented *S. cerevisiae* growth on glycerol as a sole carbon source. The pattern of *STL1* gene expression is consistent with aforementioned features of glycerol uptake; in particular, transcription of *STL1* is transiently induced after the shift to hyperosmotic medium (Posas et al. 2000; Ferreira et al.

2005) and then decreased gradually to initial state following glycerol production activation. This downregulation does not occur in a *gpd1Δ gpd2Δ* mutant, which is unable to synthesize intracellular glycerol (Ferreira et al. 2005). Other similar roles for H^+- but also Na^+-glycerol symporters have been described for the halotolerant yeasts *D. hansenii* (Lucas et al. 1990), *Pichia sorbitophila* (Lages and Lucas 1995), and *Z. rouxii* (van Zyl et al. 1990).

As mentioned before, *S. cerevisiae* shows relatively low growth rates on glycerol. Gancedo et al. (1968) speculated that glycerol uptake velocity might be a major rate-limiting step for growth on glycerol in this yeast. Homologs of *STL1* and *FPS1* genes have been identified for yeasts with the best growth performance on glycerol, which include *Y. lipolytica*, *P. tannophilus*, and *K. pastoris* (Dujon et al. 2004; Mattanovich et al. 2009; Liu et al. 2013). The contribution of Stl1 and Fps1 homologs to glycerol utilization in other yeast species is not yet clear. There is evidence that, in contrast to *S. cerevisiae*, in other yeasts Fps1 homologs might actually play a role in glycerol uptake. For example, Liu et al. (2013) showed that the expression of *FPS2* gene from *P. tannophilus* in *S. cerevisiae* was able to suppress the growth defect on glycerol of a *stl1Δ* deletion mutant, whereas overexpression of the homologous *S. cerevisiae FPS1* gene was unable to achieve this task. ScFps1 and PtFps2 orthologs are also different with regard to their protein structure. They both have a core region with six putative transmembrane domains (TMDs), but PtFps2 is much shorter as it does not have long N- and C-terminal extensions. Overexpression in the *S. cerevisiae* strain of *PtFPS2* and similar *FPS1* homologs from different non-conventional yeast species (*C. jadinii*, *P. pastoris* and *Y. lipolytica*) resulted in a significant improvement in growth rates on glycerol. These high growth rates were retained even after *STL1* gene deletion (Klein et al. 2016). However, in order to confirm the role of these glycerol facilitators, they should be functionally studied in the original organisms.

In *S. cerevisiae*, glycerol export from the cells as previously mentioned occurs through the channel protein Fps1 (Luyten et al. 1995). At high osmolarity, this glycerol efflux aquaglyceroporin is closed and the glycerol produced is retained inside the cells (Luyten et al. 1995; Tamas et al. 1999). In silico and the experimental data suggest that the Fps1 closure is one of the first events following osmoshock (Petelenz-Kurdziel et al. 2013). After a shift from high to low osmotic strength, the Fps1 channel opens and the cells release the accumulated glycerol within a few minutes. A *fps1Δ* deletion mutant was extremely sensitive to such a shift from high to low osmolarity, indicating that the Fps1 channel is important to protect the cells from a hypo-osmotic shock (Luyten et al. 1995; Tamas et al. 1999). In anaerobic cultures, the *FPS1* expression level is higher than under aerobic conditions, as it is needed to expel the excessive glycerol produced during NADH re-oxidation (ter Linde et al. 1999). The molecular structure of Fps1 has been extensively studied in order to understand the mechanisms of its opening and closure. Fps1 has two long 230 and 250 amino acids extensions, one at the N- and the other at the C-terminal of the core domain, that have been shown to be required for Fps1 closure. Strains that have modified Fps1 with deletion of either domain become hyperactive with poor glycerol accumulation, constant excretion of glycerol into the medium, and

glycerol overproduction as cells attempt to compensate for continued glycerol loss (Ahmadpour et al. 2014). This effect can also be caused by a mutation in Thr231 in the long N-terminal extension of Fps1 which renders that location unphosphorylatable as well as certain other mutations in the well-conserved motif around this residue (Ahmadpour et al. 2014). Recent work (Lee et al. 2013) has shown that the mechanism of Fps1 closure is triggered by the phosphorylation of Rgc2, which in its unphosphorylated state binds to the C-terminal extension of Fps1 and keeps the channel open. The fast opening of Fps1 channel after hypo-osmotic shock was suggested to be mediated by the Slt2 MAPK, which is stimulated by hypo-osmotic shock, cell wall stress, and morphogenic changes (Levin 2011; Baltanas et al. 2013). It is interesting that this elaborate regulation of Fps1 opening or closure is restricted to yeasts closely related to *S. cerevisiae* (Pettersson et al. 2005). Probably it has evolved as a mean for adaptation to rapid changes in the osmotic pressure in growth medium. For a more detailed review on glycerol metabolism and transport in yeast cells, the reader is referred to Klein et al. (2017).

5.3 Glycerol Role in Yeast Cells

5.3.1 Osmoregulation

The maintenance of the cellular water balance is fundamental to all living organisms. Rapid changes in the extracellular osmolarity and water activity can be detrimental for cells, for example increased osmolarity of the environment causes water efflux and hence cell shrinkage. The accumulation of compatible solutes to compensate for water loss is a universal strategy for survival under these conditions (Yancey et al. 1982). As implied by their name, such molecules are compatible with intracellular processes and either replace water or/and revert the water concentration gradient and drive water back into cells. The most abundant compatible solutes in microorganisms are small uncharged molecules such as (1) polyols (glycerol, arabitol, trehalose, or sucrose), (2) amino acids (proline, glutamate, or glutamine), and (3) ectoines (β-hydroxyectoine) (Grant 2004). In yeast and other fungi, glycerol is the most prominent solute that regulates cell turgor under high extracellular osmolarity condition (Brown 1978; Blomberg and Adler 1992). For instance, during initial phase of a wine fermentation, *S. cerevisiae* produces increased amounts of glycerol in response to osmotic stress because of the high sugar concentration in the must. Also in *S. cerevisiae*, glycerol production increases in a medium containing high concentrations of NaCl (Blomberg and Adler 1989). Moreover, a marked rise in the level of cytoplasmic glycerol-3-phosphate dehydrogenase activity has been observed under these conditions (Blomberg and Adler 1989; Andre et al. 1991). In *S. cerevisiae* the adaptation to hyperosmotic stress is accomplished via increased glycerol accumulation and its retention inside cells. Mutants that block the synthesis of glycerol (e.g., *gpd1Δgpd2Δ*) or that cause leakage of glycerol out of the cell (e.g., with hyperactive Fps1) exhibit an osmo-sensitive phenotype (Hohmann 2002). Other yeasts might

employ slightly different mechanisms to increase intracellular glycerol concentration. For example, xerotolerant yeasts, such as *Z. rouxii*, retain and accumulate glycerol against a concentration gradient (Edgley and Brown 1983; Lages et al. 1999).

In yeast, glycerol accumulation during osmo-adaptation is controlled mainly by the high osmolarity glycerol (HOG) pathway (Fig. 5.2). Mutations that inactivate the HOG pathway render yeast cells highly sensitive to hyperosmotic stress, while mutations causing constantly activated HOG pathway are lethal. The HOG pathway is a typical example of a mitogen-activated protein kinase (MAPK) pathway that is regulated by a three-tiered cascade of kinases. Central in the HOG pathway is the MAPK, Hog1 (Brewster et al. 1993), which is activated by the MAP kinase kinase (MAPKK), Pbs2 (Brewster et al. 1993). The two independent upstream osmo-sensing pathways, the Sln1 branch and Sho1 branch, activate MAPKK Pbs2

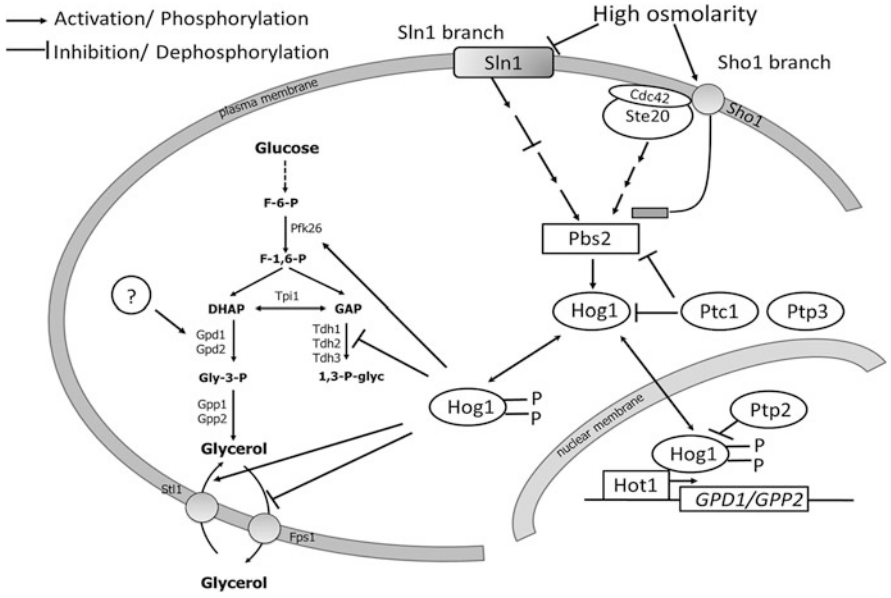

Fig. 5.2 High osmolarity glycerol (HOG) pathway in *Saccharomyces cerevisiae*. *Hog1* MAPK; *Pbs2* MAPKK; *the Sln1 branch and Sho1 branch* the two independent upstream osmo-sensing pathways; *Sln1* transmembrane protein; *Sho1* transmembrane protein with C-terminal elongation; the SH3 domain, which anchors Pbs2 to the membrane, where it interacts with the MAPKKK; *Ste20* component of the pheromone-response and filamentous MAPK pathways; *Hot1* transcriptional activator; *Ptp2, Ptp3, and Ptc1* phosphatases that inactivate Hog1; *Gpd1, Gpd2* cytosolic glycerol-3-phosphate dehydrogenases; *?* unknown protein phosphatase that dephosphorylates and activates Gpd1; *Gpp1, Gpp2* glycerol-3-phosphate phosphatases; *Tdh1, Tdh2, Tdh3* glyceraldehyde-3-phosphate dehydrogenases; *Pfk26* phosphofructo-2 kinase; *Tpi1* triose phosphate isomerase; *Stl1, Fps1* membrane glycerol transporters; *GPD1/GPP2* genes encoding glycerol-3-phosphate dehydrogenase and glycerol-3-phosphate phosphatase; *F-6-P* fructose-6-phosphate; *F-1,6-P* fructose-1,6-biphosphate; *DHAP* dihydroxyacetone phosphate; *GAP* glyceraldehyde-3-phosphate; *Gly-3-P* glycerol-3-phosphate; *1,3-P-glyc* 1,3-phosphoglycerate

(Hohmann 2002; O'Rourke et al. 2002). These two branches of the HOG pathway appear to function independently and respond to different types of stimuli. After a hyperosmotic shock, Hog1 is activated by phosphorylation in Thr174 and Tyr176 positions. The period of Hog1 phosphorylation correlates with the degree of osmotic stress and with the time it takes to initiate volume recovery after the initial volume loss (Babazadeh et al. 2013). Mutants with decreased ability in the accumulation of glycerol show prolonged HOG pathway activation, while mutants with increased glycerol accumulation show shorter periods of HOG pathway activation (Klipp et al. 2005; Krantz et al. 2004), so pathway downregulation is clearly linked to glycerol accumulation. The inactivation of the HOG pathway occurs via the dephosphorylation of Hog1 by the negative regulators Ptp2, Ptp3, and Ptc1 (Warmka et al. 2001; Wurgler-Murphy et al. 1997). The HOG pathway has been described in great detail in several recent reviews (Chen and Thorner 2007; Hohmann 2002; Saito and Posas 2012).

The pathway is activated through dual phosphorylation with MAPK Hog1 interacting with cytosolic and nuclear targets to provide cellular adaptation to osmotic stress (Westfall et al. 2004). Westfall et al. (2008) showed that cells deficient in the nuclear import of Hog1 are still capable of adapting to hyperosmotic stress, thereby highlighting the importance of cytosolic targets. The interaction of Hog1 with cytosolic targets prevents glycerol efflux and induces metabolic shift toward glycerol production at the expense of biomass formation (Petelenz-Kurdziel et al. 2013; Bouwman et al. 2011; Westfall et al. 2008).

Glycerol efflux is prevented by closure of the Fps1 channel. Although there is clear evidence that Hog1 directly interacts with Fps1 to acquire tolerance against arsenite (Thorsen et al. 2006) or acetic acid (Mollapour and Piper 2007), during a hyperosmotic shock, Hog1 appears to regulate Fps1 indirectly through phosphorylation and inhibition of the Fps1-activating proteins Rgc1 and Rgc2 (Beese et al. 2009).

Hog1 also stimulate the increase of glycolytic flux which may help in compensating for the flux that is redirected toward glycerol (Petelenz-Kurdziel et al. 2013). This is achieved via upregulation of the key glycolytic enzyme, phosphofructokinase. This enzyme is allosterically controlled by AMP and fructose 2, 6-bisphosphate, as both increase phosphofructokinase activity. The latter, fructose-2,6-bisphosphate, is the product of the phosphorylation of fructose-6-phosphate by 6-phosphofructo-2-kinase, which is encoded by *PFK26* and *PFK27*. Dihazi et al. (2004) demonstrated that Hog1 activates Pfk26 which in turn leads to higher fructose-2,6-bisphosphate production and consequently phosphofructokinase activation. Westfall et al. (2008) postulates that Hog1 triggers a decrease in activity of Tdh1 and Tdh3 that catalyze the conversion of glyceraldehyde-3-phosphate into 1,3-bisphosphoglycerate. The decrease of these two enzyme activities partially redirects the glycolytic flux to DHAP, the first intermediate of glycerol synthesis.

The rapid activation of glycerol overproduction after hyperosmotic is also caused by increased activity of Gpd1. Gpd1 is activated via dephosphorylation by an unknown protein phosphatase, in a Hog1-independent manner (Lee et al. 2012). Also hyperosmotic stress causes changes in redox status due to liberation of

protein-associated NADH. This, in turn, may trigger shifts in the cellular distribution of Gpd1 and hence its activity (Jung et al. 2010).

In the nucleus, Hog1 stimulates initiation of transcription by interacting with DNA-binding proteins, such as the activators Hot1 (Alepuz et al. 2003; Rep et al. 2000) and Smp1 (de Nadal et al. 2003) or the repressor Sko1 (Proft et al. 2001; Proft and Struhl 2002), and is also involved in subsequent steps of transcription (de Nadal et al. 2011). Changes in gene expression lead to increased amounts of glycerol biosynthetic enzymes as well as an increased capacity for glycerol uptake, especially in the absence of glucose as carbon source. Hog1 directly or indirectly stimulates expression of more than 100 genes (O'Rourke et al. 2002); among them are the genes *GPD1* and *GPP2*, and to a lesser extent also *GPP1*, as well as *STL1*. The activation of the genes *GPD1* and *GPP2* increases the cells' capacity to produce glycerol, which may play a role at later stages of the adaptation to hyperosmotic conditions, especially in sustaining the growth of the adapted cells. For more detailed review on osmoregulation in yeast cells, the reader is referred to Hohmann (2002, 2015).

5.3.2 Redox Sink During *S. cerevisiae* Growth Under Anaerobic Conditions

The coenzyme nicotinamide adenine dinucleotide (NAD^+) is an important electron transport carrier. Only catalytic amounts of this pyridine nucleotide are present in yeast and thus it must be continuously cycled between its oxidative and reduced form. The total ratio of NAD^+/NADH is approximately 3:1 in yeast (Anderson et al. 2002), including the free and bound forms of the coenzyme. Stable intracellular redox state is achieved by an accurate balancing of the NAD^+ reduction and NADH oxidation.

In a glucose medium, cytosolic reduction of NAD^+ is mainly catalyzed by the glyceraldehyde-3-phosphate dehydrogenase. The mitochondrial NADH originates from the reactions of TCA cycle. Also biosynthetic reactions during yeast growth lead to a net NADH production. The generated NADH is re-oxidized in *S. cerevisiae* via five main mechanisms, which are (1) ethanol production by the alcohol dehydrogenase, Adh; (2) glycerol production by cytosolic glycerol-3-phosphate dehydrogenase Gpd1/2; (3) respiration of cytosolic NADH via the external mitochondrial NADH dehydrogenases, Nde1/2; (4) respiration of cytosolic NADH via the glycerol-3-phosphate shuttle; and (5) oxidation of intramitochondrial NADH via a mitochondrial "internal" NADH dehydrogenase, Ndi1 (Bakker et al. 2001).

Assimilation of glucose into biomass involves formation of "excess" NADH. Most of the NADH generated in biosynthetic reactions originates from the synthesis of amino acids in the cytosol and in the mitochondrial matrix (Albers et al. 1998). As redox neutral ethanol production cannot serve as a redox sink for excess NADH, it has to be re-oxidized by other means. During growth in the presence of oxygen, this can be achieved by the respiratory chain. The external NADH dehydrogenase

(Nde) and internal NADH dehydrogenase (Ndi), both localized in the mitochondrial inner membrane, directly transfer the reducing equivalents of their compartment to the respiratory chain. But under anaerobic conditions, respiration is not possible. So under these conditions, *S. cerevisiae* re-oxidizes NADH in the glycerol-3-phosphate dehydrogenase reaction. Genes *GPD2* and *GPP1* are upregulated after a shift to anaerobic conditions, which leads to the increased glycerol production. Deletion of these genes results in a strain that is unable to grow in the absence of oxygen (Ansell et al. 1997; Bjorkqvist et al. 1997). To ensure constant activity of this route for NADH re-oxidation, the synthesized glycerol is exported from the cells via Fps1 channel.

Since the cytosolic glycerol formation is the exclusive route for NADH re-oxidation under anaerobic conditions, NADH that is produced in the mitochondrial matrix during amino acid synthesis has to be somehow transported to the cytosol. However, the mitochondrial inner membrane is virtually impermeable to pyridine nucleotide coenzymes (Von Jagow and Klingenberg 1970). That is why the anaerobic re-oxidation of intramitochondrial NADH requires a shuttle mechanism that exports redox equivalents to the cytosol. Bakker et al. (2001) proposed the possible involvement of the ethanol-acetaldehyde shuttle in the export of intramitochondrial NADH. Other existing glycerol-3-phosphate shuttles serve for transfer of redox equivalents from cytosolic NADH to the respiratory chain under aerobic conditions. This shuttle consists of two components: cytosolic NAD^+-linked glycerol-3-phosphate dehydrogenase (Gpd1 and Gpd2) and mitochondrial glycerol-3-phosphate, ubiquinone oxidoreductase, often referred to as mitochondrial glycerol-3-phosphate dehydrogenase (Gut2) (Dawson 1979).

It should be mentioned that attempt to increase glycerol production by *S. cerevisiae* (e.g., by overexpression of the gene *GPD1*) violates the redox balance in the cell and causes the production of additional oxidized by-products such as pyruvate, acetate, acetoin, 2, 3-butanediol, and succinate (Michnick et al. 1997; Roustan et al. 1999). For a more detailed review on NADH metabolism in yeast cells, the reader is referred to Bakker et al. (2001).

Because glycerol production involves dephosphorylation, it may also be involved in replenishment of the cytosolic pool of inorganic phosphate during the switch from gluconeogenic to glycolytic metabolism (Thevelein and Hohmann 1995; van Heerden et al. 2014). It has been shown that overexpression of the first enzyme in glycerol biosynthesis suppresses the growth defect of a *tps1Δ* mutant, which suffers from accumulation of sugar phosphates and depletion of inorganic phosphate pool (Thevelein and Hohmann 1995).

5.4 Methods of Glycerol Production

Glycerol can be produced either by chemical synthesis from petrochemical feedstocks or by microbial fermentation or can be recovered as a by-product of soap or biodiesel manufacture (Wang et al. 2001). The oldest among these is the process of soap production, which is known since 2800 BC when soap was obtained

for the first time by heating fats in the presence of ash (Hunt 1999). In 1811, the French chemist M. E. Chevrel defined the chemical formulas of fatty acids and the formulas of glycerin in vegetable oil and animal fat. His work was patented. It was known as the first industrial method to obtain glycerin soap by reacting fatty material with lime and alkaline material (Gesslein 1999). The process of saponification is now of lesser importance in industrial nations and many developing countries, because of the replacement of soap with detergents (Rehm 1988; Agarwal 1990). Glycerol production by microbial fermentation was discovered by Louis Pasteur (Pasteur 1858). Pasteur showed that glycerol was formed at a quantity of 2.0–3.6% (g g^{-1}) from sugar during alcoholic fermentation. That makes glycerol an important constituent of alcoholic beverages produced by fermentation (wine, beer, and others). Increasing the glycerol concentration in wine is considered desirable, as glycerol contributes to the smoothness and overall pleasant mouthfeel particularly of red wines (Eustace and Thornton 1987). But glycerol yield during fermentation was considered too low for industrial applications, so alcoholic fermentation was not considered as a source of glycerol until World War I when demand for glycerol for the manufacture of explosives exceeded the supply from the soap industry (Prescott and Dunn 1959). To meet the increased demand for glycerol, Neuberg and Reinfurth developed the so-called second form of fermentation which implies addition of steering agents (Neuberg and Reinfurth 1918). Steering agents, such as bisulfite ions, were added to the fermentation broth forming a complex with acetaldehyde. Ethanol formation, which consumes glycolytic NADH, was therefore impaired, lacking the intermediate acetaldehyde; consequently the re-oxidation of the cofactor was managed through glycerol production. Neuberg's second form of fermentation increased glycerol synthesis, reaching conversion efficiencies from 23 to 28% depending on the process conditions (Wang et al. 2001), but large amounts of sulfite-acetaldehyde complex were accumulated as a by-product (Agarwal 1990). Another process that was developed at that time consisted of running the fermentation at high pH (around 7 or above), and with this method glycerol yield was improved significantly (Connstein and Ludecke 1919). However, these wartime processes were unable to compete with chemically synthesized process developed after World War II as yields of glycerol from sugar by fermentation were low and recovery by distillation was inefficient (Vijaikishore and Karanth 1986). Chemical synthesis was responsible for considerable part of world glycerol production during the second half of the twentieth century. Since the late 1940s, and following the discovery of synthetic surfactants, glycerol has been produced chemically from epichlorohydrin that is obtained from propylene (and thus from fossil oil) as large chemical companies forecasted a glycerol shortage that stimulated more glycerol production by chemical synthetic production routes (Frost and Sullivan 2006). A significant amount of glycerol was also synthesized from allyl alcohol. But the route of glycerol production by the oxidation or chlorination of propylene has declined in relative importance since the early 1970s (Hester 2000) due to environmental concerns and the increase in cost and decrease in availability of propylene, especially in developing countries. The decline in the chemical glycerol production led to the renewed interest in glycerol

production by microbial fermentation. To fulfill this task, new strains of *S. cerevisiae* and new species of yeasts were proposed. These will be discussed in greater detail in the next sections.

In recent years, the increased focus on renewable and sustainable energy has boosted the production of biodiesel from rapeseed oil, cooking oil, and animal fats. Biodiesel is a viable fuel and as a replacement for sulfur containing diesel serves as an additive to reduce sulfur content and emissions that are progressively being lowered to meet tighter environmental legislation. Glycerol as a by-product of biodiesel production has also seen a dramatic increase in production. Approximately 10% of the reaction volume in a given biodiesel production process ends up as raw or crude glycerol (Johnson and Taconi 2007). This low-grade glycerol also contains water, salts, and other organic materials, including residual methanol as well as free fatty acids. These components vary quite a bit in content depending on the raw material used (Thompson and He 2006). Purification of the crude glycerol fraction from biodiesel production to obtain food-grade glycerol is quite expensive (Yang et al. 2012). More preferable is bioconversion of crude glycerol into liquid biofuels, green chemicals, and bioenergy on the basis of fermentation processes, which can provide an efficient solution for sustainable management of glycerol and help improve the economics of biodiesel industries (Lynd et al. 2005).

5.5 Glycerol Production by the Yeast *S. cerevisiae*

As already mentioned, during both World Wars, wild-type strains of *S. cerevisiae* were used on an industrial scale to produce glycerol from carbohydrate-containing feedstocks using a sulfite-based process (Agarwal 1990; Rehm 1988). But this process is inefficient and causes environmental problems. Rapidly developing metabolic engineering tools offer new means for redirecting carbon flux toward glycerol instead of ethanol production. In general, five different approaches for metabolic engineering of *S. cerevisiae* for improvement of glycerol production can be distinguished: (1) enhancement of glycerol-3-phosphate dehydrogenase activity, (2) improvement of glycerol efflux from the cells, (3) impairment or reduction of triose phosphate isomerase activity, (4) cutoff or attenuation of the route that leads to ethanol production, and (5) shifting of NAD^+/NADH ratio in order to increase the amount of NADH available for glycerol-3-phosphate dehydrogenase or changing cofactor specificity of glycerol-3-phosphate dehydrogenase. Most of the published studies have combined several or all of these approaches.

To enhance the activity of glycerol-3-phosphate dehydrogenase, the *GPD1* gene was overexpressed resulting in a fourfold increase in glycerol production at the expense of ethanol (Michnick et al. 1997). The substitution of all four phosphorylation sites renders glycerol-3-phosphate dehydrogenase twofold more active than that of the wild type (Oliveira et al. 2012). The evolutionary engineering of bicistronic artificial operon containing both yeast *GPD1* and *GPP2* genes in a heterologous system of *Escherichia coli* resulted in obtaining of a fused gene *GPD1-GPP2* (Salles et al. 2007). The product of this gene had both of the catalytic

sites of glycerol-3-phosphate dehydrogenase and glycerol-3-phosphate phosphatase and was able to convert DHAP into glycerol faster than separated glycerol-3-phosphate dehydrogenase and glycerol-3-phosphate phosphatase that is more likely due to substrate channeling between the two active sites.

Increase of glycerol production could be also achieved by elevation of its outflow from yeast cell. It was shown that a modified form of the Fps1 protein from which 76–230 amino acid residues were removed failed to retain the produced glycerol inside the cell under hyperosmotic stress (Karlgren et al. 2004). The corresponding strain with an engineered Fps1 glycerol channel showed poor growth and enhanced glycerol production during fermentation. Overexpression of the *GPD1* in this strain further increased the amount of the produced glycerol, however, with pronounced growth impairment (Remize et al. 2001).

Decreased ethanol formation was achieved by the deletion of one or several *ADH* genes coding for alcohol dehydrogenases (Drewke et al. 1990; Cordier et al. 2007) or *PDC1* and *PDC5* genes coding for pyruvate decarboxylases (Nevoigt and Stahl 1996). Mutants that are defective in alcohol dehydrogenase, however, accumulate large amounts of acetaldehyde and acetic acid, which are toxic to cells. Acetaldehyde could be partially detoxified by the overexpression *ALD3* coding for aldehyde dehydrogenase (Cordier et al. 2007). The mutants defective in pyruvate decarboxylase cannot grow without adding exogenous ethanol or acetate as the last enzyme is the only source of the cytosolic two-carbon compounds that are required primarily for lipid biosynthesis (Pronk et al. 1996). The most promising results have been reported for mutants of *S. cerevisiae* with a deletion of the *TPI1* gene coding for triose phosphate isomerase (Overkamp et al. 2002). Unfortunately, such mutants had a severe growth defect, especially on glucose (Compagno et al. 2001). This growth defect was later attributed to an accumulation of DHAP, which has been shown to inactivate *myo*inositol-3-phosphate synthase and thereby renders the cells auxotrophic for inositol (Shi et al. 2005). Addition of inositol partly restored growth of the *tpi1Δ* mutant. The growth defect was also partly overcome by the additional deletion of the two isogenes *NDE1* and *NDE2*, encoding mitochondrial external NADH dehydrogenase, and *GUT2*, encoding the FAD^+-dependent mitochondrial glycerol dehydrogenase (Overkamp et al. 2002). As these enzymes are primarily responsible for mitochondrial re-oxidation of cytosolic NADH, their deletion increased the availability of cytosolic NADH. The *tpi1Δ nde1Δ nde2Δ gut2Δ* quadruple mutant was able to grow on glucose but with a very low specific growth rate. Evolutionary engineering of the quadruple mutant in glucose-containing medium partially relieved this growth defect and resulted in a strain with glycerol yield which is very close to the theoretical maximum (Overkamp et al. 2002). Another approach, which was also based on a *tpi1Δ* mutant, involved also the overexpression of *GPD1*, the deletion of *ADH1*, and the overexpression of *ALD3* (Cordier et al. 2007).

An original approach to increase glycerol production, with a simultaneous drop in ethanol titer, was described by the expression of the cytosolic acetolactate synthase Ilv2. The constructed strain has a decrease in intracellular pyruvate concentration and a fourfold increase in glycerol production, with a concomitant

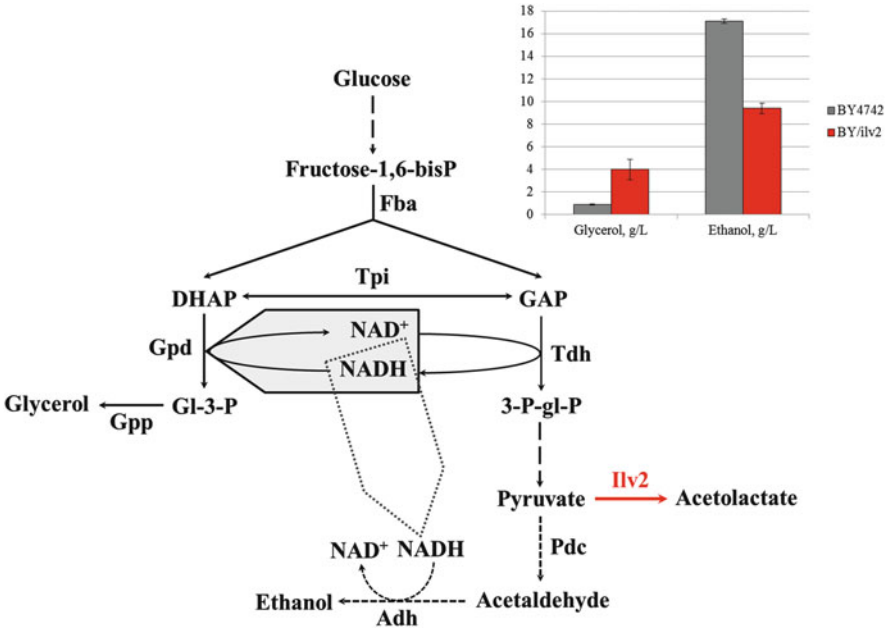

Fig. 5.3 Glycerol production by *S. cerevisiae* recombinant strain with overexpression of a truncated gene *ILV2* (Murashchenko et al. 2016). Due to less pyruvate and ethanol production, NADH predominantly directed in the reaction catalyzed by Gpd instead of the Adh reaction, resulting in increased glycerol production. *DHAP* dihydroxyacetone phosphate, *GAP* glyceraldehyde-3-phosphate, *G-3-P* glycerol 3-phosphate, *1,3-bisPG* 1,3-bisphosphoglycerate, *Fba* aldolase, *Tpi* triose phosphate isomerase, *Gpd* glycerol-3-phosphate dehydrogenase, *Gpp* glycerol-3-phosphate phosphatase, *Ilv2* acetolactate synthase, *Pdc* pyruvate decarboxylase, *Adh* alcohol dehydrogenase

1.8-fold decrease in ethanol production when compared to that of parental strain (Murashchenko et al. 2016) (Fig. 5.3). We constructed strains with a constitutively decreased expression of *TPI1* and overexpression of a fused gene for *GPD1-GPP1*, the modified gene *FPS1*, and a truncated gene *ILV2*. The constructed strain combining the above genetic manipulations grew on glucose as the sole carbon and energy source under anaerobic conditions and accumulated up to 9 times more glycerol under microaerobic conditions and up to 4.7 times more glycerol under anaerobic conditions relative to the parental wild-type strain (unpublished data) (Fig. 5.4).

The theoretical maximum for glycerol production in *S. cerevisiae* is limited by the fact that only half of the glucose carbon can be used for NADH-consuming glycerol production, whereas the other half is required for NADH regeneration via glyceraldehyde-3-phosphate dehydrogenase in the "pyruvate branch" of glycolysis. This problem was addressed by Geertman et al. (2006). They used a *pdc1Δ pdc5Δ pdc6Δ nde1Δ nde2Δ gut2Δ* deletion mutant strain and combined *GPD2* overexpression with overexpression of the gene *FDH1*, encoding a NAD^+-

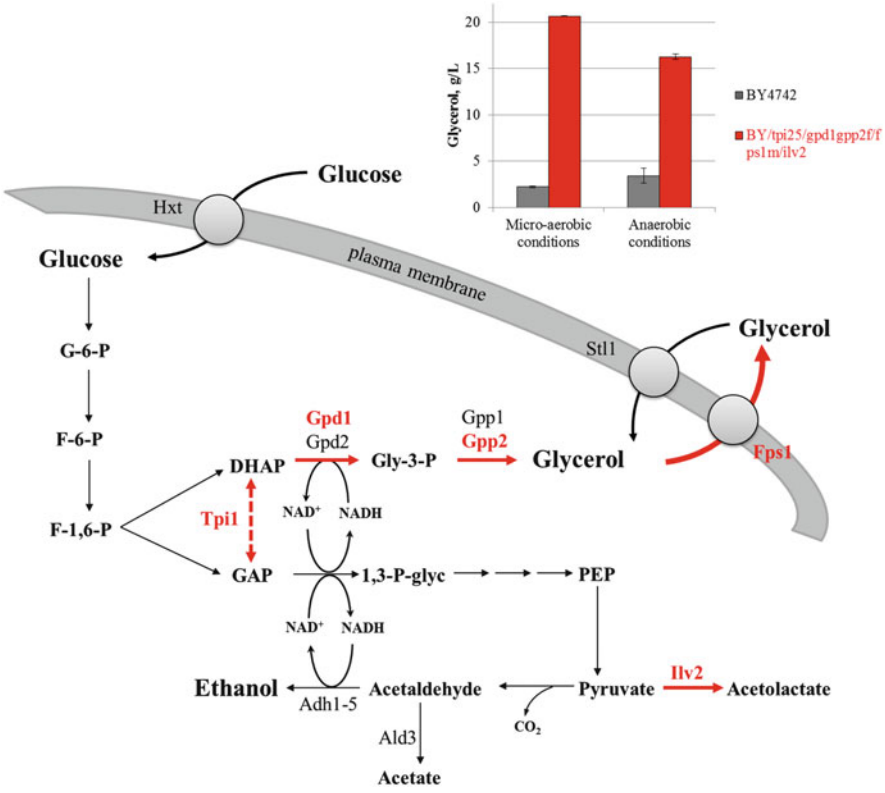

Fig. 5.4 Glycerol production in microaerobic and anaerobic conditions by *S. cerevisiae* recombinant strain with decreased expression of *TPI1* and overexpression of a fused gene for *GPD1-GPP1*, the modified gene *FPS1*, and truncated gene *ILV2*. *Red arrows* depict steps of glycerol metabolism that were targeted in the study. *G-6-P* glucose-6-phosphate, *F-6-P* fructose-6-phosphate, *F-1,6-P* fructose-1,6-biphosphate, *DHAP* dihydroxyacetone phosphate, *GAP* glyceraldehyde-3-phosphate, *Gly-3-P* glycerol-3-phosphate, *1,3-P-glyc* 1,3-phosphoglycerate, *PEP* phosphoenolpyruvate, *Ac-CoA* acetyl coenzyme A, *TCA* tricarboxylic acid cycle, *Gpd1,2* cytosolic glycerol-3-phosphate dehydrogenase, *Gpp1,2* glycerol-3-phosphate phosphatase, *Adh1-5* alcohol dehydrogenases, *Ald3* aldehyde dehydrogenase, *Ilv2* acetolactate synthase, *Hxt, Stl1, Fps1* membrane transporters

dependent formate dehydrogenase. The cells supplied with an additional source of cytosolic NADH achieved a glycerol yield that exceeded the theoretical maximum; however, this strategy required formate co-feeding. In order to avoid co-feeding, they proposed to switch the redox factor specificity of glycerol formation in *S. cerevisiae* from NADH to NADPH by introducing an NADPH-dependent DHAP reductase and redirecting the main carbon flux through the pentose phosphate pathway by reducing phosphoglucose isomerase activity. The *Bacillus subtilis gpsA* gene, encoding a NADPH-dependent DHAP reductase, was successfully introduced into an *S. cerevisiae pgi1* mutant but that resulted in only 35%

increase of glycerol formation, probably due to the low specific activity of the bacterial *gpsA* gene product in the yeast transformant (Nevoigt 2008).

5.6 Glycerol Production by Osmotolerant Yeasts

Glycerol production by osmotolerant yeasts was first described by Nickerson and Carroll (1945) for the yeast *Zygosaccharomyces acidifaciens*. This study together with the following reports on the production of glycerol and other polyols (e.g., erythritol, D-arabitol, and mannitol) by Spencer and Shu (1957) and Onishi et al. (1961) stimulated a comprehensive investigation of osmotolerant or osmophilic yeasts with the expectation that an organism producing glycerol without a need for a steering agent might be found. However, these investigations did not lead to commercialization due to the difficulties in recovering glycerol from the fermentation broth and the availability of less expensive chemical synthetic sources for glycerol from petroleum sources. Later, the development of separation techniques, such as reverse osmosis, ultrafiltration, ion exchange, and ion exclusion, provided the means for the economic industrialization of glycerol production by osmotolerant yeast fermentation.

Most osmotolerant yeast species considered for glycerol production belong to the genera *Candida*, *Debaryomyces*, *Hansenula*, *Pichia*, *Saccharomyces*, *Schizosaccharomyces*, *Torulaspora*, and *Zygosaccharomyces* (Onishi 1963; Zhang et al. 1963; Zhuge 1973; Kumar et al. 1989; Petrovska et al. 1999). All of these yeasts require oxygen for growth, which considerably increases production costs due to additional expenses needed for bioreactor aeration and cooling (Cueto-Rojas et al. 2015).

Glycerol yields as high as 40% have been reached using a strain of *C. magnoliae* (Hajny et al. 1960). *C. magnoliae* is isolated from honeycomb and also known as an erythritol producer (Kim et al. 1996). *C. magnoliae* is able to grow in the presence of up to 50% (w/v) sugars and produces erythritol, mannitol, and glycerol in response to high sugar concentrations (Yu et al. 2006). When *C. magnoliae* was grown in high concentrations of fructose, it produced a significant amount of glycerol comparable to the amount of erythritol (Yu et al. 2006). Similarly, intracellular glycerol accumulation is also critical for *C. magnoliae* to maintain osmolarity as is the case for *S. cerevisiae*.

An intensive effort has been made in China since the 1970s to develop a glycerol process based on osmotolerant yeasts. Zhuge (1973) isolated a strain from among 5000 osmotolerant yeast isolates with considerable promise for glycerol production. This strain isolated from glazed fruit in Southern China was later identified as *C. glycerinogenes* (Wang et al. 1999). *C. glycerinogenes* has been commercially exploited to produce glycerol (Zhuge et al. 2001). Compared with other yeasts, *C. glycerinogenes* has several useful properties, such as tolerance to high glucose concentration, rapid growth, and the ability to accumulate high extracellular glycerol concentration.

Z. rouxii and *Hansenula anomala* are other osmotolerant yeasts that produce a significant amount of glycerol under high osmotic conditions (Groleau et al. 1995; Parekh and Pandey 1985).

5.7 Glycerol Application in the Industry

The applications of glycerol in industry are linked primarily to its physical properties: for example, owing to its humectant property, glycerol is used in the preparation of gels and ointments. Glycerol can also be used as in formulations that require a plasticizer to enhance the dispersion of the coat over beads, granules, and tablets in cosmetics and pharmaceutics. Glycerol is widely used in preprocessed tobacco to prevent tobacco from crumbling. It is also used in the healthcare industry to maintain pressure in the eyeball and central nervous system. Glycerol is hygroscopic in nature and therefore is extensively used in glues and adhesives to prevent them from drying too fast. Glycerol is also used in the textile industry for softening of fibers, in flexible foams, and related applications. At low temperatures, glycerol tends to super cool instead of crystallize. This is why aqueous glycerol solutions resist freezing and is widely used as antifreeze in cooling systems. Glycerol is also used as a stabilizing agent for storage and shipment of cells, organs, and proteins.

5.8 Glycerol Bioconversion to High-Value Chemicals

In the past decade, the world production of biodiesel as an alternative to petroleum has significantly increased (Chen and Liu 2016). This increase has resulted in large amounts of glycerol being generated as a by-product from biodiesel production (about 10% by weight) through transesterification with methanol that is catalyzed by the addition of NaOH or KOH (Garlapati et al. 2016). As a by-product, glycerol can contribute a significant revenue stream to the biodiesel producers (Willke and Vorlop 2008). Chemical uses of glycerol have also increased demand for this chemical. Of interest is the use of glycerol as a central raw material for the production of the fine chemicals: glyceric acid, dihydroxyacetone, ketomalonic (or mesoxalic) acid, poly(ketomalonate), 1,2,3-tri-tert-butyl glycerol, propylene glycol (1,2-propanediol), acrolein, and epichlorohydrin (Pagliaro et al. 2007). But chemical synthesis is often accompanied by the using of hazardous compounds and contributes to environmental pollution. Due to these considerations, the microbial conversion of glycerol, especially as a cheap by-product of biodiesel industry, to value-added compounds represents a more environmentally-friendly approach. Some existing or plausible biotechnological applications that use glycerol as a substrate for conversion or as a source of carbon and energy for growth of the microorganisms are listed as follows:

1. Production of dihydroxyacetone (DHA). DHA is predominantly used as coloring agent in sunless tanning creams and lotions (Levy 1992). Its chemical synthesis is expensive and involves dealing with hazardous reactants (Hekmat et al. 2003).

Biotechnological production of DHA employs the bacterium *Gluconobacter oxydans*, which has a membrane-bound pyrroloquinoline quinone-dependent glycerol dehydrogenase that catalyzes the oxidation of the secondary hydroxyl group of glycerol to DHA (Matsushita et al. 1994). Other bacteria *Gluconobacter melanogenus* (Flickinger and Perlman 1977) and *Acetobacter xylinum* (Nabe et al. 1979) have also been reported to synthesize DHA from glycerol.

2. Production of 1,3-propanediol. 1,3-Propanediol (1,3-PDO) is used for the production of polyesters, polyethers, polyurethanes, adhesives, composites, laminates, coatings, and moldings. In addition, 1,3-PDO and terephthalic acid are widely used for production of valuable biodegradable polymer polytrimethylene terephthalate. Chemical synthesis of 1,3-PDO has many issues, such as high energy consumption, toxic intermediates, and expensive catalysts. Fortunately, 1,3-PDO can be biosynthesized from glycerol in two sequential reactions: first, glycerol dehydratase or diol dehydratase converts glycerol into 3-hydroxypropionaldehyde (3-HPA) (Schneider and Pawelkiewicz 1966; Toraya et al. 1978); in the second step, 3-HPA is reduced to 1,3-PDO by NADPH- or NADH-dependent 1,3-PDO dehydrogenase. The production of 1,3-PDO has already been demonstrated for strains of *Klebsiella pneumoniae*, *Clostridium butyricum*, and *Clostridium acetobutylicum* (Forage and Foster 1982; Biebl 1991).
3. Production of ethanol. Fuel ethanol can be used as an additive to gasoline for biofuel for car engines. In the past decade, an exponential growth in the production of fuel ethanol has occurred worldwide (Schubert 2006). Fuel ethanol is primarily produced from sugarcane, corn, or sugar beets (da Silva et al. 2009). Considerable efforts have been made toward the development of profitable technology for ethanol production from lignocellulosic feedstocks (Dellomonaco et al. 2010). Raw glycerol (by-product of biodiesel production) is also considered as an inexpensive feedstock for fuel ethanol production (Licht 2010). Unfortunately, the most frequently used ethanol producer *S. cerevisiae* does not exhibit acceptable growth rates in a synthetic glycerol medium if no growth-supporting medium supplements (such as amino acids and nucleic bases) are added (Swinnen et al. 2013). The growth of *S. cerevisiae* on glycerol may be considerably improved, by the selection of glycerol-adapted *S. cerevisiae* strains (Ochoa-Estopier et al. 2011), expression of heterologous glycerol transporters (Klein et al. 2016), etc. Also raw glycerol can be converted to ethanol by engineered strains of methylotrophic yeast *Hansenula polymorpha* (Hong et al. 2010; Kata et al. 2016). Elementary mode analysis and metabolic evolution of *E. coli* mutants have led to substantial improvement of ethanol production from glycerol (Trinh and Srienc 2009). Also some new microorganisms with good ability to grow and produce ethanol on crude glycerol have been recently isolated, e.g., bacterium *Kluyvera cryocrescens* (Choi et al. 2011) and yeast *P. tannophilus* (Liu et al. 2012).
4. Production of 2,3-butanediol. 2,3-Butanediol (2,3-BDO) is used as a fuel, solvent, and for the production of polymers (Perego et al. 2003; Saha and Bothast 1999). Biosynthesis of 2,3-BDO occurs from pyruvate in three steps.

First, acetolactate synthase catalyzes the condensation of two pyruvate molecules to form acetolactate and CO_2. Then, acetolactate is decarboxylated to acetoin by acetolactate decarboxylase. And finally acetoin is reduced to 2,3-butanediol by 2,3-BDO dehydrogenase or acetoin reductase (Juni 1952). Thus for 2,3-BDO production, all substrates have to be converted to pyruvate. Many substrates have been used for the production of 2,3-BDO, e.g., starch hydrolysates, raw and decolored molasses, and corncob hydrolysates (Perego et al. 2000; Ma et al. 2010). Glycerol might also be a good carbon source for 2,3-BDO production, because 2,3-BDO is a known by-product of 1,3-propanediol production from glycerol by *K. pneumoniae* (Biebl et al. 1998). Production of 2,3-BDO from glycerol by *K. pneumoniae* G31 resulted in high product yields under intense aeration and alkaline pH conditions (Petrov and Petrova 2010).

5. Production of glyceric acid. Glyceric acid is an expensive promising chemical used for pharmaceutical purposes. Glyceric acid is a known by-product of dihydroxyacetone production from glycerol with *G. oxydans*. The biosynthesis of glyceric acid is funneled from glycerol via two dehydrogenases: first, glycerol is oxidized to glyceraldehyde which is subsequently oxidized further to glyceric acid (Habe et al. 2009b). Among various acetic acid bacteria, *Acetobacteri tropicalis* was identified as the best glyceric acid-producing organism (Habe et al. 2009a).
6. Production of biosurfactants. Surfactants are used as cleaners, emulsifiers, laundry detergents, or in paints. Biosurfactants consist of a hydrophilic part (consists of a sugar, peptide, or protein) and a hydrophobic/lipophilic part (contains fatty acids or fatty alcohols). The biosurfactants have advantages over chemically produced detergents because they are biodegradable, less toxic, and can be produced from renewable resources. Natural biosurfactant producers are bacteria, yeast, and fungi (Mulligan 2005). Pure or raw glycerol is often used in biosurfactant production, as it constitutes the backbone of the lipid component of biosurfactants. Among microorganisms that can carry out the production of biosurfactants using glycerol are the yeast *Pseudozyma antarctica* (Morita et al. 2007), *Candida bombicola* (Ashby and Solaiman 2010), and the bacterium *Pseudomonas aeruginosa* (Wu et al. 2008).
7. Several more or less successful attempts have been done to use raw glycerol as a source of carbon for the production of succinate, citrate, and amino acids. For more details, the reader is referred to an excellent recent review (Wendisch et al. 2011).

Thus, glycerol is the valuable compound with important role in nature and vast application in different branches of the industry. It can be obtained in several ways including biotechnological processes, yeast alcoholic fermentation and biodiesel production. Glycerol is widely used in the cosmetic, paint, automotive, food, and pharmaceutical industries and for the production of value-added compounds. In yeast cells, glycerol production plays a role in protection against osmotic stress, maintenance of redox balance, and pool of cytoplasmic inorganic phosphate. Glycerol also can be used by yeast cells as a carbon and energy source.

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References

Adler L, Blomberg A, Nilsson A (1985) Glycerol metabolism and osmoregulation in the salt-tolerant yeast *Debaryomyces hansenii*. J Bacteriol 162:300–306

Agarwal GP (1990) Glycerol. In: Fiechter A (ed) Advances in biochemical engineering/biotechnology 41: microbial bioproducts. Springer, Berlin, pp 95–128

Ahmadpour D, Geijer C, Tamas MJ, Lindkvist-Petersson K, Hohmann S (2014) Yeast reveals unexpected roles and regulatory features of aquaporins and aquaglyceroporins. Biochim Biophys Acta 1840:1482–1491

Albers E, Liden G, Larsson C, Gustafsson L (1998) Anaerobic redox balance and nitrogen metabolism in *Saccharomyces cerevisiae*. Recent Res Dev Microbiol 2:253–279

Albertyn J, Hohmann S, Thevelein JM, Prior BA (1994) *GPD1*, which encodes glycerol-3-phosphate dehydrogenase is essential for growth under osmotic stress in *Saccharomyces cerevisiae* and its expression is regulated by the high-osmolarity glycerol response pathway. Mol Cell Biol 14:4135–4144

Alepuz PM, de Nadal E, Zapater M, Ammerer G, Posas F (2003) Osmostress-induced transcription by Hot1 depends on a Hog1-mediated recruitment of the RNA Pol II. EMBO J 22:2433–2442

Anderson RM, Bitterman KJ, Wood JG, Medvedik O, Cohen H, Lin SS, Manchester JK, Gordon JI, Sinclair DA (2002) Manipulation of a nuclear NAD^+ salvage pathway delays aging without altering steady-state NAD^+ levels. J Biol Chem 277:18881–18890

Andre L, Hemming A, Adler L (1991) Osmoregulation in *Saccharomyces cerevisiae*: studies on the osmotic induction of glycerol production and glycerol-3-phosphate dehydrogenase (NAD^+). FEBS Lett 286:13–17

Ansell R, Granath K, Hohmann S, Thevelein JM, Adler L (1997) The two isoenzymes for yeast NAD^+-dependent glycerol 3-phosphate dehydrogenase encoded by *GPD1* and *GPD2* have distinct roles in osmoadaptation and redox regulation. EMBO J 16:2179–2187

Ashby RD, Solaiman DK (2010) The influence of increasing media methanol concentration on sophorolipid biosynthesis from glycerol-based feedstocks. Biotechnol Lett 32:1429–1437

Babazadeh R, Adiels CB, Smedh M, Petelenz-Kurdziel E, Goksor M, Hohmann S (2013) Osmostress-induced cell volume loss delays yeast Hog1 signaling by limiting diffusion processes and by Hog1-specific effects. PLoS One 8:e80901

Bakker BM, Overkamp KM, van Maris AJ, Kotter P, Luttik MA, van Dijken JP, Pronk JT (2001) Stoichiometry and compartmentation of NADH metabolism in *Saccharomyces cerevisiae*. FEMS Microbiol Rev 25:15–37

Baltanas R, Bush A, Couto A, Durrieu L, Hohmann S, Colman-Lerner A (2013) Pheromone-induced morphogenesis improves osmoadaptation capacity by activating the HOG MAPK pathway. Sci Signal 6:ra26

Beese SE, Negishi T, Levin DE (2009) Identification of positive regulators of the yeast fps1 glycerol channel. PLoS Genet 5(11):e1000738

Biebl H (1991) Glycerol Fermentation of 1,3-Propanediol by *Clostridium butyricum*—measurement of product inhibition by use of a Ph-Auxostat. Appl Microbiol Biotechnol 35:701–705

Biebl H, Zeng AP, Menzel K, Deckwer WD (1998) Fermentation of glycerol to 1,3-propanediol and 2,3-butanediol by *Klebsiella pneumoniae*. Appl Microbiol Biotechnol 50:24–29

Bjorkqvist S, Ansell R, Adler L, Liden G (1997) Physiological response to anaerobicity of glycerol 3-phosphate dehydrogenase mutants of *Saccharomyces cerevisiae*. Appl Environ Microbiol 63:128–132

Blomberg A (2000) Metabolic surprises in *Saccharomyces cerevisiae* during adaptation to saline conditions: questions, some answers and a model. FEMS Microbiol Lett 182:1–8

Blomberg A, Adler L (1989) Roles of glycerol and glycerol-3-phosphate dehydrogenase (NAD^+) in acquired osmotolerance of *Saccharomyces cerevisiae*. J Bacteriol 171:1087–1092

Blomberg A, Adler L (1992) Physiology of osmotolerance in fungi. Adv Microb Physiol 33:145–212

Bouwman J, Kiewiet J, Lindenbergh A, van Eunen K, Siderius M, Bakker BM (2011) Metabolic regulation rather than de novo enzyme synthesis dominates the osmo-adaptation of yeast. Yeast 28:43–53

Brewster JL, de Valoir T, Dwyer ND, Winter E, Gustin MC (1993) An osmosensing signal transduction pathway in yeast. Science 259:1760–1763

Brown AD (1978) Compatible solutes and extreme water stress in eukaryotic micro-organisms. Adv Microb Physiol 17:181–242

Chen Z, Liu D (2016) Toward glycerol biorefinery: metabolic engineering for the production of biofuels and chemicals from glycerol. Biotechnol Biofuels 9:205

Chen RE, Thorner J (2007) Function and regulation in MAPK signaling pathways: lessons learned from the yeast *Saccharomyces cerevisiae*. Biochim Biophys Acta 1773:1311–1340

Chen X, Fang H, Rao Z, Shen W, Zhuge B, Wang Z, Zhuge J (2008) Cloning and characterization of a NAD+-dependent glycerol-3-phosphate dehydrogenase gene from *Candida glycerinogenes*, an industrial glycerol producer. FEMS Yeast Res 8:725–734

Choi WJ, Hartono MR, Chan WH, Yeo SS (2011) Ethanol production from biodiesel-derived crude glycerol by newly isolated *Kluyvera cryocrescens*. Appl Microbiol Biotechnol 89:1255–1264

Compagno C, Brambilla L, Capitanio D, Boschi F, Ranzi BM, Porro D (2001) Alterations of the glucose metabolism in a triose phosphate isomerase-negative *Saccharomyces cerevisiae* mutant. Yeast 18(7):663–670

Connstein W, Ludecke K (1919) Uber Glycerin-Gewinnung durch Garung. Ber Dtsch Chem Ges 52:1385–1391

Cordier H, Mendes F, Vasconcelos I, Francois JM (2007) A metabolic and genomic study of engineered *Saccharomyces cerevisiae* strains for high glycerol production. Metab Eng 9:364–378

Costenoble R, Valadi H, Gustafsson L, Niklasson C, Franzen CJ (2000) Microaerobic glycerol formation in *Saccharomyces cerevisiae*. Yeast 16:1483–1495

Cueto-Rojas HF, van Maris AJ, Wahl SA, Heijnen JJ (2015) Thermodynamics-based design of microbial cell factories for anaerobic product formation. Trends Biotechnol 33(9):534–546

da Silva GP, Mack M, Contiero J (2009) Glycerol: a promising and abundant carbon source for industrial microbiology. Biotechnol Adv 27:30–39

Dawson AG (1979) Oxidation of cytosolic NADH formed during aerobic metabolism in mammalian cells. Trends Biochem Sci 4:171–176

de Nadal E, Casadome L, Posas F (2003) Targeting the MEF2-like transcription factor Smp1 by the stress-activated Hog1 mitogen-activated protein kinase. Mol Cell Biol 23:229–237

de Nadal E, Ammerer G, Posas F (2011) Controlling gene expression in response to stress. Nat Rev Genet 12:833–845

de Vries RP, Flitter SJ, van de Vondervoort PJ, Chaveroche MK, Fontaine T, Fillinger S, Ruijter GJ, d'Enfert C, Visser J (2003) Glycerol dehydrogenase, encoded by *gldB* is essential for osmotolerance in *Aspergillus nidulans*. Mol Microbiol 49:131–141

Dellomonaco C, Fava F, Gonzalez R (2010) The path to next generation biofuels: successes and challenges in the era of synthetic biology. Microb Cell Fact 9:3

Dihazi H, Kessler R, Eschrich K (2004) High osmolarity glycerol (HOG) pathway-induced phosphorylation and activation of 6-phosphofructo-2-kinase are essential for glycerol accumulation and yeast cell proliferation under hyperosmotic stress. J Biol Chem 279:23961–23968

Drewke C, Thielen J, Ciriacy M (1990) Ethanol formation in adh0 mutants reveals the existence of a novel acetaldehyde-reducing activity in *Saccharomyces cerevisiae*. J Bacteriol 172 (7):3909–3917

Dujon B, Sherman D, Fischer G, Durrens P, Casaregola S, Lafontaine I, De Montigny J, Marck C, Neuvéglise C, Talla E, Goffard N, Frangeul L, Aigle M, Anthouard V, Babour A, Barbe V, Barnay S, Blanchin S, Beckerich JM, Beyne E, Bleykasten C, Boisramé A, Boyer J, Cattolico L, Confanioleri F, De Daruvar A, Despons L, Fabre E, Fairhead C, Ferry-Dumazet H, Groppi A, Hantraye F, Hennequin C, Jauniaux N, Joyet P, Kachouri R, Kerrest A, Koszul R, Lemaire M, Lesur I, Ma L, Muller H, Nicaud JM, Nikolski M, Oztas S, Ozier-Kalogeropoulos-O, Pellenz S, Potier S, Richard GF, Straub ML, Suleau A, Swennen D, Tekaia F, Wésolowski-Louvel M, Westhof E, Wirth B, Zeniou-Meyer M, Zivanovic I, Bolotin-Fukuhara M, Thierry A, Bouchier C, Caudron B, Scarpelli C, Gaillardin C, Weissenbach J, Wincker P, Souciet JL (2004) Genome evolution in yeasts. Nature 430:35–44

Edgley M, Brown AD (1983) Yeast water relations: physiological changes induced by solute stress in *Saccharomyces cerevisiae* and *Saccharomyces rouxii*. J Gen Microbiol 129:3453–3463

Eriksson P, Andre L, Ansell R, Blomberg A, Adler L (1995) Characterization of *GPD2*, a second gene encoding *sn*-glycerol 3-phosphate dehydrogenase (NAD^+) in *Saccharomyces cerevisiae*, and its comparison with *GPD1*. Mol Microbiol 17:95–107

Eustace R, Thornton RJ (1987) Selective hybridization of wine yeasts for higher yields of glycerol. Can J Microbiol 33:112–117

Ferreira C, van Voorst F, Martins A, Neves L, Oliveira R, Kielland-Brandt MC, Lucas C, Brandt A (2005) A member of the sugar transporter family, Stl1p is the glycerol/H+ symporter in *Saccharomyces cerevisiae*. Mol Biol Cell 16:2068–2076

Fillinger S, Ruijter G, Tamas MJ, Visser J, Thevelein JM, d'Enfert C (2001) Molecular and physiological characterization of the NAD-dependent glycerol 3-phosphate dehydrogenase in the filamentous fungus *Aspergillus nidulans*. Mol Microbiol 39:145–157

Finley D, Ulrich HD, Sommer T, Kaiser P (2012) The ubiquitin-proteasome system of *Saccharomyces cerevisiae*. Genetics 192:319–360

Flickinger MC, Perlman D (1977) Application of oxygen-enriched aeration in the conversion of glycerol to Dihydroxyacetone by *Gluconobacter melanogenus* IFO 3293. Appl Environ Microbiol 33:706–712

Forage RG, Foster MA (1982) Glycerol fermentation in *Klebsiella pneumoniae*: functions of the coenzyme B12-dependent glycerol and diol dehydratases. J Bacteriol 149:413–419

Frost and Sullivan (2006) R&D creating new avenues for glycerol. https://www.frost.com/prod/servlet/market-insight-top.pag?docid=77264824. Accessed 4 Aug 2006

Gancedo C, Gancedo JM, Sols A (1968) Glycerol metabolism in yeasts. Pathways of utilization and production. Eur J Biochem 5:165–172

Garlapati VK, Shankar U, Budhiraja A (2016) Bioconversion technologies of crude glycerol to value added industrial products. Biotechnol Rep 9:9–14

Geertman JM, van Maris AJ, van Dijken JP, Pronk JT (2006) Physiological and genetic engineering of cytosolic redox metabolism in *Saccharomyces cerevisiae* for improved glycerol production. Metab Eng 8:532–542

Gesslein BW (1999) Humectants in personal care formulation: a practical guide. In: Schueller R, Romanowski P (eds) Conditioning agents for hair and skin. Marcel Dekker, New York, pp 95–96

Grant WD (2004) Life at low water activity. Philos Trans R Soc Lond Ser B Biol Sci 359:1249–1266; discussion 1266–1247

Groleau D, Chevalier P, Yuen TLSTH (1995) Production of polyols and ethanol by osmophilic yeast *Zygosaccharomyces rouxii*. Biotechnol Lett 17:315–320

Habe H, Fukuoka T, Kitamoto D, Sakaki K (2009a) Biotransformation of glycerol to D-glyceric acid by *Acetobacter tropicalis*. Appl Microbiol Biotechnol 81:1033–1039

Habe H, Shimada Y, Yakushi T, Hattori H, Ano Y, Fukuoka T, Kitamoto D, Itagaki M, Watanabe K, Yanagishita H, Matsushita K, Sakaki K (2009b) Microbial production of glyceric acid, an organic acid that can be mass produced from glycerol. Appl Environ Microbiol 75:7760–7766

Hajny GJ, Hendershot WF, Peterson WH (1960) Factors affecting glycerol production by a newly isolated osmophilic yeast. Appl Microbiol 8:5–11

Hekmat D, Bauer R, Fricke J (2003) Optimization of the microbial synthesis of dihydroxyacetone from glycerol with *Gluconobacter oxydans*. Bioprocess Biosyst Eng 26:109–116
Heller KB, Lin EC, Wilson TH (1980) Substrate specificity and transport properties of the glycerol facilitator of *Escherichia coli*. J Bacteriol 144:274–278
Heredia CF, Sols A, DelaFuente G (1968) Specificity of the constitutive hexose transport in yeast. Eur J Biochem 5:321–329
Hester A (2000) Microbial glycerol. Ind Bioprocess 22(4):3–5
Hohmann S (2002) Osmotic stress signaling and osmoadaptation in yeasts. Microbiol Mol Biol Rev 66:300–372
Hohmann S (2015) An integrated view on a eukaryotic osmoregulation system. Curr Genet 61 (3):373–382
Holst B, Lunde C, Lages F, Oliveira R, Lucas C, Kielland-Brandt MC (2000) *GUP1* and its close homologue *GUP2*, encoding multimembrane-spanning proteins involved in active glycerol uptake in *Saccharomyces cerevisiae*. Mol Microbiol 37:108–124
Hong WK, Kim CH, Heo SY, Luo LH, Oh BR, Seo JW (2010) Enhanced production of ethanol from glycerol by engineered *Hansenula polymorpha* expressing pyruvate decarboxylase and aldehyde dehydrogenase genes from *Zymomonas mobilis*. Biotechnol Lett 32:1077–1082
Hunt JA (1999) A short history of soap. Pharm J 263(7076):985–989
Johnson DT, Taconi KA (2007) The glycerin glut: options for the value-added conversion of crude glycerol resulting from biodiesel production. Environ Prog 26:338–348
Jung S, Marelli M, Rachubinski RA, Goodlett DR, Aitchison JD (2010) Dynamic changes in the subcellular distribution of Gpd1p in response to cell stress. J Biol Chem 285:6739–6749
Jung JY, Kim TY, Ng CY, Oh MK (2012) Characterization of *GCY1* in *Saccharomyces cerevisiae* by metabolic profiling. J Appl Microbiol 113:1468–1478
Juni E (1952) Mechanisms of formation of acetoin by bacteria. J Biol Chem 195:715–726
Karlgren S, Filipsson C, Mullins JGL, Bill RM, Tamas MJ, Hohmann S (2004) Identification of residues controlling transport through the yeast aquaglyceroporin Fps1 using a genetic screen. Eur J Biochem 271:771–779
Kata I, Semkiv MV, Ruchala J, Dmytruk KV, Sibirny AA (2016) Overexpression of the genes *PDC1* and *ADH1* activates glycerol conversion to ethanol in the thermotolerant yeast *Ogataea (Hansenula) polymorpha*. Yeast 33(8):471–478
Kim SY, Park SS, Jeon YJ, Seo JH (1996) Analysis of fermentation characteristics for production of erythritol by *Candida sp*. Korean J Food Sci Technol 28:935–939
Klein M, Z-u I, Knudsen PB, Carrillo M, Swinnen S, Workman M, Nevoigt E (2016) The expression of glycerol facilitators from various yeast species improves growth on glycerol of *Saccharomyces cerevisiae*. Metab Eng Commun 3:252–257
Klein M, Swinnen S, Thevelein J, Nevoigt E (2017) Glycerol metabolism and transport in yeast and fungi: established knowledge and ambiguities. Environ Microbiol 19(3):878–893
Klipp E, Nordlander B, Kruger R, Gennemark P, Hohmann S (2005) Integrative model of the response of yeast to osmotic shock. Nat Biotechnol 23:975–982
Krantz M, Nordlander B, Valadi H, Johansson M, Gustafsson L, Hohmann S (2004) Anaerobicity prepares *Saccharomyces cerevisiae* cells for faster adaptation to osmotic shock. Eukaryot Cell 3:1381–1390
Kumar J, Agarwal SC, Basu SK, Spivastava OP (1989) Isolation, evaluation and taxonomic characterization of some glycerol producing osmotolerant yeasts. Biol Membr 15:129–135
Kurtzman CP, Fell JW, Boekhout T (eds) (2011) The yeasts—a taxonomic study. Elsevier, Amsterdam
Lages F, Lucas C (1995) Characterization of a glycerol/H+ symport in the halotolerant yeast *Pichia sorbitophila*. Yeast 11:111–119
Lages F, Silva-Graca M, Lucas C (1999) Active glycerol uptake is a mechanism underlying halotolerance in yeasts: a study of 42 species. Microbiology 145(9):2577–2585
Larsson K, Ansell R, Eriksson P, Adler L (1993) A gene encoding sn-glycerol-3-phosphate dehydrogenase (NAD^+) complements an osmosensitive mutant of *Saccharomyces cerevisiae*. Mol Microbiol 10:1101–1111

Larsson C, Pahlman IL, Ansell R, Rigoulet M, Adler L, Gustafsson L (1998) The importance of the glycerol 3-phosphate shuttle during aerobic growth of *Saccharomyces cerevisiae*. Yeast 14:347–357

Lee DH, Kim MD, Ryu YW, Seo JH (2008) Cloning and characterization of *CmGPD1*, the *Candida magnoliae* homologue of glycerol-3-phosphate dehydrogenase. FEMS Yeast Res 8:1324–1333

Lee YJ, Jeschke GR, Roelants FM, Thorner J, Turk BE (2012) Reciprocal phosphorylation of yeast glycerol-3-phosphate dehydrogenases in adaptation to distinct types of stress. Mol Cell Biol 32:4705–4717

Lee J, Reiter W, Dohnal I, Gregori C, Beese-Sims S, Kuchler K, Ammerer G, Levin DE (2013) MAPK Hog1 closes the *S. cerevisiae* glycerol channel Fps1 by phosphorylating and displacing its positive regulators. Genes Dev 27:2590–2601

Levin DE (2011) Regulation of cell wall biogenesis in *Saccharomyces cerevisiae*: the cell wall integrity signaling pathway. Genetics 189:1145–1175

Levy SB (1992) Dihydroxyacetone-containing sunless or self-tanning lotions. J Am Acad Dermatol 27:989–993

Licht FO (2010) World ethanol and biofuels report, vol 8

Liu X, Jensen PR, Workman M (2012) Bioconversion of crude glycerol feedstocks into ethanol by *Pachysolen tannophilus*. Bioresour Technol 104:579–586

Liu X, Mortensen UH, Workman M (2013) Expression and functional studies of genes involved in transport and metabolism of glycerol in *Pachysolen tannophilus*. Microb Cell Fact 12:27

Lucas C, Da Costa M, van Uden N (1990) Osmoregulatory active sodium-glycerol cotransport in the Halotolerant yeast *Debaryomyces hansenii*. Yeast 6:187–191

Luyten K, Albertyn J, Skibbe WF, Prior BA, Ramos J, Thevelein JM, Hohmann S (1995) Fps1, a yeast member of the MIP family of channel proteins, is a facilitator for glycerol uptake and efflux and is inactive under osmotic stress. EMBO J 14:1360–1371

Lynd LR, van Zyl WH, McBride JE, Laser M (2005) Consolidated bioprocessing of cellulosic biomass: an update. Curr Opin Biotechnol 16:577–583

Ma CQ, Wang AL, Wang Y, Jiang TY, Li LX, Xu P (2010) Production of 2,3-butanediol from corncob molasses, a waste by-product in xylitol production. Appl Microbiol Biotechnol 87:965–970

Maeda T, Wurgler-Murphy SM, Saito H (1994) A two-component system that regulates an osmosensing MAP kinase cascade in yeast. Nature 369:242–245

Marshall JH, Kong YC, Sloan J, May JW (1989) Purification and properties of glycerol: $NADP^+$ 2-oxidoreductase from *Schizosaccharomyces pombe*. J Gen Microbiol 135:697–701

Matsushita K, Toyama H, Adachi O (1994) Respiratory chains and bioenergetics of acetic acid bacteria. Adv Microb Physiol 36:247–301

Matsuzawa T, Ohashi T, Hosomi A, Tanaka N, Tohda H, Takegawa K (2010) The *gld1* gene encoding glycerol dehydrogenase is required for glycerol metabolism in *Schizosaccharomyces pombe*. Appl Microbiol Biotechnol 87:715–727

Mattanovich D, Graf A, Stadlmann J, Dragosits M, Redl A, Maurer M, Kleinheinz M, Sauer M, Altmann F, Gasser B (2009) Genome, secretome and glucose transport highlight unique features of the protein production host *Pichia pastoris*. Microb Cell Fact 8:29

Merico A, Ragni E, Galafassi S, Popolo L, Compagno C (2011) Generation of an evolved *Saccharomyces cerevisiae* strain with a high freeze tolerance and an improved ability to grow on glycerol. J Ind Microbiol Biotechnol 38:1037–1044

Michnick S, Roustan JL, Remize F, Barre P, Dequin S (1997) Modulation of glycerol and ethanol yields during alcoholic fermentation in *Saccharomyces cerevisiae* strains overexpressed or disrupted for *GPD1* encoding glycerol 3-phosphate dehydrogenase. Yeast 13:783–793

Mollapour M, Piper PW (2007) Hog1 mitogen-activated protein kinase phosphorylation targets the yeast Fps1 aquaglyceroporin for endocytosis, thereby rendering cells resistant to acetic acid. Mol Cell Biol 27:6446–6456

Morita T, Konishi M, Fukuoka T, Imura T, Kitamoto D (2007) Microbial conversion of glycerol into glycolipid biosurfactants, mannosylerythritol lipids, by a basidiomycete yeast, *Pseudozyma antarctica* JCM 10317(T). J Biosci Bioeng 104:78–81

Mulligan CN (2005) Environmental applications for biosurfactants. Environ Pollut 133:183–198

Murashchenko L, Abbas C, Dmytruk K, Sibirny A (2016) Overexpression of the truncated version of *ILV2* enhances glycerol production in *Saccharomyces cerevisiae*. Yeast 33(8):463–469

Nabe K, Izuo N, Yamada S, Chibata I (1979) Conversion of glycerol to dihydroxyacetone by immobilized whole cells of *Acetobacter xylinum*. Appl Environ Microbiol 38:1056–1060

Neuberg C, Reinfurth E (1918) Natürliche und erzwungene Glycerinbildung bei der alkoholischen Gärung. Biochem Z 92:32

Neves L, Lages F, Lucas C (2004) New insights on glycerol transport in *Saccharomyces cerevisiae*. FEBS Lett 565:160–162

Nevoigt E (2008) Progress in metabolic engineering of *Saccharomyces cerevisiae*. Microbiol Mol Biol Rev 72:379–412

Nevoigt E, Stahl U (1996) Reduced pyruvate decarboxylase and increased glycerol-3-phosphate dehydrogenase [NAD^+] levels enhance glycerol production in *Saccharomyces cerevisiae*. Yeast 12:1331–1337

Nickerson WJ, Carroll WR (1945) On the metabolism of *Zygosaccharomyces*. Arch Biochem 7:257–271

Norbeck J, Blomberg A (1997) Metabolic and regulatory changes associated with growth of *Saccharomyces cerevisiae* in 1.4 M NaCl. Evidence for osmotic induction of glycerol dissimilation via the dihydroxyacetone pathway. J Biol Chem 272:5544–5554

Norbeck J, Pehlman AK, Akhtar N, Blomberg A, Adler L (1996) Purification and characterization of two isoenzymes of dl-glycerol-3-phosphatase from *Saccharomyces cerevisiae*. Identification of the corresponding *GPP1* and *GPP2* genes and evidence for osmotic regulation of Gpp2p expression by the osmosensing mitogen-activated protein kinase signal transduction pathway. J Biol Chem 271:13875–13881

O'Rourke SM, Herskowitz I, O'Shea EK (2002) Yeast go the whole HOG for the hyperosmotic response. Trends Genet 18:405–412

Ochoa-Estopier A, Lesage J, Gorret N, Guillouet SE (2011) Kinetic analysis of a *Saccharomyces cerevisiae* strain adapted for improved growth on glycerol: implications for the development of yeast bioprocesses on glycerol. Bioresour Technol 102(2):1521–1527

Oliveira R, Lucas C (2004) Expression studies of *GUP1* and *GUP2*, genes involved in glycerol active transport in *Saccharomyces cerevisiae*, using semi-quantitative RT-PCR. Curr Genet 46:140–146

Oliveira R, Lages F, Silva-Graca M, Lucas C (2003) Fps1p channel is the mediator of the major part of glycerol passive diffusion in *Saccharomyces cerevisiae*: artefacts and re-definitions. Biochim Biophys Acta 1613:57–71

Oliveira AP, Ludwig C, Picotti P, Kogadeeva M, Aebersold R, Sauer U (2012) Regulation of yeast central metabolism by enzyme phosphorylation. Mol Syst Biol 8:623

Onishi H (1963) Osmophilic yeasts. Adv Food Res 12:53–94

Onishi H, Saito N, Koshiyama I (1961) Studies on osmoplilic yeasts. Part XI. Various factors affecting on polyalcohol production by *Pichia miso*. Agric Biol Chem 25:124–130

Overkamp KM, Bakker BM, Kotter P, Luttik MA, Van Dijken JP, Pronk JT (2002) Metabolic engineering of glycerol production in *Saccharomyces cerevisiae*. Appl Environ Microbiol 68:2814–2821

Pagliaro M, Ciriminna R, Kimura H, Rossi M, Della Pina C (2007) From glycerol to value-added products. Angew Chem Int Ed Engl 46:4434–4440

Pahlman AK, Granath K, Ansell R, Hohmann S, Adler L (2001) The yeast glycerol 3-phosphatases Gpp1p and Gpp2p are required for glycerol biosynthesis and differentially involved in the cellular responses to osmotic, anaerobic, and oxidative stress. J Biol Chem 276:3555–3563

Parekh SR, Pandey NK (1985) Production of glycerol by *Hansenula anomala*. Biotechnol Bioeng 27:1081–1091

Pasteur ML (1858) Production constante de glyce'rine dans la fermentation alcoolique. C R Acad Sci 46:857

Pavlik P, Simon M, Schuster T, Ruis H (1993) The glycerol kinase (*GUT1*) gene of *Saccharomyces cerevisiae*: cloning and characterization. Curr Genet 24:21–25

Perego P, Converti A, Del Borghi A, Canepa P (2000) 2,3-butanediol production by *Enterobacter aerogenes*: selection of the optimal conditions and application to food industry residues. Bioprocess Eng 23:613–620

Perego P, Converti A, Del Borghi M (2003) Effects of temperature, inoculum size and starch hydrolyzate concentration on butanediol production by *Bacillus licheniformis*. Bioresour Technol 89:125–131

Petelenz-Kurdziel E, Kuehn C, Nordlander B, Klein D, Hong KK, Jacobson T, Dahl P, Schaber J, Nielsen J, Hohmann S, Klipp E (2013) Quantitative analysis of glycerol accumulation, glycolysis and growth under hyper osmotic stress. PLoS Comput Biol 9:e1003084

Petrov K, Petrova P (2010) Enhanced production of 2,3-butanediol from glycerol by forced pH fluctuations. Appl Microbiol Biotechnol 87:943–949

Petrovska B, Winkelhausen E, Kuzmanova S (1999) Glycerol production by yeasts under osmotic and sulfite stress. Can J Microbiol 45:695–699

Pettersson N, Filipsson C, Becit E, Brive L, Hohmann S (2005) Aquaporins in yeasts and filamentous fungi. Biol Cell 97:487–500

Posas F, Chambers JR, Heyman JA, Hoeffler JP, de Nadal E, Arino J (2000) The transcriptional response of yeast to saline stress. J Biol Chem 275:17249–17255

Prescott SC, Dunn CG (1959) Industrial microbiology, 3rd edn. McGraw-Hill, New York, pp 208–217

Proft M, Struhl K (2002) Hog1 kinase converts the Sko1-Cyc8-Tup1 repressor complex into an activator that recruits SAGA and SWI/SNF in response to osmotic stress. Mol Cell 9:1307–1317

Proft M, Pascual-Ahuir A, de Nadal E, Arino J, Serrano R, Posas F (2001) Regulation of the Sko1 transcriptional repressor by the Hog1 MAP kinase in response to osmotic stress. EMBO J 20:1123–1133

Pronk JT, Yde Steensma H, van Dijken JP (1996) Pyruvate metabolism in *Saccharomyces cerevisiae*. Yeast 12(16):1607–1633

Quispe C, Coronado C, Carvalho J Jr (2013) Glycerol: production, consumption, prices, characterization and new trends in combustion. Renew Sust Energ Rev 27:475–493

Redkar RJ, Locy RD, Singh NK (1995) Biosynthetic pathways of glycerol accumulation under salt stress in *Aspergillus nidulans*. Exp Mycol 19:241–246

Rehm H-J (1988) Microbial production of glycerol and other polyols. In: Rehm H-J, Reed HJ (eds) Biotechnology, vol. 6B. Special microbial processes. VCH, Verlagsgesellschaft, Weinheim, pp 51–69

Remize F, Barnavon L, Dequin S (2001) Glycerol export and glycerol-3-phosphate dehydrogenase, but not glycerol phosphatase, are rate limiting for glycerol production in *Saccharomyces cerevisiae*. Metab Eng 3:301–312

Rep M, Krantz M, Thevelein JM, Hohmann S (2000) The transcriptional response of *Saccharomyces cerevisiae* to osmotic shock. Hot1p and Msn2p/Msn4p are required for the induction of subsets of high osmolarity glycerol pathway-dependent genes. J Biol Chem 275:8290–8300

Romano AH (1986) Microbial sugar transport systems and their importance in biotechnology. Trends Biotechnol 4:207–213

Ronnow B, Kielland-Brandt MC (1993) Gut2, a gene for mitochondrial glycerol-3-phosphate dehydrogenase of *Saccharomyces cerevisiae*. Yeast 9:1121–1130

Roustan JL, Salblayrolles JM, Barre P, Dequin S (1999) Glycerol overproduction by engineered *Saccharomyces cerevisiae* wine yeast strains leads to substantial changes in by-product formation and to a stimulation of fermentation rate in stationary phase. Appl Environ Microbiol 65:143–149

Ruijter GJ, Visser J, Rinzema A (2004) Polyol accumulation by *Aspergillus oryzae* at low water activity in solid-state fermentation. Microbiology 150:1095–1101

Saha BC, Bothast RJ (1999) Production of 2,3-butanediol by newly isolated *Enterobacter cloacae*. Appl Microbiol Biotechnol 52:321–326

Saito H, Posas F (2012) Response to hyperosmotic stress. Genetics 192:289–318

Salles IM, Forchhammer N, Croux C, Girbal L, Soucaille P (2007) Evolution of a *Saccharomyces cerevisiae* metabolic pathway in *Escherichia coli*. Metab Eng 9:152–159

Schneider Z, Pawelkiewicz J (1966) The properties of glycerol dehydratase isolated from *Aerobacter aerogenes*, and the properties of the apoenzyme subunits. Acta Biochim Pol 13:311–328

Schubert C (2006) Can biofuels finally take center stage? Nat Biotechnol 24:777–784

Schuurink R, Busink R, Hondmann DH, Witteveen CF, Visser J (1990) Purification and properties of $NADP^{(+)}$-dependent glycerol dehydrogenases from *Aspergillus nidulans* and *A. niger*. J Gen Microbiol 136:1043–1050

Shi Y, Vaden DL, Ju S, Ding D, Geiger JH, Greenberg ML (2005) Genetic perturbation of glycolysis results in inhibition of de novo inositol biosynthesis. J Biol Chem 280:41805–41810

Spencer JFT, Shu P (1957) Polyhydric alcohol production by osmophilic yeasts: effect of oxygen tension and inorganic phosphate concentration. Can J Microbiol 3:559

Sprague GF, Cronan JE (1977) Isolation and characterization of *Saccharomyces cerevisiae* mutants defective in glycerol catabolism. J Bacteriol 129:1335–1342

Sutherland FC, Lages F, Lucas C, Luyten K, Albertyn J, Hohmann S, Prior BA, Kilian SG (1997) Characteristics of Fps1-dependent and -independent glycerol transport in *Saccharomyces cerevisiae*. J Bacteriol 179:7790–7795

Swinnen S, Klein M, Carrillo M, McInnes J, Nguyen HT, Nevoigt E (2013) Re-evaluation of glycerol utilization in *Saccharomyces cerevisiae*: characterization of an isolate that grows on glycerol without supporting supplements. Biotechnol Biofuels 6:157

Swinnen S, Ho PW, Klein M, Nevoigt E (2016) Genetic determinants for enhanced glycerol growth of *Saccharomyces cerevisiae*. Metab Eng 36:68–79

Tamas MJ, Luyten K, Sutherland FC, Hernandez A, Albertyn J, Valadi H, Li H, Prior BA, Kilian SG, Ramos J, Gustafsson L, Thevelein JM, Hohmann S (1999) Fps1p controls the accumulation and release of the compatible solute glycerol in yeast osmoregulation. Mol Microbiol 31:1087–1104

Tani T, Yamada K (1987) Glycerol metabolism in methylotrophic yeasts. Agric Biol Chem 51:1927–1933

ter Linde JJ, Liang H, Davis RW, Steensma HY, van Dijken JP, Pronk JT (1999) Genome-wide transcriptional analysis of aerobic and anaerobic chemostat cultures of *Saccharomyces cerevisiae*. J Bacteriol 181:7409–7413

Thevelein JM, Hohmann S (1995) Trehalose synthase, guard to the gate of glycolysis in yeast? Trends Biochem Sci 20:3–10

Thome PE (2004) Isolation of a *GPD* gene from *Debaryomyces hansenii* encoding a glycerol 3-phosphate dehydrogenase (NAD+). Yeast 21:119–126

Thompson JC, He BB (2006) Characterization of crude glycerol from biodiesel production from multiple feedstocks. Appl Eng Agric 22:261–265

Thorsen M, Di Y, Tängemo C, Morillas M, Ahmadpour D, Van der Does C, Wagner A, Johansson E, Boman J, Posas F, Wysocki R, Tamás MJ (2006) The MAPK Hog1p modulates Fps1p-dependent arsenite uptake and tolerance in yeast. Mol Biol Cell 17:4400–4410

Toraya T, Honda S, Kuno S, Fukui S (1978) Coenzyme B12-dependent diol dehydratase: regulation of apoenzyme synthesis in *Klebsiella pneumoniae* (*Aerobacter aerogenes*) ATCC 8724. J Bacteriol 135:726–729

Trinh CT, Srienc F (2009) Metabolic engineering of *Escherichia coli* for efficient conversion of glycerol to ethanol. Appl Environ Microbiol 75:6696–6705

van Heerden JH, Wortel MT, Bruggeman FJ, Heijnen JJ, Bollen YJ, Planqué R, Hulshof J, O'Toole TG, Wahl SA, Teusink B (2014) Lost in transition: start-up of glycolysis yields subpopulations of nongrowing cells. Science 343:1245114

van Zyl PJ, Kilian SG, Prior BA (1990) The role of an active transport mechanism in glycerol accumulation during osmoregulation by *Zygosaccharomyces rouxii*. Appl Microbiol Biotechnol 34:231–235

van Zyl PJ, Prior BA, Kilian SG (1991) Regulation of glycerol metabolism in *Zygosaccharomyces rouxii* to osmotic stress. Appl Microbiol Biotechnol 36:369–374

Vijaikishore P, Karanth NG (1986) Glycerol production by fermentation—a review. Process Biochem 21:54–57

Von Jagow G, Klingenberg M (1970) Pathways of hydrogen in mitochondria of *Saccharomyces carlsbergensis*. Eur J Biochem 12:583–592

Wang Z-X, Zhuge J, Fang H-Y (1999) A new osmotolerant glycerol-highly-producing species of *Candida sp*.—*Candida glycerolgenesis* Zhuge sp. nov. Acta Microbiol Sin 39:68–74

Wang Z-X, Zhuge J, Cao Y, Chen J, Fang H-Y (2000) The key enzymes of metabolism of glycerol in *Candida glycerolgenesis*. Acta Microbiol Sin 40:180–187

Wang Z-X, Zhuge J, Fang H, Prior BA (2001) Glycerol production by microbial fermentation: a review. Biotechnol Adv 19:201–223

Warmka J, Hanneman J, Lee J, Amin D, Ota I (2001) Ptc1, a type 2C Ser/Thr phosphatase, inactivates the HOG pathway by dephosphorylating the mitogen-activated protein kinase Hog1. Mol Cell Biol 21:51–60

Watanabe Y, Nagayama K, Tamai Y (2008) Expression of glycerol 3-phosphate dehydrogenase gene (*CvGPD1*) in salt-tolerant yeast *Candida versatilis* is stimulated by high concentrations of NaCl. Yeast 25:107–116

Wendisch VF, Lindner SN, Meiswinkel TM (2011) Chapter 18: Use of glycerol in biotechnological applications. In: Montero G, Stoytcheva M (eds) Biodiesel—quality, emissions and by-products. ISBN 978-953-307-784-0, Published: 16 Nov 2011

Westfall PJ, Ballon DR, Thorner J (2004) When the stress of your environment makes you go HOG wild. Science 306:1511–1512

Westfall PJ, Patterson JC, Chen RE, Thorner J (2008) Stress resistance and signal fidelity independent of nuclear MAPK function. Proc Natl Acad Sci U S A 105:12212–12217

Willke T, Vorlop K (2008) Biotransformation of glycerol into 1,3-propanediol. Eur J Lipid Sci Technol 110:831–840

Wu JY, Yeh KL, Lu WB, Lin CL, Chang JS (2008) Rhamnolipid production with indigenous *Pseudomonas aeruginosa* EM1 isolated from oil-contaminated site. Bioresour Technol 99:1157–1164

Wurgler-Murphy SM, Maeda T, Witten EA, Saito H (1997) Regulation of the *Saccharomyces cerevisiae* HOG1 mitogen-activated protein kinase by the PTP2 and PTP3 protein tyrosine phosphatases. Mol Cell Biol 17:1289–1297

Yancey PH, Clark ME, Hand SC, Bowlus RD, Somero GN (1982) Living with water stress: evolution of osmolyte systems. Science 217:1214–1222

Yang F, Hanna MA, Sun R (2012) Value-added uses for crude glycerol—a byproduct of biodiesel production. Biotechnol Biofuels 5:13

Yu JH, Lee DH, Oh YJ, Han KC, Ryu YW, Seo JH (2006) Selective utilization of fructose to glucose by *Candida magnoliae*, an erythritol producer. Appl Biochem Biotechnol 129–132:870–879

Zhang S-Z, Yang X-W, Wang H-L (1963) A study on production of glycerol and arabitol by osmophilic yeasts: II. The condition for production of arabitol by *Hansenula arabitolgenes*. Fang Acta Microbiol Sin 9:134–139

Zhuge J (1973) A study on glycerol production by fermentation with a new strain of osmophilic yeast. Kexue Tongbao (Sci Bull Sin) 18:188–189

Zhuge J, Fang HY, Wang ZX, Chen DZ, Jin HR, Gu HL (2001) Glycerol production by a novel osmotolerant yeast *Candida glycerinogenes*. Appl Microbiol Biotechnol 55:686–692

Lipids of Yeasts and Filamentous Fungi and Their Importance for Biotechnology

6

Volkmar Passoth

Abstract

Lipids are essential for any organism. They are hydrophobic compounds and include, for instance, acylglycerides, free fatty acids, sterols and isoprenoids. Their synthesis starts from acetyl-CoA, which is converted to lipids by a variety of complex biochemical reactions. Lipids consist of highly reduced molecules, and the redox factor providing electrons for their synthesis is NADPH, which is mainly produced by the pentose phosphate pathway and malic enzyme. There are a variety of fungi that can accumulate lipids; oleaginous fungi can accumulate 20 to more than 80% of their biomass as lipids. Many of them can convert carbon sources derived from second-generation substrates or low-value residues, such as lignocellulose hydrolysate or crude glycerol from biodiesel production. There are also substantial efforts to genetically manipulate fungi to produce lipids or lipid-derived compounds with high-efficiency and from low-value substrates. Lipids have broad potential in biotechnological applications. Polyunsaturated fatty acids are essential for human nutrition, and they can be produced in high amounts by certain zygomycetous species or by genetically modified yeasts. Acylglycerides can be converted to biodiesel and high-value chemicals. It is possible to obtain biodiesel, jet fuel, specific chemicals and carotenes as chemicals and food and feed additives from isoprenoids. Lipids can be combined with other biomolecules. Glycolipids have a high biotechnological potential as biosurfactants. Lipoproteins are essential for the viability of any eukaryotic cell and can be the target for developing novel antifungal drugs. As most lipids are stored intracellularly, there is also a high demand for process development towards a sustainable production of lipid-derived fuels and

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chemicals. Lipid metabolism of fungi and its biotechnological utilisation has recently obtained huge attention and is a rapidly developing field.

Contents

6.1 Introduction

Lipids are regarded as one of the three macronutrients, apart from carbohydrates and proteins. They are important as energy storage substance and essential for cell integrity as the cell membrane and all membranes of organelles are mainly formed by lipids. From this background it might be surprising that there is no real commonly accepted definition of the term “lipid”. Usually lipids are just defined by their solubility in organic solvents such as chloroform, hydrocarbons, alcohols, etc. There are a variety of lipid classes, including acylglycerols, free fatty acids, hydrocarbons (including isoprenoids), sterols and phospholipids (Akpina-Bayizit 2014). In terms of potential biotechnological applications of microbial lipids, the term “microbial oils” is frequently used, which refers to intracellular storage lipids mainly comprising triacylglycerols (Ochsenreither et al. 2016). A variety of fungi, so-called oleaginous fungi, have the ability to accumulate lipids to large amounts, i.e. more than 20% up to 80% and more of the total biomass (Ratledge and Wynn 2002). The ability of accumulating lipids is attracting growing attention, as these lipids can be used in a broad range of biotechnological applications, for instance, to generate biofuels, chemicals or food and feed additives. Specific chemicals such as Ω-3 fatty acids, fatty alcohols, alkanes or carotenes can be derived from the lipid metabolism (Mata-Gomez et al. 2014; Sitepu et al. 2014; Béligon et al. 2016;

Ochsenreither et al. 2016; Passoth 2017). Lipids can be bound to other biomolecules, for instance, carbohydrates or proteins, forming glycolipids and lipoproteins, respectively. Lipoproteins are essential for cell viability of all eukaryotes and may represent novel targets for antifungal drugs (Santiago-Tirado and Doering 2016). Glycolipids form biosurfactants, which have a broad potential of applications, for instance, in bioremediation of polluted soils and as antimicrobial agents (Paulino et al. 2016).

Oleaginous organisms have been isolated to enable production of lipid-derived compounds from a variety of substrates. Methods have been established to genetically modify lipid-accumulating fungi to improve lipid accumulation from different substrates or to convert non-oleaginous fungi to oil-accumulating organisms (Beopoulos et al. 2009; Chen et al. 2013). Production of high-value compounds from low-value residues, such as lignocellulose hydrolysate or residual glycerol from biodiesel production, would be an important step towards a sustainable society that is largely independent on fossil fuels. This chapter aims to summarise recent progress in understanding fungal lipid metabolism and its biotechnological application.

6.2 Oleaginous Fungi

A number of fungi have been identified that are able to accumulate more than 20% of their biomass as lipids. These fungi are quite diverse and belong to at least three fungal phyla: asco- and basidiomycetes (mainly yeasts) and zygomycetes. *Lipomyces starkeyi*, *Lipomyces lipofer* and *Yarrowia lipolytica* are examples of ascomycetous oleaginous yeasts. Basidiomycetous yeasts belong, for instance, to the genera *Rhodosporidium*/*Rhodotorula*, *Phaffia*, *Sporobolomyces* and *Solicoccozyma* (Flores-Cotera et al. 2001; Jin et al. 2015; Ochsenreither et al. 2016). There are ongoing activities to identify novel oleaginous fungi. Recently, oleaginous yeasts have been found among cryophilic yeasts, with *Leucosporidium creatinivorum* being the most promising one, accumulating lipids to more than 70% of the cell dry weight with a yield of 0.2 g lipids per gram consumed glucose (Filippucci et al. 2016). Accordingly, these organisms also have a broad diversity in utilising substrates for lipid accumulation, including glucose, xylose, glycerol, organic acids and polymers such as starch, and hydrophobic compounds such as alkanes (Tanimura et al. 2014; Jin et al. 2015). Filamentous fungi usually are able to produce longer polyunsaturated fatty acids, whereas yeasts can accumulate more oil in their cells; in fact, oleaginous yeasts are among the highest known producers of lipids and can reach lipid proportions of more than 85% of their dry weight (Ochsenreither et al. 2016). Table 6.1 provides some examples of oleaginous fungi.

Table 6.1 Examples of oleaginous fungal species

Phylum	Taxonomic placement	Species	Lipid content (% DM)	Remarks[a]
Zygomycetes	Order *Mucorales*	*Cunninghamella echinulata*	24–54	17–22% of total FA GLA
		Cunninghamella blakesleeana	41	22% of total FA PUFA
		Mucor circinelloides	15–36	15–26% of total FA PUFA
		Thamnidium elegans	71	Chatzifragkou et al. (2011)
	Order *Mortierellales*	*Mortierella elongata*	24	58% of total FA ARA, 13% EPA
		Mortierella alpina	50	43% of total FA ARA
	Order *Umbelopsidales*	*Umbelopsis isabellina* (syn. *Mortierella isabellina*)	71	11% of total FA ALA, 2.5 GLA
Basidiomycetes	Subphylum Pucciniomycotina Class *Cystobasidiomycetes* Order *Cystobasidiales*	*Rhodotorula minuta*	25	
	Class Microbotryomycetes Order *Leucosporidiales*	*Leucosporidium creatinivorum*	49–61, 70	The latter value was obtained at 20 °C in a test of cryophilic yeasts (Filippucci et al. 2016)
	Order *Sporidiobolaceae*	*Rhodotorula colostri*	27	
		Rhodotorula mucilaginosa	28–33	
		Rhodosporidium paludigenum	31	
		Rhodosporidium sphaerocarpum	15–43	
		Rhodosporidium babjevae	65	
		Rhodosporidium diobovatum	20–41	

		Rhodosporidium toruloides	20–60, 67.5	67.5% in high cell-density culture (Li et al. 2007)
		Rhodotorula glutinis	35–57	
		Rhodotorula graminis	40	
		Rhodosporidium fluviale	25	
		Rhodotorula glacialis	68	
	Order uncertain	*Rhodotorula terpenoidalis*	27	
	Subphylum Agaricomycotina Class Tremellomycetes Order *Filobasidiales*	*Solicoccozyma terricola* (syn. *Cryptococcus terricolus*)	69	
		Solicoccozyma terreus (syn. *Cryptococcus terreus*)	51	
		Naganishia adeliensis	53	Obtained at 20 °C in a test of cryophilic yeasts (Filippucci et al. 2016)
	Order *Cystofilobasidiales*	*Phaffia rhodozyma*	37	Flores-Cotera et al. (2001)
	Order *Trichosporonales*	*Cutaneotrichosporon cutaneum*	31	Liu et al. (2013)
Ascomycetes	Subphylum Saccharomycotina Class Saccharomycetes Order *Saccharomycetales* Family *Lipomycetaceae*	*Myxozyma melibiosi*	23	
		Lipomyces lipofer	40	
		Lipomyces tetrasporus	66	
		Lipomyces starkeyi	62	
		Lipomyces doorenjongii	72	
		Lipomyces kockii	78	
	Family *Metschnikowiaceae*	*Kodamaea ohmeri*	53	
		Metschnikowia pulcherrima	30	

(continued)

Table 6.1 (continued)

Phylum	Taxonomic placement	Species	Lipid content (% DM)	Remarks[a]
	Family *Wickerhamomycetacaea*	*Cyberlindnera jadinii*	20–30	
		Cyberlindnera saturnus	25	
	Family *Debaryomycetaceae*	*Schwanniomyces occidentalis*	23	
		Candida diddensiae	37	
		Kurtzmaniella cleridarum	33	
	Family *Dipodascaceae*	*Geotrichum histeridarum*	34	
		Galactomyces candidus	50	
	Family *Saccharomycetaceae*	*Torulaspora delbrueckii*	40	
		Saccharomyces cerevisiae	45	A strain engineered in multiple genes of lipid metabolism (Kamisaka et al. 2013)
	Uncertain family	*Yarrowia lipolytica*	55, 85	85% in a *mga2-G643R* mutant strain overexpressing *DGA1* (Liu et al. 2015a, b)

If no other reference is given, information was compiled from Sitepu et al. (2014) for oleaginous yeasts and from Béligon et al. (2016) and Bellou et al. (2016) for oleaginous zygomycetes

[a]*FA* fatty acids, *ARA* arachidonic acid, *EPA* eicosapentaenoic acid, *ALA* α-linolenic acid, *GLA* γ-linolenic acid, *PUFA* polyunsaturated fatty acids

6.3 Biochemistry of Lipid Synthesis

Fatty acids for microbial oils of oleaginous fungi are synthesised in the cytoplasm from acetyl-CoA, following a reversed β-oxidation. Acetyl-CoA carboxylase synthesises ATP-dependent malonyl-CoA from acetyl-CoA, which is the substrate of the fatty acid synthase (FAS)—enzyme complex. FAS elongates the acyl-CoA chain by two carbons in each step, consuming NADPH. Usually, synthesis starts with acetyl-CoA, resulting in even-numbered fatty acids. However, it is also possible that propionyl-CoA is used in the first condensation, which results in odd-numbered fatty acids (Tehlivets et al. 2007). Odd-numbered fatty acids have been observed in some yeast strains (e.g. Olstorpe et al. 2014). Two NADPH are consumed per elongation step to reduce the acetyl groups; thus, for the synthesis of 1 mole of C18 saturated fatty acid (stearic acid), 8 mole ATP and 16 mole NADPH are required (Ratledge 2014). When glucose is the carbon source, the acetyl-CoA is derived from citric acid, which is exported out of the mitochondria. This happens when there is a surplus of carbon and energy for the cell, which implies that there is a limitation for another essential compound, such as nitrogen, phosphorus or sulphur (Ratledge and Wynn 2002; Wu et al. 2010, 2011). Under those circumstances, AMP, which is an essential cofactor of isocitrate dehydrogenase (ICDH), is degraded, and thus ICDH is no longer able to perform the conversion of isocitrate to α-ketoglutarate. As a result, isocitrate accumulates and levels out with citrate, which is then exported, usually in exchange with malate, to the cytoplasm. In the cytoplasm, citrate is converted to oxaloacetate and acetyl-CoA by ATP citrate lyase (ACL), consuming ATP and coenzyme A (Tang et al. 2015). ACL has up to now been found in all known oleaginous fungi (Ratledge and Wynn 2002).

It has been pointed out that supply of NADPH is the limiting factor in lipid synthesis. The major pathways for NADPH generation are the pentose phosphate pathway (PPP) with the two NADPH-generating enzymes glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH) and the pyruvate-oxaloacetate-malate (POM) or transhydrogenase cycle. In this cycle, pyruvate is carboxylated to oxaloacetate by pyruvate carboxylase (PC), and then the oxaloacetate is reduced to malate, which consumes one mole NADH per mole oxaloacetate, and then malic enzyme (ME) converts the malate to pyruvate, CO_2 and NADPH. There is also a cytoplasmic, $NADP^+$-dependent ICDH in many eukaryotic organisms, which may have a role in generating NADPH during lipid synthesis (Ratledge 2014).

Malic enzyme has been suggested to be the major provider of NADPH for lipid production. A physical interaction between malic enzyme and other lipid synthesis enzymes has been proposed (Ratledge and Wynn 2002; Ratledge 2014). However, in many oleaginous yeasts, no cytoplasmic malic enzyme has been found. *Lipomyces starkeyi* possesses a putative cytoplasmic malic enzyme, but it has a preference for NAD^+ instead of $NADP^+$ (Tang et al. 2010; Vorapreeda et al. 2013). *Y. lipolytica* contains only a mitochondrial ME, and its overexpression did not affect lipid accumulation (Beopoulos et al. 2011). Similarly, the overexpression of a $NADP^+$-dependent ME from *Mortierella alpina* did not result in increased lipid production in this yeast (Zhang et al. 2013). Metabolic flux analysis showed that

there is no NADPH production by ME in this yeast and that the PPP, although not significantly upregulated under lipid accumulation conditions, was obviously sufficient for the production of reducing cofactors for lipid synthesis. This might also be due to the fact that *Y. lipolytica* accumulates rather low amounts of lipids compared to other oleaginous fungi (Zhang et al. 2016a). On the other hand, by overexpressing genes of acetyl-CoA carboxylase (*ACC1*) and diacylglycerol acyltransferase (*DGA1*), the final step of triacylglyceride synthesis, the lipid content in *Y. lipolytica* could be increased to more than 60% of cell dry weight, without further manipulation of NADPH-generating enzymes (Tai and Stephanopoulos 2013).

However, in other fungi a clear impact of NADPH-generating enzymes on lipid synthesis has been shown. In the oleaginous yeast *Cutaneotrichosporon cutaneum* (syn. *Trichosporon cutaneum*, Liu et al. 2015b), ME was upregulated under lipid accumulation conditions (Liu et al. 2013). Overexpression of a ME from *Mucor circinelloides* resulted in substantially increased lipid contents both in *M. circinelloides* (Zhang et al. 2007) and in *Rhodotorula glutinis* (Della-Bianca et al. 2013). On the other hand, overexpressing a native ME gene in *R. toruloides* did not have a large effect on lipid accumulation (Zhang et al. 2016b). In *M. circinelloides*, both PPP and ME contribute to NADPH formation during lipid synthesis (Tang et al. 2015; Zhao et al. 2015). *M. alpina*, which accumulates significantly more lipids than *M. circinelloides*, has a substantially higher and more constant ME activity than *M. circinelloides*. Most probably, oxaloacetate, which is formed in the cytoplasm as a result of the ACL reaction, is NADH dependently reduced to malate, which is then converted to pyruvate, CO_2 and NADPH by ME. Pyruvate is subsequently transported into the mitochondrion by the pyruvate/malate/citrate shuttle system. In *M. circinelloides*, a substantial part of the malate is also transported back to the mitochondria, since there is an accumulation of cytoplasmic malate due to the low activity of ME (Zhao et al. 2016). Conversion of cytoplasmic malate to pyruvate by ME and transport of pyruvate back to mitochondria would, however, result in a lack of oxaloacetate in the mitochondria of filamentous fungi, as there is obviously no mitochondrial PC (Wynn et al. 2001), and thus pyruvate can only be converted to acetyl-CoA by pyruvate dehydrogenase (PDH). Additional OA can, however, be generated by the glyoxylate cycle, where isocitrate is first converted to glyoxylate and succinate by isocitrate lyase (ICL). Glyoxylate can then be condensed with acetyl-CoA, resulting in malate, which can be transported out from the mitochondria, serving as additional substrate for ME, generating more NADPH (Vongsangnak et al. 2013). Indeed, increased activities of the glyoxylate cycle have been demonstrated in *M. circinelloides* under lipid accumulation conditions (Zhao et al. 2015). Yeasts have both cytoplasmic and mitochondrial PC activities, and in *T. cutaneum*, an increased amount of mitochondrial oxaloacetate synthesized by mitochondrial PC has been observed, while cytoplasmic oxaloacetate was mainly produced by ACL (Liu et al. 2013).

When all NADPH has to be provided by the PPP, as it is possibly the case in yeasts lacking $NADP^+$-dependent cytoplasmic ME activity, the maximum lipid yield per gram glucose would be 0.276 g lipids (Ratledge 2014), and, indeed, in one study a yield of 0.27 g lipids was reported in engineered *Y. lipolytica* (Tai and

Stephanopoulos 2013). Recently, the simultaneous overexpression of a glyceraldehyde-3-phosphate dehydrogenase with a preference for $NADP^+$ instead of NAD^+ and a ME from *M. circinelloides* and overexpression of a NAD^+/NADH kinase resulted in a maximum lipid yield of 0.28 g/g glucose (Qiao et al. 2017). This is, however, still not reaching the maximum theoretical lipid yield from glucose, when NADPH is not exclusively generated by PPP, which was calculated to be 0.316 g per gram consumed sugar (Ratledge 2014). Still, it is considerably higher than the yields that have been obtained until now, where values higher than 0.22 g/g seldomly were obtained (Ratledge 2014; Jin et al. 2015).

However, it has been pointed out that NADPH availability is not necessarily the only limiting factor in lipid synthesis. Rodríguez-Frómeta and colleagues discovered that a *M. circinelloides* strain overexpressing a gene encoding cytosolic ME produced more lipids than the original mutant strain but not more than a control strain, which was only transformed with the selection marker LeuA, encoding α-isopropylmalate isomerase, a step in leucine biosynthesis. Adding external leucine to the growth medium did not have a positive effect on lipid synthesis, indicating that the leucine synthesis pathway itself is activating lipid synthesis (Rodríguez-Frómeta et al. 2013). A similar phenomenon has been observed in *S. cerevisiae*, where complementing of a *LEU2* mutation resulted in a higher lipid content of the cells (Kamisaka et al. 2007). The mechanism behind this activation of lipid production by an active leucine synthesis pathway is not completely clear. It is possible that acetyl-CoA is released from leucine degradation, which can serve as substrate for lipid synthesis. The substrate of α-isopropylmalate isomerase, α-isopropylmalate, which accumulates in *leuA* mutants, plays a pivotal role in leucine metabolism in yeasts. It binds to Leu3p, which turns this protein from a transcriptional repressor to an activator of leucine biosynthesis. One may speculate that similar regulatory phenomena also occur in *M. circinelloides* and that accumulation of α-isopropylmalate in the *leuA* mutants activates synthesis and prevents degradation of leucine and by this supply of acetyl-CoA from this pathway (Rodríguez-Frómeta et al. 2013).

Other substrates than glucose have also been considered for single cell oil production. This includes, for instance, xylose, which is the major component of hemicellulose and the second most abundant sugar in nature. Xylose is present in lignocellulose hydrolysates in considerable amounts. Substantial efforts have been taken to engineer strains of *Saccharomyces cerevisiae* to perform ethanol production from xylose; however, only recently strains capable of surviving the harsh industrial conditions have been obtained. Alternative to ethanol production, the natural ability of many oleaginous fungi could be harnessed to generate lipids from xylose (Passoth 2014). Xylose is metabolised via the PPP. To achieve this, the sugar is first converted to xylulose, which in most fungi occurs in a two-step reaction. At first, xylose is reduced to xylitol by xylose reductase (XR). In most fungi, this reaction is NADPH dependent; only few yeasts, those that can form ethanol from xylose, have a dual NADPH-/NADH-dependent activity. In the second step, xylitol is reoxidised by xylitol dehydrogenase (XDH) to xylulose, which is then phosphorylated and enters the PPP. Due to the demand for NADPH in the first step, cells grown on xylose would need more NADPH, and thus, more hexose

generated in the PPP would have been recycled through the PPP to generate this NADPH. Under those circumstances, 1 mole acetyl-CoA would be formed out of 100 g xylose (about 0.66 moles, thus, about 1.5 moles would be formed per mole xylose). Therefore, an alternative pathway of xylose utilisation has been postulated, phosphorolytic cleavage of xylulose-5-P by phosphoketolase. This enzyme generates acetyl phosphate and glyceraldehyde-3-phosphate. Indeed, the corresponding enzyme has been found in a variety of yeast species grown on xylose medium without glucose, including oleaginous species such as *Lipomyces starkeyi* and *Rhodosporidium toruloides* (Evans and Ratledge 1984). Via this pathway, lipid yield on weight basis from xylose would be slightly higher than from glucose. For xylose, 1.2 moles acetyl-CoA would be formed out of 100 g xylose, corresponding to 0.34 g lipids per gram sugar. For glucose, 1.1 moles acetyl-CoA would be formed out of 100 g (0.56 moles) of glucose, corresponding to 0.32 g lipids per gram sugar (Evans and Ratledge 1984; Papanikolaou and Aggelis 2011; Jin et al. 2015). There are varying results about the real yields obtained from xylose; however, there are examples where higher lipid yields have been obtained from xylose compared to those on glucose (Fakas et al. 2009). Glycerol is another interesting substrate for lipid production, as it is generated as a side product of transesterification in biodiesel production. Glycerol is phosphorylated by glycero-kinase and then oxidised to dihydroxyacetone phosphate or glyceraldehyde phosphate, which are further assimilated following the glycolytic pathway. The theoretical lipid yield for glycerol has been estimated with 0.3 g/g, but in practice, usually values of 0.1 g/g are obtained (Papanikolaou and Aggelis 2011). In two cases, for *Aspergillus* sp. and *Thamnidium elegans* lipid yields of about 0.2 g/g were obtained (André et al. 2010; Chatzifragkou et al. 2011). Organic acids, especially acetic acid, have also been considered as potential substrates for lipid production. Acetic acid is a side product of a variety of processes and also frequently found in lignocellulosic hydrolysate (Papanikolaou and Aggelis 2011; Passoth 2014). Acetic acid is converted to acetyl-CoA and can be assimilated via the assimilatory glyoxylate cycle (Barnett and Kornberg 1960). The pathways of lipid formation from different carbon sources are summarised in Fig. 6.1.

From the initially saturated, mainly C16 and C18 fatty acids, unsaturated fatty acids are synthesized at the endoplasmic reticulum by the action of desaturases and elongases (Bellou et al. 2016). Eukaryotic systems use an aerobic pathway of desaturation (Fig. 6.2). This pathway consists of three membrane-bound proteins, NAD(P)H-cytochrome b_5 reductase, cytochrome b_5 and the terminal cyanide-sensitive desaturase. The desaturase complex is bound to the endoplasmic reticulum (Certik and Shimizu 1999). It has been stated that in fungi, the desaturases are dependent on NADPH (Kendrick and Ratledge 1992; Ratledge 2014); however, for the yeast *Y. lipolytica* and the filamentous fungus *M. circinelloides*, a dual cofactor dependency of NADH and NADPH has been found, for the latter even a higher activity with NADH (Pugh and Kates 1979; Jackson et al. 1998).

Desaturation starts with palmitic (C16) and stearic (C18) acids and the first double bound is generated at the Δ9 position, i.e. palmitoleic acid (C16:1 *cis* 9) and oleic acid (C18:1 *cis* 9) are generated. From oleic acid, further desaturations may occur (Fig. 6.3), at Δ12 position to generate linoleic acid (C18:2), which may be

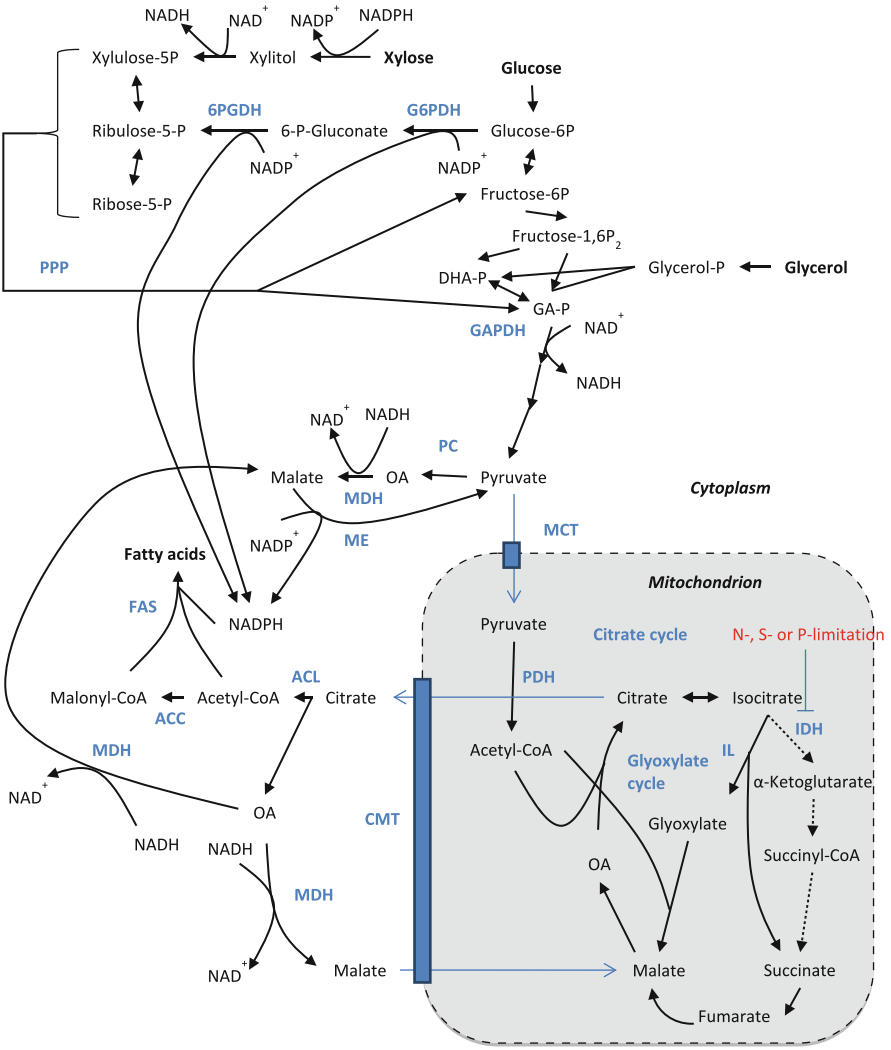

Fig. 6.1 Simplified scheme of fatty acid biosynthesis from glucose, xylose and glycerol. Acetyl-CoA is provided from the ATP citrate lyase (ACL) reaction, NADPH from malic enzyme (ME) and the pentose phosphate pathway (PPP) enzymes, glucose-6-P dehydrogenase (G6PDH) and 6-P-gluconate dehydrogenase (6PGDH). Citrate-malate can be transported by citrate-malate translocase (CMT) or specific transporters, pyruvate by monocarboxylic acid transporter (MCT). *DHA-P* dihydroxyacetone-P, *GA-P* glycerol aldehyde-P, *GAPDH* glycerol aldehyde-P dehydrogenase, *PC* pyruvate carboxylase, *OA* oxaloacetate, *MDH* malate dehydrogenase, *FAS* fatty acid synthetase, *ACC* acetyl-CoA carboxylase, *PDH* pyruvate dehydrogenase, *IL* isocitrate lyase, *IDH* isocitrate dehydrogenase

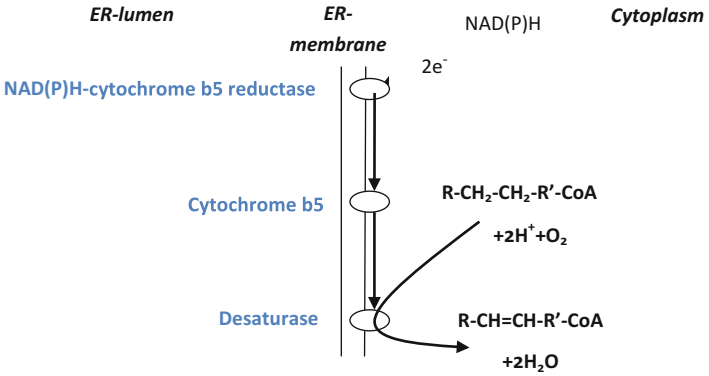

Fig. 6.2 Aerobic desaturation of fatty acids in fungi. Electrons from NAD(P)H are transported along a membrane-bound electron transport chain at the membrane of the endoplasmic reticulum (ER) and, finally, together with two electrons from the saturated binding, transferred to O_2 as final electron acceptor, generating an unsaturated fatty acid and H_2O

further desaturated at Δ15 position, generating the Ω-3 fatty acid α-linolenic acid (ALA, C18:3, *cis* 9,12,15). Oleic, linoleic and linolenic acid can be converted further by Δ6 desaturase, leading to the Ω-9, Ω-6 and Ω-3 series of fatty acids (Certik and Shimizu 1999). Yeasts usually have a limited ability to form a great variety of fatty acids. The major fatty acids are usually C16:0 and C18:1, and fatty acids longer than C18 are only produced in traces. Usually low amounts of C18:2 and C18:3 are produced (dependent on culture conditions, up to 10% of the total fatty acids), in basidiomycetes such as *R. toruloides* usually a bit more than in ascomycetes; still, C18:2 and C18:3 have also been found in ascomycetous yeasts (Ratledge 2004; Olstorpe et al. 2014; Brandenburg et al. 2016). However, some filamentous zygomycetes can also synthesise longer polyunsaturated fatty acids (PUFA). In the Ω-9 series of fatty acids, after the Δ6 desaturase step, one elongase and Δ5 desaturase reaction result in the production of mead acid (MA, 20:3 *cis* 9,12,15). In the Ω-6 and Ω-3 series of fatty acids, elongase and Δ5 desaturase reactions generate arachidonic acid (AA, 20:4 *cis* 5,8,11,14) and eicosapentaenoic acid (EPA, 20:5 *cis* 5,8,11,14,17), respectively. EPA can also be synthesised from AA by a Δ17 desaturase, which especially happens at low temperatures (Certik and Shimizu 1999). Up to now, no fungi are known which are able to further elongate the fatty acids to produce the longer-chain Ω-3 fatty acid docosahexaenoic acid (DHA, 22:6 *cis* 4,7,10, 13,16,19). Thraustochytrids, a group of heterotrophic marine microbes originally classified as fungi but now aligned with heterokont algae (Béligon et al. 2016), are commercially used to produce DHA as a food additive and produce fatty acids by using a different pathway, based on the polyketide synthase route (Metz et al. 2001; Ratledge 2012).

Free fatty acids are then converted to triacylglycerides (TAG) in a series of enzymatic steps starting from glycerol phosphate or dihydroxyacetone phosphate, which are in several steps converted to phosphatidic acid, which is then

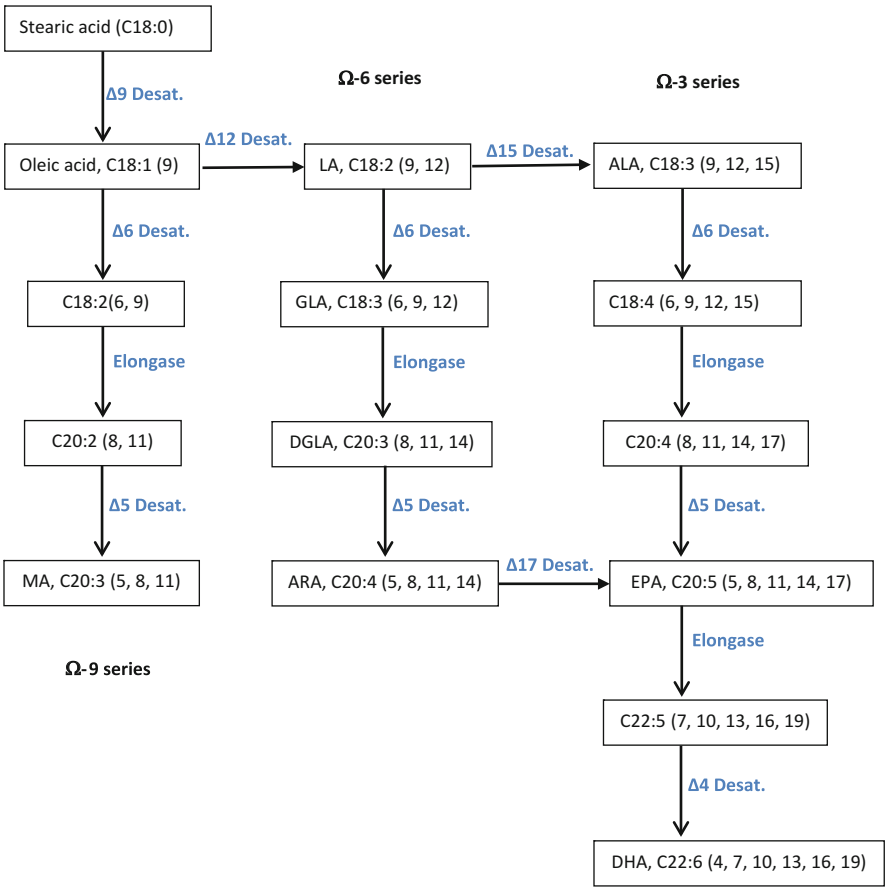

Fig. 6.3 Biosynthesis of unsaturated fatty acids. Production of C18 and longer unsaturated fatty acids starts by Δ9 desaturation of stearic acid by an according desaturase (Desat.). Δ17 desaturase is activated by low temperatures. By a series of desaturations and elongations, fatty acids of Ω-9, Ω-6 and Ω-3 series are generated. *MA* mead acid, *LA* linoleic acid, *GLA* γ-linolenic acid, *DGLA* dihomo-γ-linolenic acid, *ARA* arachidonic acid, *ALA* α-linolenic acid, *EPA* eicosapentaenoic acid, *DHA* docosahexaenoic acid. Currently, no fungi are known that can naturally produce DHA

dephosphorylated to diacylglycerol and then acylated to triacylglycerol (Sorger and Daum 2003). Storage lipids are localised in lipid bodies in the cell, and recent research has demonstrated that lipid body formation is a highly organised process. In yeasts, lipid bodies are generated between the two leaflets of the lipid bilayer of the endoplasmic reticulum (ER) (Garay et al. 2014). There are a variety of proteins associated with lipid bodies, most of them involved in lipid metabolism, and many of them also associated with the ER. Among them there are the enzymes for TAG synthesis, which are essential for lipid body formation (Wang 2015; Zhu et al. 2015). A lipid body-specific protein has been identified in *R. toruloides*, and its overexpression in *S. cerevisiae* resulted in giant lipid bodies (Zhu et al. 2015). Lipid

bodies seem to have a variety of important cellular functions, including protecting cells from lipid toxicity and allowing lipid mobilisation in a coordinated way (Wang 2015).

6.4 Production of Polyunsaturated Fatty Acids (PUFAs) by Fungi

Yeast oils are quite similar to vegetable oils in their composition. Oleaginous yeasts grown on residues from the paper industry (spent sulphite liquor) were used to produce fat for food purposes (fat for baking or as butter replacement) in Germany during both World Wars. However, these processes were too expensive and were stopped immediately when vegetable lipids could be imported (Sitepu et al. 2014).

Currently, microbial oils are regarded as an alternative source of PUFA for human nutrition. Higher plants cannot produce fatty acids longer than C18, and thus they can only provide the essential fatty acids linoleic acid (C18:2) and ALA (C18:3). In principle, animals including humans can synthesise the longer PUFAs AA, EPA and DHA from linoleic and linolenic acid, but very slowly. Specific PUFAs have been demonstrated to have positive effects on the prevention of cardiovascular and inflammatory diseases, cancer, brain disorders, obesity and diabetes. A variety of studies have shown that there should be a balanced relation between Ω-6 and Ω-3 fatty acids in a healthy diet. Currently, the Ω-6/Ω-3 ratio is usually very high in modern diets. This should be reduced to avoid negative effects on health (Simopoulos 2006; Bellou et al. 2016).

Traditionally, fish oils have been the main sources of Ω-3 fatty acids. However, there are several limitations to extensive utilisation of fish as the main source of Ω-3 fatty acids. Caught fish is a limited resource, and wild fish populations are declining (Golden et al. 2016). Farmed fish requires the addition of Ω-3 fatty acids to the feed, since fish, like most animals, have a limited capacity to generate longer-chain fatty acids. In their natural habitats, fish take up PUFAs from aquatic microorganisms, which have the ability to synthesise long-chain PUFAs. For cultured fish, those PUFAs have to be added to the feed. These PUFAs are mainly derived from fish oil; aquaculture currently consumes about 70% of all globally produced fish oil, and about 90% of the available fish oil is obtained from caught fish. It is clear that, against a background of growing demand for seafood, this is not a sustainable practice (Tocher 2015). Fish oils also have an undesirable odour and may contain contaminants such as mutagenic compounds and heavy metals (Bellou et al. 2016). This implies that there is a crucial demand for alternative sources of PUFA, especially of the longer-chained Ω-3 fatty acids EPA and DHA.

Microbes, especially fungi and microalgae, have been regarded as potential PUFA producers. As mentioned above, certain filamentous oleaginous zygomycetes are able to produce EPA. However, yeasts have also been regarded as resources of PUFAs. Although yeasts usually do not produce longer-chain fatty acids, they are able to accumulate lipids to much higher amounts compared to filamentous fungi, and thus they are promising hosts to be genetically engineered

towards production of long-chain PUFAs (Béligon et al. 2016). If PUFAs are produced by filamentous fungi, production systems are frequently based on solid-state fermentations, implying specific parameters to be optimised (Asadi et al. 2015). For the production of unsaturated fatty acids, temperature plays a key role. An increase in desaturation upon decreasing temperatures has been observed in a variety of yeasts, although there are obviously different processes at work in the low-temperature response, which can in some cases even result in a lower degree of desaturation at lower temperatures (e.g. Olstorpe et al. 2014). However, for several fungi belonging to the genus *Mortierella*, low cultivation temperature (12 °C) during lipid accumulation significantly increased EPA production, because Δ17 desaturase was activated by cold (Shimizu et al. 1988; Certik and Shimizu 1999). The addition of certain precursors can also increase the production of long-chain PUFAs. If ALA was added, EPA was produced even at standard growth temperatures, due to the temperature-independent activity of the Ω-3 desaturation route; however, lower temperatures additionally increased the production to 1.88 g/l of culture broth (Shimizu et al. 1989).

As already mentioned above, a variety of genera of Zygomycota have a tremendous ability to produce commercially interesting PUFAs. Among them, the genus *Mortierella* has been found to have a great potential to produce a variety of different PUFAs. The strain *M. alpina* 1S-4 was originally isolated because of its high production of AA. A range of desaturase mutants have been generated from this strain by random mutagenesis, e.g. in the Δ12 desaturase, overproducing MA; Δ6 desaturase, overproducing dihomo-γ-linolenic acid (DGLA, C20:3 *cis*-8, 11, 14), a fatty acid of the Ω-6-series; or Δ5 desaturase, overproducing AA. These strains are not only interesting from a commercial point of view but also for general understanding of lipid metabolism. Methods for metabolic engineering of this fungus have been developed, including transformation systems based on complementation of an *URA3* mutation or on resistance markers. Transformation could be achieved using biolistic methods or an *Agrobacterium tumefaciens*-based system (Sakuradani et al. 2013). Overexpression of elongases increased the production of AA, supporting the finding that its production is limited in the elongation step from C18:3 to C20:3 (Wynn and Ratledge 2000; Takeno et al. 2005a). Moreover, RNA interference was also introduced in *M. alpina*, and inactivating Δ12 desaturase indeed resulted in a change in the fatty acid profile, demonstrating that it is possible to influence the fatty acid profile by molecular methods (Takeno et al. 2005b).

A variety of fungi have already been commercialised for the production of PUFAs. From 1985 to 1990, γ-linolenic acid (C18:3, Ω-6) was produced with *M. circinelloides* by J&E Sturge (UK), with a volume of 5–10 tons per year. DHA is commercially produced by a dinoflagellate alga, as well as by certain species of thraustochytrids. Upon application of DHA to food, it has been observed that portions of DHA can lose a C2 unit, resulting in EPA. EPA, although in general regarded positive for health, was contraindicated for having positive effects on babies. However, it has been discovered that the presence of AA can prevent the degradation of DHA to EPA. AA is produced using *M. alpina*, and the process has been commercialised by Gist-brocades (the Netherlands), now taken over by DSM

(the Netherlands). AA-rich oil is also produced by Suntory (Japan) and Cargill Alking Bioengineering Co. Ltd (China) (Ratledge 2013). EPA is produced by some companies using algae as production organisms. However, an alternative has been developed by DuPont (USA), using genetically modified *Y. lipolytica* (Ratledge 2013; Xue et al. 2013). For reaching this aim, a Δ9 elongase, a Δ8 desaturase, a Δ5 desaturase and a Δ17 desaturase were expressed in the yeast, and β-oxidation was impaired by inactivating a peroxisomal gene (*PEX10*). The final strain was able to produce EPA to 15% of the cell dry weight, and the total oil content was 56.6% (Xue et al. 2013).

6.5 Lipid Modification of Other Biomolecules

6.5.1 Fungal Glycolipids as Biosurfactants

Glycolipids are derivatives from the lipid metabolism, and they have biotechnological potential as biosurfactants. They consist of a long-chain fatty acid, representing the hydrophobic part, and a carbohydrate moiety, which is hydrophilic. The carbohydrate component can be glucose, trehalose, mannose, galactose, sophorose, cellobiose or rhamnose (Roelants et al. 2014; Paulino et al. 2016). Both rhamno- and sophorolipids are produced commercially; however, rhamnolipids are produced by the opportunistically pathogenic bacterium *Pseudomonas aeruginosa*, whereas sophorolipids are produced by non-pathogenic yeast strains (Van Bogaert et al. 2011; Paulino et al. 2016).

Sophorolipids (Fig. 6.4) are synthesised from C16 or C18 fatty acids, with a single or a twofold desaturation. At first, the fatty acid is hydroxylated at Ω-1 or Ω-2 position by cytochrome P450 monooxygenase. Then, by two glycosyltransferase steps, glucose is connected to the molecule, consuming one UDP-glucose per step. These glycosylations are performed by two different glycosyltransferases, UgtA1 and UgtB1 (Saerens et al. 2011a, c). The first glucose is β-glycosidic bound to the hydroxyl group. The second glucose is bound β-1,2 glycosidic to the first glucose, forming sophorose. The resulting molecule can be acetylated by an acetyltransferase (Saerens et al. 2011b). Acetylation usually happens at the 6′ and/or the 6″ position of the sophorose (Van Bogaert et al. 2011). The sophorolipid is then transported out of the cell by an ABC transporter (Mdr) into the extracellular space (Van Bogaert et al. 2013), where it can be further lactonised by a cell wall-bound lactonesterase. Interestingly, this enzyme has homology to a biotechnologically interesting lipase of *Pseudozyma antarctica* (Ciesielska et al. 2014). Sophorolipids can have a free fatty acid tail (acid form), or an internal esterification can happen between the carboxyl group of the fatty acid and the 4″ of the sophorose (lactonic form). In some cases, lactonisations can also happen between the carboxyl group and the 6′ or 6″ position of the sophorose. Sophorolipids are synthesised as a mixture of all these different forms. To some extent the proportion of these forms can be influenced by the fermentation conditions (Van Bogaert et al. 2011); however, a clear species dependency in the production pattern has also been

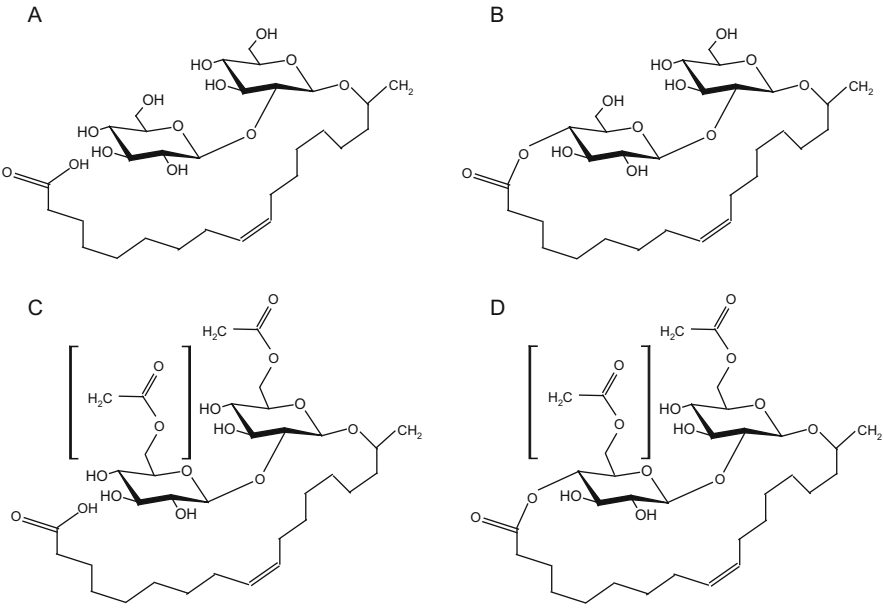

Fig. 6.4 Sophorolipids are formed from sophorose (hydrophilic part), a β-1,2 diglucose and a terminal or subterminal hydroxylated fatty acid (hydrophobic part), often hydroxyoleate or hydroxystearate. The fatty acid is β-glycosidically linked to the sophorose via its hydroxyl group. Sophorolipids can have an acidic form (**A**, **C**) or a lactonic form (**B**, **D**); both these forms can be mono- or diacetylated (**C**, **D**)

demonstrated, with production of the lactone form by *Starmerella bombicola* and *Candida apiculata* and production of the acid form by *Candida stellata* and other closely related strains (Kurtzman et al. 2010).

Biosurfactants have a number of biotechnological applications. Initially, biosurfactants were mainly applied for bioremediation of polluted soil and water (Mulligan 2005). However, a variety of novel applications are now possible. In the food industry, they can serve as emulsifiers, for foaming and wetting, and as solubilisers. They also have antimicrobial activities and can act against bacteria, fungi and even viruses. Apart from this, they are less toxic than synthetically synthesised surfactants, are biodegradable and are even more effective than chemically synthesised surfactants, as their critical micelle concentration (CMC) is about 10–40 times lower (Nitschke and Costa 2007; Mnif and Ghribi 2016).

A variety of yeast species are able to produce sophorolipids. The current major producer of sophorolipids is *S. bombicola*, usually designated by its anamorph name *Candida bombicola*. There are a variety of other yeast species, which are closely related to *S. bombicola*, including *C. stellata*, *Candida floricola*, *Candida riodocensis*, *Candida apicola* and *Candida batistae*. However, sophorolipids have also been found to be produced by phylogenetically more distant species, including *Wickerhamomyces anomalus* and *Wickerhamiella domercqiae*, and even the

basidiomycetous yeasts *Rhodotorula bogoriensis* and *Cryptococcus* spp. *C. apicola* was the first yeast species in which sophorolipid production was discovered, and similar final concentrations and product yields have been obtained compared to *S. bombicola*; however, research and commercialisation efforts have mainly focused on the latter species during the last years (Van Bogaert et al. 2011; Paulino et al. 2016).

S. bombicola generated lipid concentrations of more than 400 g/l, and 70% of the substrate was converted to sophorolipids. Accordingly, *S. bombicola* has been commercialised, and a range of patents have been filed (Van Bogaert et al. 2011).

For the production of sophorolipids, usually a sugar and a hydrophobic compound are used as carbon sources. Standard substrates consist of 100 g/l glucose, taking into account that *S. bombicola* tolerates quite high sugar concentrations and a fatty acid or a triglyceride (Van Bogaert et al. 2011). Alkanes have also successfully been used, as this yeast can also assimilate alkanes, and their presence triggers the production of sophorolipids (Inoue and Ito 1982). Glucose can be kept in excess, while the hydrophobic substrate is usually added in a controlled fed-batch mode (Davila et al. 1992). Alternative substrates have also been regarded. When non-glucose sugars are used, sophorolipids are still produced but much less than on glucose. Using fatty acids or alkanes with the right chain length (C16–C18) greatly increases surfactant production. Free fatty acids are better converted than their respective oils, but methyl esters of the free fatty acids are better converted than the corresponding free fatty acids (Asmer et al. 1988; Van Bogaert et al. 2011). Cheaper, alternative substrates can also be used for sophorolipid production, including molasses to replace the sugar or glycerol as hydrophobic substrate; however, production of sophorolipids is rather low (Van Bogaert et al. 2011). Waste products such as biodiesel waste streams, used frying oil or restaurant waste have also been tested to replace the standard hydrophobic compounds. The results were difficult to evaluate, as comparison with standard substrates were missing; nevertheless, a better yield has been obtained compared to fermentations without adding the hydrophobic substrates, and, thus, a potential valorisation of these waste products seems possible (Nitschke and Costa 2007; Van Bogaert et al. 2011; Paulino et al. 2016).

Nitrogen sources usually include yeast extract or corn steep liquor; however, other N sources such as ammonium or urea are also possible. Some minerals should also be present. Similar to lipid accumulation, sophorolipid production starts when the cells enter stationary phase due to nitrogen limitation. Stationary phase can be maintained for about 20 days, but typical fermentations last for 10 days. The process tolerates a broad temperature range; typical fermentations are running at 25–30 °C, although 21 °C has been determined as optimal. In contrast, C/N ratio, pH and oxygenation have to be controlled carefully. At low C/N ratio, the formed sophorolipids are metabolised, indicating that they are formed as a kind of external storage material (Van Bogaert et al. 2011). Indeed, the genes encoding sophorolipid synthesis are induced by nitrogen limitation. This is also true for gene clusters encoding mannosylerythritol and cellobiose lipids (Roelants et al. 2014). The pH decreases during fermentation to 3.5 and should thereafter be held at this value.

Oxygen is required by the cytochrome P450 monooxygenase, and the oxygen transfer rate should be kept between 50 and 80 mM O_2 per litre and hour (Van Bogaert et al. 2011). The highest production of sophorolipids (422 g/l) was obtained in a two-step process, where whey was initially converted to oily biomass by the oleaginous yeast *Cryptococcus curvatus*, and in the second step, the disintegrated yeast biomass was used as nutrient source for *S. bombicola* (Daniel et al. 1999). This represents also a way of introducing residues into the production process, which are usually not metabolised by *S. bombicola* (such as lactose from whey).

The genes involved in sophorolipid production in *S. bombicola* have been identified. Similar to the synthesis pathways of many other secondary metabolites, they are organised in a subtelomeric cluster (Van Bogaert et al. 2013); only the gene for lactonisation is located elsewhere in the genome (Ciesielska et al. 2014). Moreover, a few years ago, techniques have been established for genetic engineering of *S. bombicola*. Transformation was possible both by electroporation and Li acetate treatment; two selection markers have been established, *URA3*, complementing the corresponding auxotrophic mutation, and a resistance marker against hygromycin (Van Bogaert et al. 2008a, b). This enables the production of tailored sophorolipids, for instance, cells can be manipulated to produce non-acetylated sophorolipids by deleting the acetyltransferase gene (Saerens et al. 2011b). Non-acetylated sophorolipid forms have, for instance, been shown to have antiviral activity (Shah et al. 2005). The majority of patents on yeast-based glycolipids are on sophorolipids, probably due to a highly efficient production system. Apart from occasional applications for bioremediation of hydrocarbons and aromatic compounds in soil and liquids (Mulligan 2005), sophorolipids are used, for instance, in cosmetics, ecological cleaning products or dishwasher products (Paulino et al. 2016). They also have a pronounced antibiotic effect and have been shown to inhibit bacteria (Díaz De Rienzo et al. 2015), fungi (Haque et al. 2016) and cancer cells (Van Bogaert et al. 2011; Paulino et al. 2016, and references therein). Further experiments have shown that *S. bombicola* can be manipulated to produce other compounds, such as a cellobiose lipid and the bioplastic polyhydroxyalkanoate. Although there are still many factors to optimise, *S. bombicola* has obviously a great potential for the production of high-value, lipid-related compounds (Roelants et al. 2013).

Apart from sophorolipids, also other glycolipids are produced by fungi, including mannosylerythritol lipids (MEL) and cellobiose lipids (CBL) (Roelants et al. 2014). MEL consist of a mannosylerythritol disaccharide, which is acetylated in the positions R4 and R6 and acylated with short-chain (C2–C8) and medium-chain (C10–C18) fatty acids in the positions R2 and R3 (Fig. 6.5). A variety of different MEL has been originally identified, called ustilipids, as they are produced by fungi belonging to *Ustilaginales* (Kurz et al. 2003). In a recent review, four different MEL have been distinguished, MEL-A (fully acetylated), MEL-B (acetylated in R6), MEL-C (acetylated in R4) and MEL-D (completely deacetylated). A variety of fungi can produce MEL, including *Schizonella melanogramma*, several *Pseudozyma* species, the dimorphic fungus *Ustilago maydis* and *Geotrichum candidum* (Roelants et al. 2014). The highest-produced concentrations of MEL

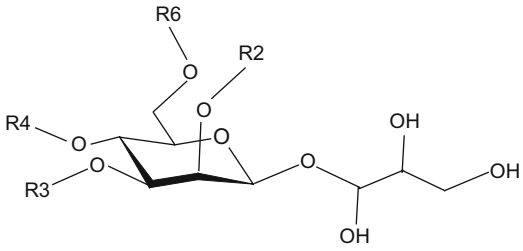

Fig. 6.5 Structure of mannosylerythritol lipids (MEL). R2, R3—fatty acid (C2–C18); MEL-A, R4, R6—$COCH_3$; MEL-B, R4—H, R6—$COCH_3$; MEL-C, R4—$COCH_3$, R6—H; MEL-D no acetylation in R4 and R6

have been obtained with *Pseudozyma aphidis* and *Pseudozyma antarctica* (165 and 140 g/l, respectively) (Kitamoto et al. 2001; Rau et al. 2005). In some fungi of the order *Ustilaginales*, special MEL have been found. The species *Pseudozyma churashimaensis* formed, apart from "usual" MEL-A, also a specific MEL-A2, which was triacetylated at R2, R4 and R6 (Morita et al. 2011). In *Pseudozyma antarctica*, even novel glycolipids were identified, where the erythrose moiety was replaced by mannitol, arabitol or ribitol (Morita et al. 2009, 2012).

The genes involved in biosynthesis of MEL have been identified in *U. maydis*; it was actually the first gene cluster responsible for the production of glycolipids identified in fungi. Five genes encode the whole pathway. The first gene (*emt1*) encodes a glucosyltransferase, which performs a stereospecific mannosylation from GDP-mannose of meso-erythritol at the C4 position. The second gene (*mat1*) is an acetyltransferase gene, which results in the acetylation at the R4- and R6 positions of mannose. Acetylation of R4 happens after acylation at R2 with a short-chain fatty acid (performed by an acyltransferase encoded by *mac1*) or with a medium- to long (C10–C18)-chain fatty acid (catalysed by an acyltransferase encoded by *mac2*). Both acyltransferases are highly specific, both in terms of the regioselectivity and in terms of the fatty acid chain length. Acylation was obviously essential for MEL secretion. The fifth gene (*mmf1*) encodes a protein of the family of the major facilitators, which is essential for the secretion of the glycolipid (Hewald et al. 2006). Gene clusters in different species of *Ustilaginales* encoding for MEL seem to be very similar, as a MEL biosynthesis gene cluster with a high degree of identity to the *U. maydis* cluster has also been found in *P. antarctica* (Morita et al. 2013).

MEL are highly valued in a range of applications, from the treatment of schizophrenia and/or diseases caused by dopamine metabolic dysfunction to the utilisation as chemical tools for the purification of proteins or as anti-agglomeration agents of ice slurry (Kitamoto et al. 2000, 2001; Im et al. 2003; Roelants et al. 2014). A variety of forms with different degrees of acetylation or acylation with uncommon fatty acids have been generated and resulted in a number of additional potential applications (Roelants et al. 2014 and references therein). MEL with antifungal activities have also been identified (Kitamoto et al. 1993).

Cellobiose lipids (CBL) are produced by a small number of basidiomycetous yeasts, mainly belonging to *Ustilaginales*, a few also to the *Trichosporonales*. Ustilagic acid (Fig. 6.6) contains a cellobiose moiety, esterified with an acetyl group (at 6′) and a short-chain (C6 or C8) β-hydroxy fatty acid (at 2″). The

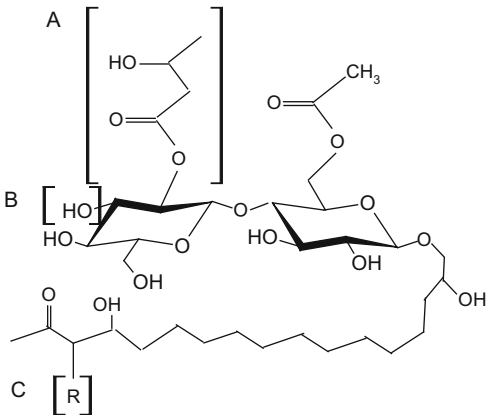

Fig. 6.6 Structure of the cellobiose lipids (CBL) ustilagic acid and flocculosin. Ustilagic acid: (A) β-hydroxyhexanoic or octanoic acid, B-OH, C-H or –OH. Flocculosin: (A) β-hydroxyoctanoic acid, B-acetyl, C-H

cellobiose is O-glycosidically linked to the terminal hydroxyl group of (2, 15, 16) tri- or (15, 16) dihydroxy palmitic acid (Kulakovskaya et al. 2010; Roelants et al. 2014). Decoration of the sugar and hydroxylation of the fatty acid may vary between different species. *Pseudozyma flocculosa* produces flocculosin, a cellobiose lipid with antifungal activity. Flocculosin (Fig. 6.6) consists of (3, 15, 16) trihydroxy palmitic acid as lipid component. The cellobiose is acetylated at the 6′ and 3″ positions; the short-chain fatty acid is exclusively C8 β-hydroxy fatty acid (Teichmann et al. 2011). Similar to the MEL synthesis genes, the genes for CBL are organised in clusters, and the clusters in *U. maydis* and *P. flocculosa* are highly similar: the former has a size of 40 kb and the latter 60 (Teichmann et al. 2007, 2011). They contain two genes responsible for double hydroxylation of palmitic acid and a glycosyltransferase gene for glycosylation of the terminal hydroxyl group of the hydroxy fatty acid by two subsequent additions of glucose from UDP-glucose. It is also possible that one cellobiose moiety is added in a single step, but this is still not proven. There are genes encoding acetyl- and acyltransferases, and there are genes for the synthesis of the short-chain fatty acid and its β-hydroxylation. There is an additional acetyltransferase in *P. flocculosa*, which does not have a homologue in *U. maydis*. This gene is responsible for the additional acetylation in flocculosin. In *U. maydis*, α-hydroxylation of the fatty acid is the last step of glycolipid synthesis. In *P. flocculosa*, α-hydroxylation is already performed before the other hydroxylations, and the gene responsible for this step is still unknown. Both clusters also contain an ABC transporter (in contrast to the MEL clusters, which contain a major facilitator), which is required for secretion of the glycolipid (Teichmann et al. 2007, 2011; Roelants et al. 2014).

Cellobiose lipids have pronounced antifungal activities and are regarded as alternative fungicides. Many species of the genus *Pseudozyma* have been shown to have antifungal activities (Passoth and Schnürer 2003), and a biocontrol product based on *P. flocculosa* for the treatment of powdery mildew has been commercialised. The involvement of a cellobiose lipid in antifungal activity of *P. flocculosa* has been demonstrated (Cheng et al. 2003).

The field of glycolipids is an excellent example of the huge biotechnological potential of fungal lipid metabolism. It is rapidly evolving towards novel potential applications in cleaning, pharmaceuticals, cosmetics, food and agricultural industry; novel strains with high potential for glycolipid production are isolated, and novel glycolipids are identified and synthesised (Mnif and Ghribi 2016; Van Bogaert et al. 2016).

6.5.2 Lipid Modification of Proteins

Lipid modification of proteins plays an essential role in all kingdoms of life. Adding hydrophobic groups to proteins can influence their folding, function and localisation. Four main classes of lipid modification have been found in fungi, including myristoylation, isoprenylation, S-acylation and glycosylphosphatidylinositol (GPI) modification (Santiago-Tirado and Doering 2016).

N α-myristoylation is the binding of myristate, a saturated C14 fatty acid, to an N-terminal glycine, which has been exposed to the N-terminus by removal of the initial N-terminal methionine. Binding occurs via an amide bond to the α-amino group of the glycine and is irreversible. It is found in a variety of fungal proteins, for instance, at the Rpt2 subunit of the 26S proteasome of *S. cerevisiae*. When the glycine is removed or replaced by another amino acid, protease activity is not influenced, but localisation is disturbed. Corresponding mutants showed phenotypes consistent with reduced degradation of misfolded proteins. In the case of the *S. cerevisiae* proteasome, the probable role of myristoylation is nuclear anchoring (Hirano et al. 2016). Myristoylation is catalysed by N-myristoyltransferase, which is present from yeast to humans, and it has been shown to be essential in a variety of fungi (Santiago-Tirado and Doering 2016).

Isoprenylation (also called prenylation) is the addition of isoprenoids (farnesyl or geranylgeranyl, see below) to a cysteine residue in a C-terminal CAAX consensus sequence, where C stands for cysteine, A for any aliphatic amino acid, and X for any amino acid. The AAX is removed after prenylation. Prenylation is performed by protein prenyltransferases, and the reaction is irreversible. The protein is additionally capped by methylation (Nguyen et al. 2010). Isoprenylation is an essential process, and a variety of proteins have been found to be prenylated. Farnesylated proteins were firstly discovered when studying mating pheromones in the basidiomycetous yeast *R. toruloides* (Kamiya et al. 1978). Prenylated pheromones are common among both basidio- and ascomycetes. The *S. cerevisiae* a-factor is the best-studied prenylated fungal pheromone. Prenylation makes the resulting molecule highly hydrophobic, requiring a specific secretion mechanism, involving an ABC transporter (encoded by *STE6*). Secretion of the a-factor is thus completely different from that of the α-factor, which is hydrophilic and exported using the "classical" protein secretion pathway via endoplasmic reticulum (ER) and Golgi apparatus. Basidiomycetes produce exclusively hydrophobic pheromones, indicating that their mating mechanism is different from that of ascomycetes

(Michaelis and Barrowman 2012). GTP-binding proteins of the Ras family are another example of isoprenylated proteins, indicating that isoprenylation plays an essential role in cell signalling, stress response and signal transduction (Omer and Gibbs 1994).

Protein S-acylation is the binding of a fatty acid residue, in most cases palmitate (palmitoylation), to a cysteine residue of the polypeptide. There is no known recognition sequence for S-acylation, and both soluble and membrane-bound proteins can be palmitoylated. Palmitoylation of soluble proteins can mediate interaction with plasma membranes, sometimes in connection with other lipid modification of the protein. For membrane-bound proteins, it regulates the protein stability and the intracellular trafficking (Santiago-Tirado and Doering 2016).

GPI modification, or GPI anchoring, is the transfer of a glycolipid to a protein at its C-terminus by transamidation. The glycolipid is synthesised at the membrane of the ER and consists of a phosphatidylinositol linked to a linear glycan composed of glucosamine, three mannose residues and a phosphoethanolamine, the latter being required for binding to the protein. Further species-specific modifications with additional ethanolamine, sugars or lipid groups may occur (Muñiz and Zurzolo 2014; Santiago-Tirado and Doering 2016). GPI modification is evolutionarily conserved and essential in all eukaryotes. GPI-anchored proteins are placed on the outer surface of the cell. Those proteins have two signal sequences, one signal sequence at the N-terminus targeting it to the ER and one GPI-attachment site at the C-terminus (designated the ω-site). Both signal sequences are removed during processing (Orlean and Menon 2007). GPI anchoring is irreversible, but under certain circumstances, the protein can be released by the action of a phosphatidylinositol-specific phospholipase. GPI proteins may also be transferred from the membrane to the cell wall, which may be determined by additional sequences surrounding the GPI anchor site (Frieman and Cormack 2004). Pathogenic fungi use GPI-anchored proteins to attach to tissues, and they are involved in resistance to the immune response of the host (Santiago-Tirado and Doering 2016).

As lipid modification is essential for eukaryotic cells, it is regarded as potential target for developing antifungal drugs. Prenylation is essential for the activation of small G proteins, including several oncogenic Ras proteins. Therefore, a variety of prenylation inhibitors, especially farnesyltransferases, have been developed, originally for anticancer treatment. The structure of the farnesyltransferase of the pathogenic dimorphic fungus *Cryptococcus neoformans* has been studied in detail, and, for instance, a substrate-induced conformational change has been observed, which does not occur in human farnesyltransferases and may thus represent a potential therapeutic target (Hast et al. 2011). Palmitoylation is also a potential therapeutic target. Many of the proteins potentially involved in pathogenesis are palmitoylated by the protein *S*-acyltransferase encoded by *PFA4*, including Ras1, which enables high-temperature growth. Pfa4 thus seems to be a promising target for novel anti-*C. neoformans* drugs, especially from the background that the closest human homologue, DHHC6, is to only 21% identical to *C. neoformans* Pfa4 on the amino acid level (Nichols et al. 2015; Santiago-Tirado and Doering 2016). N-myristoylation is another potential target for antifungal drugs. In

C. neoformans, there is one N-myristoyltransferase, and the deletion of its gene is lethal. N-myristoyltransferases from different origins have been shown to be conserved regarding their acyl-CoA substrates; however, they have very specific peptide substrates. Based on this, N-myristoyltransferase inhibitors have been developed, mimicking the N-terminal structure of one of the N-myristoyltransferase targets, the ADP-ribosylation factor Arf1 (Lodge et al. 1998). As mentioned above, the GPI core structure is conserved among eukaryotes, but there are species-specific modifications, which include additional sugars, lipids, phosphoethanolamines or acylation of the inositol ring. Accordingly, specific inhibitors of fungal GPI anchoring have been identified that may have a great potential in antifungal therapy (McLellan et al. 2012; Watanabe et al. 2012; Mann et al. 2015). Another potential application of the GPI-anchoring system is to display enzymes on the surface of fungal cells. *S. cerevisiae* was manipulated to display cellulose- and hemicellulose-degrading enzymes on its surface by connecting those enzymes to a GPI anchor. Such so-called armed strains were able to directly produce ethanol from lignocellulose (Hasunuma and Kondo 2012).

6.6 Microbial Biodiesel Production by Yeasts

6.6.1 Lipid Production from Lignocellulose and Crude Glycerol

Biodiesel is currently the second most abundant biofuel in the world, after ethanol. It is produced from oil plants, such as soy, oil palms or rapeseed. For generating biodiesel, the triglycerides of the vegetable oils are in a transesterification reaction esterified with methanol, replacing the glycerol and generating fatty acid methyl esters (FAME). Transesterification with ethanol instead of methanol is also possible but is currently less applied. Glycerol is thus a side product of the biodiesel process. Biodiesel utilisation has increased in Europe during the last years, and there is potential for further growth especially in Europe, because of the expanding use of diesel engines for transportation fleets (Gnansounou 2010). However, oil plants are grown on arable land, and their energy yield per hectare is relatively low. Thus, a substantial area of arable land is required for the replacement of large proportions of mineral oil-derived diesel. Moreover, oil plants are grown on areas of cleared rain forest, and deforestation to enable cultivation of oil plants has been reported (Azócar et al. 2010; Graham-Rowe 2011).

Oils from oleaginous fungi, especially yeasts, may represent an alternative to biodiesel from oil plants. Most oleaginous yeasts can convert a variety of substrates to lipids, including sugars and acids from lignocellulose hydrolysates and glycerol (Sitepu et al. 2014). On glucose, fairly high lipid concentrations have been reached. In high cell-density cultures of *R. toruloides* on glucose, a final lipid content of 72 g/l was obtained (Li et al. 2007). Metabolically engineered *R. toruloides* reached 89 g/l (Zhang et al. 2016b), and in engineered *Y. lipolytica*, even 99 g/l was reached (Qiao et al. 2017). These values are close to theoretical limits, which, according to the National Renewable Energy Laboratory (NREL) have to be reached for

obtaining economically viable biodiesel production by oleaginous yeasts (http://www.nrel.gov/docs/fy15osti/62498.pdf). However, sustainable biodiesel production should be performed preferably using residual substrates such as lignocellulose or crude glycerol from biodiesel production.

The C/N ratio of lignocellulose is high (Hyvönen et al. 2000; Reinertsen et al. 1984), which triggers lipid accumulation in fungi (see above). However, lignocellulose hydrolysate contains inhibitors that are toxic for microbes cultivated in it (Jönsson et al. 2013). Hydroxymethylfurfural (HMF) had a slight impact on oleaginous yeasts; it influenced lipid accumulation only at higher concentrations and only in some strains. Nevertheless, a prolonged lag phase upon exposure to HMF has been observed in several strains. Furfural and vanillin showed a strong inhibition of oleaginous yeasts (Chen et al. 2009; Hu et al. 2009). Acetic acid at a concentration of 5 g/l completely inhibited growth of strains belonging to *L. starkeyi*, *R. glutinis* and *R. toruloides*. *Trichosporon cutaneum* showed some growth at this concentration (Chen et al. 2009). However, the effect of acetic acid and other weak organic acids depends on the pH of the medium. Different degrees of inhibition have been observed in cultures of *L. starkeyi* in hemicellulose hydrolysate at pH 5 and 6 (Brandenburg et al. 2016).

Inhibitors in lignocellulose may be one important reason why fermentations of lignocellulose hydrolysates in the best case reach only values slightly above 10 g/l. On sugar cane bagasse, 15.7 g/l lipids were obtained using *Trichosporon fermentans* (Huang et al. 2012), on rice straw 11.5 g/l (Huang et al. 2009). On corn cob hydrolysate, 12.3 g/l was achieved by *Trichosporon cutaneum* (Gao et al. 2014). Recently, the ability of a variety of yeast strains to convert corn stover hydrolysate [hydrolysed by ammonia fibre expansion (AFEX)] and switchgrass hydrolysate (hydrolysed by dilute acid hydrolysis) was tested. Switchgrass hydrolysate was more inhibitory, and only a few of the tested strains could grow on it. Three strains, belonging to *Lipomyces kononenkoae*, *Lipomyces tetrasporus* and *Rhodosporidium toruloides*, were able to convert the acid hydrolysate to lipids (at 15–20% solid load at pH 6–7 and some amendments with nitrogen) yielding final lipid concentrations of 25–30 g/l. Similar values were reached by these strains as well as a *Saitoella coloradoensis* strain on corn stover hydrolysate. The achieved yields were 0.13–0.15 g lipids per consumed carbon source, and the production rates 0.12–0.22 g per litre and hour (Slininger et al. 2016).

These values are still far below those identified as minimum to obtain competitive fuel production (final lipid concentration 90 g/l, yield 0.28 g lipid/g carbon source, production rate 1.3 g/l h, http://www.nrel.gov/docs/fy15osti/62498.pdf). System analysis calculated a minimum selling price of $ 9.55 per gasoline gallon equivalent (about $ 2.50 per litre gasoline equivalent), when only producing fuel. In this scenario, a lipid yield of 0.24 g/g available sugar was assumed, a lipid productivity of 0.34 g/l h and a cellular lipid content of 60%. Conversion of the pentose fraction of the lignocellulose to succinate had a great impact on the total costs and could decrease the minimum selling price to $ 5.28 per gasoline gallon equivalent (about $ 1.40 per litre gasoline equivalent). Increasing lipid yield, decreasing extraction costs and obtaining higher lipid contents of the biomass

were some critical factors to improve the process (Biddy et al. 2016). On the other hand, conversion of the hexose fraction to ethanol is an established process; thus, lipid production from only the hemicellulose fraction may be another strategy to make biofuel production more efficient. Acid pretreatment generates a liquid and a solid fraction, and the hemicellulose is almost completely present in the liquid fraction. Thus, it can be separated relatively easily from cellulose/lignin. Since the liquid fraction also contains most of the inhibitors released during pretreatment, its conversion to lipids can be complicated. This problem might be circumvented by establishing fed-batch cultivation. Recently, the hemicellulose fraction of birch wood hydrolysate was converted to lipids using *L. starkeyi*. The yeast co-consumed acetic acid and xylose, enabling establishment of a pH-regulated fed-batch fermentation. A final concentration of 8 g/l and a yield of 0.1 g/g carbon source were obtained, which are currently the highest values achieved with the hemicellulose fraction of lignocellulose (Brandenburg et al. 2016). If one takes into account biogas production from the residues, i.e. connects biolipid production to another biofuel production process, lipid yield and proportion of the yeast biomass become less critical for the total energy efficiency of the process. However, since an aerobic fermentation process requires more energy than, for instance, the more or less anaerobic production of ethanol, rapid fermentation of the sugars is one of the crucial issues to improve the efficiency of microbial lipid production (Karlsson et al. 2016). Isolation of rapidly lipid-accumulating strains is, therefore, one of the important tasks of current research on oleaginous organisms. Another approach may be represented by remodelling the metabolism towards producing more NADPH at the cost of NADH, which, apart from providing redox equivalents for fatty acid synthesis, decreases the demands for oxygen in respiration (Qiao et al. 2017).

Utilising cheap, locally available substrates can also reduce the price of biodiesel. In a recent study, sugar cane juice was tested as substrate for *R. toruloides*-based biodiesel production in Brazil. Final production costs were estimated to about $ 0.50 per litre biodiesel. Interestingly, the amount of biodiesel produced per hectare when converting sugar cane juice using the oleaginous yeast was more than six times higher than the biodiesel production per hectare obtained from soybean oil (Soccol et al. 2016).

Another approach for improving the energy efficiency of biodiesel production would be utilisation of the crude glycerol which is produced during transesterification (Karlsson et al. 2016). Although pure glycerol is a high-value compound, used, for instance, in production of foods, beverages, pharmaceutics or cosmetics, crude glycerol is highly problematic: it contains salt, methanol and other toxic compounds, which prevent any direct use of the crude glycerol as platform chemical and are inhibitory to any fermentation process. Crude glycerol is produced at about 10% (w/w) of the generated biodiesel, and the increase in biodiesel volumes has saturated the marked for crude glycerol, making expensive cleaning procedures for this side product economically unfavourable (Haas et al. 2006; Johnson and Taconi 2007). Consequently, a variety of attempts have been made to utilise crude glycerol as carbon source for biolipid production. Cultivation of

Cryptococcus curvatus was optimised in terms of temperature, pH and crude glycerol concentration. Based on these parameters, fed-batch cultivation was designed, and a final lipid concentration of 22 g/l was obtained after 12 days of cultivation (Cui et al. 2012). In another attempt, glycerol from recycled restaurant oil (yellow grease) was tested as substrate for *C. curvatus*, and a final lipid concentration of 17.1 g/l was obtained after 12 days of fed-batch cultivation (Liang et al. 2010). Uçkun Kiran et al. tested crude glycerol and rapeseed meal, another problematic residue from biodiesel production, as nitrogen source. The rapeseed meal was pre-fermented with the proteolytic fungus *Aspergillus oryzae*, to make the nitrogen available for the oleaginous yeast in the subsequent fermentation. Lipid production was tested with *R. toruloides* Y4, and after 120 h of fed-batch cultivation, a maximum lipid concentration of 13 g/l was obtained, corresponding to a lipid yield of 0.19 g/g glycerol. When yeast extract was used as nitrogen source, final lipid concentration was only 9.4 g/l. This may indicate that rapeseed meal is a better nitrogen source, or that it provides some additional carbon source. Interestingly, higher final lipid contents and yields were obtained with 50 g/l glycerol compared to 100 g/l glycerol, potentially due to some inhibitory effects of the glycerol on the yeast (Uçkun Kiran et al. 2013). Another study showed that thin stillage has superior characteristics as nitrogen source compared to yeast extract. Lipid production by *R. glutinis* resulted in 5.4 g lipids per litre, compared to 2.3 g/l on yeast extract (Yen et al. 2012). Tchakouteu et al. tested a variety of strains belonging to *C. curvatus*, *Y. lipolytica*, *R. toruloides* and *L. starkeyi*. They found both *R. toruloides* and *L. starkeyi* to be best for lipid production, generating more than 12 g/l lipids in shake flask cultures. This was reached after 395 and 470 h, respectively, at an initial glycerol concentration of 120 g/l. The lipid yields were about 0.1 g/g consumed glycerol. At a lipid concentration of 50 g/l, *R. toruloides* reached a final lipid concentration of 7.9 g/l after 195 h and a lipid yield of 0.17 g/g. *L. starkeyi* did not have an increased lipid yield at lower initial glycerol concentrations (Tchakouteu et al. 2015). The potential of *R. glutinis* to co-produce lipids and carotenoids on crude glycerol was investigated. In an optimised fermentation (optimised in terms of C/N ratio, initial glycerol concentration and Tween 20 addition), maximum concentrations of 6 g lipid per litre and 135 mg carotenes per litre (yields of 0.033 g/g and 0.7 mg/g, respectively) were obtained after a fermentation time of 72 h (Saenge et al. 2011). Recently, crude glycerol conversion to lipids was tested using *R. toruloides* ATCC 10788. In these experiments, a lipid yield of 0.44 g/g consumed glycerol was obtained. This is beyond the theoretical maximum, which was explained by the consumption of additional carbon sources from the crude glycerol, such as fatty acid methyl esters, soap, diglycerides, monoglycerides and residual triglycerides (Uprety et al. 2017). The results obtained so far indicate that a variety of strains are able to metabolise crude glycerol from biodiesel production. However, huge differences with regard to growth rate, lipid production rate and yield indicate that not all factors of lipid accumulation from crude glycerol are well understood. Yields and production rates are far from those that would enable commercially viable lipid production; thus, there is an urgent demand for further research in this field.

6.6.2 Manipulating Yeasts for Improving Lipid Production

Until recently, for most of the above-mentioned oleaginous yeast species, methods for genetic engineering were unavailable or only poorly developed, with the exception of *Y. lipolytica* (Beopoulos et al. 2009). *Y. lipolytica* can convert a variety of carbon sources to lipids, including glucose and glycerol, but not xylose (or only to a small extent by certain strains), the major sugar of hemicellulose. However, recent efforts have been directed to generating xylose-assimilating strains. By complementing the corresponding *Escherichia coli* mutants, Rodriguez et al. (2016) identified a cryptic xylose assimilation pathway in *Y. lipolytica*. Obviously, the genes for xylose assimilation are present in the yeast but expressed only at a very low level. This obstacle could be overcome by expressing the genes from a strong promoter. In another attempt, xylose reductase and xylitol dehydrogenase from the xylose-fermenting yeast *S. stipitis* and the native xylulokinase were overexpressed in *Y. lipolytica*. Resulting strains were able to grow and produce lipids on xylose; in a bioreactor fed-batch cultivation, a final lipid concentration of 20.1 g/l was reached, with a lipid yield of 0.08 g/g and a production rate of 0.19 g/l h. On mixed carbon source (xylose and glycerol), a final lipid concentration of 50.5 g/l was achieved, the lipid yield was 0.12 g per gram consumed carbon source and the production rate is 0.23 g/l h. Apart from lipids, citric acid and some xylitol were also produced (Ledesma-Amaro et al. 2016b). A variety of genetic engineering efforts to improve the growth on further carbon sources such as glycerol, disaccharides, starch or inulin, and their conversion to lipids and other valuable compounds by *Y. lipolytica* have been performed (summarised in Ledesma-Amaro and Nicaud 2016).

Several attempts have also been made to increase the central lipid metabolism of *Y. lipolytica*. Inactivation of *GUT2*, which encodes for a glycerol-3-P dehydrogenase converting glycerol-3-P to dihydroxyacetone phosphate, and peroxisomal genes involved in β-oxidation of fatty acids, significantly increases lipid production by *Y. lipolytica* (Beopoulos et al. 2008). Recently, further improvements have been achieved. Overexpression of *DGA1*, encoding diacylglycerol acyltransferase, and *ACC1*, encoding acetyl-CoA carboxylase, resulted in a lipid content of 62% of biomass dry weight, a lipid yield of 0.27 and a production rate of 0.253 g/l h during the lipid production phase. Overexpression was achieved by using an intron-containing TEF promoter, which ensured a 17-fold overexpression compared to the intron-less promoter (Tai and Stephanopoulos 2013). Comparison of gene expression in lipid-accumulating human tissues resulted in the identification of Δ9 stearoyl-CoA desaturase (SCD) as a potential target for manipulation towards higher lipid concentrations. Indeed, overexpressing this protein in *Y. lipolytica* (in a background overexpressing *ACC1* and *DGA1*) resulted in a final lipid concentration of 55 g/l and a lipid yield of 0.266 g/g glucose. Sugar uptake rate was enhanced in the transformant strain. The metabolic background of this effect was probably the removal of the saturated fatty acids, palmitic and stearic acid, which can act as inhibitors of ACS (Qiao et al. 2015). A similar effect was discovered in a mutant with a modified regulator protein *mga2-G643R*, in which the glycine at the amino

acid residue 643 is exchanged by an arginine. The resulting mutant had an increased production of unsaturated fatty acid on the expense of the corresponding saturated fatty acids. *MGA2* is a transcriptional activator of the Δ9 desaturase, and it was demonstrated that Δ9 desaturase was upregulated in a strain expressing the *mga2-G643R* variant. Moreover, also glycolytic genes were upregulated but not those of the citrate cycle. This was probably generating an imbalance between glycolysis and citric acid cycle, resulting in the higher production of precursors for lipid production. When expression of the *mga2-G643R* variant was combined with the overexpression of *DGA1*, a final lipid titre of 25 g/l, a productivity of 0.145 g/l h, a yield of 0.213 g lipids per gram glucose and an intracellular lipid content of about 85% could be reached (Liu et al. 2015a). In another attempt, *Y. lipolytica*, apart from overexpressing *DGA1* and *ACC1*, was engineered to generate NADPH instead of NADH during glycolysis by introducing a $NADP^+$-dependent glyceraldehyde phosphate dehydrogenase (Qiao et al. 2017). NADH kinase, generating NADPH out of NADH, was also overexpressed. The resulting strain had a similar yield as the strain constructed by Tai and Stephanopoulos (2013). However, its production rate (1.2 g/l h) was substantially higher. The final lipid concentration in a bioreactor was 99 g/l, which comes close to the values that are postulated to be required for commercial production (Qiao et al. 2017).

The red yeast *R. toruloides* has been identified as a potential production organism for biodiesel, as it can grow on a broader range of carbon sources than *Y. lipolytica*, and because it can accumulate much more lipid than the wild type of *Y. lipolytica*. Until recently, very limited molecular tools for genetic engineering of this yeast were available. However, genetic manipulation is now possible based on an *Agrobacterium tumefaciens* system. Overexpressing *DGA1* and *ACC1* enabled significantly increased lipid production. Interestingly, overexpression of *DGA1* had a relative reproducible effect of increased lipid formation, whereas *ACC1* expression in some transformants caused a significant increase in lipid production, while others showed no difference to the wild type. Nevertheless, highest lipid production was demonstrated in a strain overexpressing both genes, i.e. on glucose and xylose, respectively; final concentration was 16.4 g/l and 9.5 g/l, cellular lipid contents 61.1% and 43.4%, lipid production rates 0.28 g/l and 0.11 g/l and lipid yields 0.23 g/g and 0.14 g/g (Zhang et al. 2016c). Further attempts overexpressed a variety of enzymes, malic enzyme, pyruvate carboxylase, glycerol-3-P dehydrogenase and stearoyl-CoA desaturase (SCD). Additionally, genes involved in β-oxidation were inactivated. However, only overexpression of stearoyl-CoA desaturase and malic enzyme had an effect on lipid production. In a background where *DGA1* and *ACC1* were overexpressed, only overexpression of SCD enabled further increased lipid production. The final lipid concentration reached by this strain was 89.4 g/l (cellular lipid content 75.6%), a productivity of 0.62 g/l h and a yield of 0.22 g/g (Zhang et al. 2016b).

Procedures for manipulating oleaginous yeasts are currently rapidly developing. Recently, novel strong promoters have been identified in *R. toruloides*. These promoters contain introns, and obviously these introns can substantially increase gene expression. These are valuable tools for gene overexpression in *R. toruloides*

and other red yeasts (Liu et al. 2016). For *Y. lipolytica*, a CRISPR-Cas9-mediated system for markerless targeted gene integration has been developed, enabling the stable integration of several genes into the genome (Schwartz et al. 2016). Genetic manipulation of *L. starkeyi* seems to be difficult; however, a transformation system has been established (Calvey et al. 2014).

Methods for manipulating oleaginous yeasts other than *Y. lipolytica* became available only recently. Therefore, much effort has gone into generating a lipid-producing *S. cerevisiae*. *S. cerevisiae* is a non-oleaginous yeast; but it is an established industrial organism that is stress tolerant and can perform well in industrial fermentations. Molecular manipulation is very well developed in this yeast, so it may have potential for production of biodiesel and other lipid-derived chemicals. In *S. cerevisiae*, the precursor of lipid synthesis, acetyl-CoA, cannot be transferred from the mitochondria to the cytoplasm via the citrate-oxaloacetate shuttle (Beopoulos et al. 2011); it is produced in the cytoplasm by the reactions of pyruvate decarboxylase (PDC), aldehyde dehydrogenase (AlDH) and acetyl-CoA synthase (Holzer and Goedde 1957). In Pdc^- mutants, C2-compounds such as ethanol or acetate have to be provided to enable growth on glucose (Pronk et al. 1996). *S. cerevisiae* is a fermentative yeast, which means that its metabolism is directed towards converting the final products of glycolysis to ethanol rather than to acetyl-CoA. Redirecting the metabolic flux from ethanol towards acetyl-CoA production is thus one of the major challenges when manipulating *S. cerevisiae* for high lipid production (Buijs et al. 2013; Lian and Zhao 2015). When *ALD6*, encoding aldehyde dehydrogenase, and a modified ACS from *Salmonella enterica* were expressed, the level of acetyl-CoA was increased (Shiba et al. 2007). Additional overexpression of *ADH2*, encoding the assimilatory alcohol dehydrogenase of *S. cerevisiae*, also increased the availability of acetyl-CoA (Chen et al. 2013). By overexpressing *FAS1* and *FAS2*, *ACC1* and *DGA1*, encoding FAS, acetyl-CoA carboxylase and DAG, a strain was obtained that could accumulate up to 17% lipids per dry matter (Runguphan and Keasling 2014). By further manipulations, including expression of ACL, disruption of isocitrate dehydrogenase genes, overexpression of a fatty acyl-CoA synthetase, overexpression of a modified *DGA1* and disruption of the transcription factor *SNF2* (which is involved in activation of glucose-repressed genes), *S. cerevisiae* could be converted to an oleaginous yeast, accumulating up to 45% lipids per cell dry matter (Kamisaka et al. 2006, 2013; Tang et al. 2013; Zhou et al. 2014).

6.7 Production of Specific Chemicals and Fuels Derived from Lipid Metabolism

Apart from producing triglycerides, and thus a biodiesel replacement, generation of advanced lipid-derived chemicals and biofuels has been investigated. These compounds include free fatty acids, fatty alcohols, fatty acid ethyl esters and alkanes (Lian and Zhao 2015). Another important class of compounds are terpenes, which have potential as high-value chemicals and biofuels (Ye et al. 2016).

6.7.1 Fatty Alcohols

About two million tons of fatty alcohols are currently produced worldwide, and their production is expected to grow. The major demand is for short-chain fatty acids (C11–C14), which are key intermediates for surfactants present in detergents, lubricants, cosmetics, personal care products and pharmaceuticals. There is also a growing demand for longer-chain fatty alcohols (C16–C22), which are used as lubricant bases, emollients and thickeners in cosmetics and foods. Moreover, they are used as polymerisation agents in plastics. Unsaturated fatty alcohols can also be used for the mentioned purposes, and they have some advantages compared to saturated fatty alcohols; since they have a lower melting point, a higher solubility in water and functional groups can be introduced (Fillet and Adrio 2016). Several approaches have been used to enable and increase the fatty alcohol production in yeasts. In all studies, a fatty acid reductase was expressed in the yeasts. When manipulating *S. cerevisiae*, enzymes improving lipid production were also overexpressed, for instance, Runguphan and Keasling (2014) overexpressed *ACC1*, *FAS1* and *FAS2* from *S. cerevisiae*, a thioesterase (tesA) from *E. coli*, which releases the fatty acid from CoA and a ME from *M. alpina*. At the same time, genes involved in β-oxidation were disrupted. The resulting strain produced 98 mg fatty alcohols per litre. In another study with *S. cerevisiae*, apart from overexpressing a fatty acid reductase from barn owl (*Tyto alba*), *ACC1* (from *S. cerevisiae*) and ACL (from *Y. lipolytica*) were overexpressed, and *RPD3*, a negative regulator of the *INO1* gene involved in phospholipid synthesis, was disrupted. The resulting strain was able to produce 1.1 g/l 1-hexadecanol (Feng et al. 2015). In the same background, a xylose pathway (XR from *Scheffersomyces shehatae*, XDH from *Candida tropicalis* and XKS from *Komagataella pastoris*) was expressed. Promoter engineering and evolving the strain by serial cultivation on xylose as sole carbon source resulted in 1.2 g/l 1-hexadecanol from xylose (Guo et al. 2016). In a strain engineered for the overproduction of free fatty acids (see below), further manipulation also enabled fatty alcohol production. Overexpressing a fusion protein of carboxylic acid reductase (CAR) from *Mycobacterium marinum* and yeast *ADH5*, and another copy of the CAR gene, deletion of *HFD1*, encoding long-chain fatty aldehyde dehydrogenase and deletion of *ADH6*, resulted in increased titres of fatty alcohols. In a fed-batch culture, 1.5 g/l fatty alcohol was produced. This is in the order of magnitude of what is attained using *E. coli*; however, the yield in yeast is still lower (Zhou et al. 2014).

Oleaginous yeasts have also been manipulated to produce fatty alcohols. For *Y. lipolytica*, for a long time, only patents were available, describing fatty alcohol production up to 0.8 g/l (Fillet and Adrio 2016). Recently, *Y. lipolytica* was manipulated to produce fatty alcohol by expressing a fatty aldehyde reductase from the bacterium *Marinobacter aquaeolei* (encoded by *Maqu_2220*). The resulting strain produced 167 mg fatty alcohols per litre. The same gene was also expressed in *L. starkeyi*, resulting in 770 mg/l fatty alcohol. Overlaying the culture with dodecane resulted in extraction of a major part of the formed fatty alcohols into the dodecane overlay (Wang et al. 2016). In another study, *Maqu_2220* was

co-expressed with an *E. coli* fatty acyl-CoA synthetase in *Y. lipolytica*, and the resulting strain produced 2.15 g/l fatty alcohols in a bioreactor fermentation (Xu et al. 2016). *Maqu_2220* has been expressed before in *R. toruloides*, resulting in the best known fatty alcohol production by yeasts observed so far. The resulting strain was able to produce fatty alcohols from glucose, fructose, sucrose and xylose, as well as from a medium with mixed carbon sources and inhibitors (glucose, xylose, acetic acid, furfural and formic acid). In fed-batch cultivation with sucrose and corn steep liquor, 8 g/l was obtained. Interestingly, the major proportion of the fatty alcohols was excreted into the medium, making it easy to extract the fatty alcohols (Fillet et al. 2015).

Fatty acid ethyl esters (FAEE) can be directly used as biodiesel. For producing biodiesel, triglycerides from plant oil are transesterified to usually fatty acid methyl or ethyl esters (FAME or FAEE), which are then used as biodiesel. Direct production of these esters by the production organism would therefore be advantageous. Moreover, FAEE are naturally excreted from yeast cells, and thus no lipid extraction would be required. In a variety of attempts for manipulating *S. cerevisiae* to produce FAEE, most of them were based on episomal plasmids, resulting in unstable production patterns (summarised in Lian and Zhao 2015). Based on multicopy integration of a heterologous wax ester synthase, 34 mg/l could be produced. Loss of the FAEE phenotype in non-selective conditions could be prevented by deleting *RAD52*, which is crucial for homologous recombination. Overexpressing acetyl-CoA-binding protein, which is important for the transport of acyl-CoA esters and the reduction of feedback inhibition of acyl-CoA on FAS, ACC and long-chain acyl-CoA synthetase, and a $NADP^+$-dependent glyceraldehyde-3-phosphate dehydrogenase increased FAEE production to 48 mg/l (Shi et al. 2014). The highest concentrations obtained to date with *S. cerevisiae* have been generated on glycerol and oleic acid as carbon sources. The glycerol assimilation pathway and a glycerol transporter were overexpressed. Moreover, genes for dihydroxyacetone phosphate degradation and glycerol export were deleted. This forced ethanol production from glycerol. Overexpressing a bacterial acyltransferase (from *Acinetobacter baylyi*) resulted in the condensation of the formed ethanol and the externally added oleic acid, with a final ethyl oleate concentration of 0.52 g/l (Yu et al. 2012). *Y. lipolytica* was also engineered to produce FAEE, and the results demonstrated that targeting the enzymes to the respective compartments greatly influences the final product formation. When the *Acinetobacter baylyi* wax ester synthase was expressed in the cytoplasm, only 7.1 mg/l FAEEs was synthesised. However, FAEE production was significantly increased, when the gene was targeted to ER or peroxisome, achieving FAEE concentrations of 136.5 and 110.9 mg/l, respectively (Xu et al. 2016).

6.7.2 Free Fatty Acids

Free fatty acids are another example of advanced chemicals that can be derived from lipid metabolism. Similar to fatty alcohols, they have a broad potential to be

converted to chemicals that can replace oleochemicals derived from fossil resources, including high-energy fuels such as diesel or jet fuels. They can also be used for the production of cleaning agents, agrochemicals, biocidal agents, agents for textile processing or polymer additives (Lennen and Pfleger 2013). It has been discovered that after inactivation of acyl-CoA synthetases and *FAA1* and *FAA4*, yeast cells start to secrete free fatty acids into the growth medium (Scharnewski et al. 2008). Acyl-CoA synthetases (ACS) are enzymes that generate acyl-CoA from free fatty acids, which is essential to further metabolise them. In *S. cerevisiae*, there are four long-chain ACS, encoded by *FAA1-4* (Black and DiRusso 2007). Very recently, a number of studies have been performed to engineer *S. cerevisiae* for overproduction of free fatty acids. Simultaneous disruption of *FAA1* and *FAA4*, the genes of the major acyl-CoA synthases, together with overexpression of an acyl-ACP thioesterase from *E. coli* and a general enhancement of fatty acid synthesis by overexpressing FAS and ACC increased free fatty acid production up to 400 mg/l (Runguphan and Keasling 2014). Recently, ^{13}C flux analysis of lipid production in this constructed strain revealed further limitations. ACL overexpression alone did not result in a large improvement in lipid production. This was due to the action of malate synthase, which diverted acetyl-CoA to malate synthesis. Knocking out the respective gene (*MLS1*) resulted in a very slowly growing mutant. In contrast, when the gene was only downregulated (under control of a mutated version of the *TEF1* promoter), the cells grew well, and lipid production was significantly enhanced. Further improvement of lipid production could be reached by inactivating *GPD1*, encoding glycerol-3-P dehydrogenase, which catalyses the conversion of dihydroxyacetone phosphate to glycerol-3-P. This reaction is required for triglyceride synthesis but competes with the fatty acid synthesis pathway for acetyl-CoA. Knocking out this gene also increased the flux towards fatty acid production. Together, these manipulations based on flux analysis increased the secretion of free fatty acids from the original amount to 780 mg/l (Ghosh et al. 2016). In another approach, the ACS genes *FAA1* and *FAA2* were inactivated. Moreover, genes involved in β-oxidation, *FAA2*, *PXA2* and *POX1*, were disrupted. Faa2 is the ACS in the peroxisomes, Pxa1 is the fatty acid transporter into the peroxisome, and Pox1 degrades fatty acids inside the peroxisome. The resulting strain produced 1.3 g/l of fatty acids. In this mutant strain, *DGA1* and *TGL3* were overexpressed. *TGL3* encodes the major triglyceride lipase. The resulting strain produced 2.2 g free fatty acids per litre (Leber et al. 2015). Recently, further improvement of free acid production has been reported. A strain with deleted long-chain fatty aldehyde dehydrogenase (*HFD1*) was further engineered. Additional genes involved in fatty acid degradation were disrupted, including *FAA1* and *-4* and *POX1*. A truncated *E. coli* thioesterase (tesA) was expressed, to release free fatty acids from CoA. To increase the amount of acetyl-CoA, a chimeric pathway was expressed, consisting of *Mus musculus* ACL, *R. toruloides* ME, endogenous malate dehydrogenase with removed peroxisomal signal and a citrate transporter. All overexpressed genes were integrated into the genome. The resulting strain produced 7 g/l free fatty acids in a shake flask fed-batch culture and 10.4 g/l in a glucose-limited fed-batch culture in a bioreactor

(Zhou et al. 2016). To identify additional genes that can boost lipid production, a high-throughput method based on the fluorescent dye Nile red for the detection of intracellular lipids has been developed. A *S. cerevisiae* strain, defective in storage lipid production and β-oxidation, was transformed with a cDNA library of *Y. lipolytica*, and high lipid producers were identified using the Nile red-based method. By this method, a variety of genes were discovered, including a putative glycosylphosphatidylinositol (GPI)-anchored protein, malate dehydrogenase, glyceraldehyde-3-P dehydrogenase, fatty acid hydroxylase, farnesyltransferase, anoctamin, dihydrolipoamide dehydrogenase and phosphatidylethanolamine-binding protein. The highest increase in lipid production was found by expressing the GPI protein. GPI proteins have several functions in the plasma membrane; it is not clear in which way they influence the lipid synthesis. Further research is required, and the high-throughput screening methodology may reveal novel pathways for increasing lipid production by yeasts (Shi et al. 2016).

Recently, the oleaginous yeast *Y. lipolytica* was also manipulated to produce free fatty acids. Genes involved in triglyceride and sterol ester synthesis (*DGA1*, *DGA2*, *ARE1* and *LRO1*) and in β-oxidation (*MFE1*) were deleted. Overexpression of several thioesterases increased free fatty acid excretion. Best results were obtained with a codon-optimised thioesterase (*TEII*) from *Rattus norvegicus*. When the cells were cultivated in the presence of 10% decane or dodecane, the excreted fatty acids accumulated in the alkane phase and were thus removed from the cells, preventing toxic effects of the free fatty acids on the cells. The yeast easily tolerated the alkanes, probably because *Y. lipolytica* is a yeast able to assimilate alkanes. In this study, however, alkanes were not assimilated, possibly because the high sugar concentration in the medium repressed alkane assimilation. In optimised bioreactor cultivation, the engineered strain produced 4.3 g/l free fatty acids. When 15% dodecane was added to the fermenter, the resulting strain produced 10.4 g/l. Most remarkably, the amount of secreted lipids was higher than the dry cell weight in the fermenter (120%). Thus, by enabling excretion of the free fatty acids, the maximal limit of lipid production being 100% of the biomass was exceeded. The fatty acid yield was 0.2 g per gram glucose (Ledesma-Amaro et al. 2016a). In another approach, the oleaginous yeast *C. curvatus* was grown on glucose, and the final biomass was hydrolysed via a hydrothermal treatment. Collected triglycerides were almost completely hydrolysed by the treatment. For this study, final concentrations and yields were not presented, but according to my own calculations of the final biomass and recovery ratios, the final amount of free fatty acids was about 3–4 g/l (Espinosa-Gonzalez et al. 2014).

The majority of the above-mentioned fatty acids have a chain length of C16–C18. From an industry point of view, especially for the production of biofuels, shorter chains of C6–C10 would be more attractive as they can serve as precursors for gasoline and jet fuel production (Peralta-Yahya et al. 2012). However, yeasts naturally mainly produce fatty acids with a chain length of C14–C18, and there is a certain cytotoxic effect of short-chain fatty acids, making their microbial production quite challenging (Jarboe et al. 2013). Production of short-chain fatty acids may be achieved, when a short-chain thioesterase (TE) removes the growing fatty

acid from the FAS. Fungal FAS has a scaffold structure, making it kinetically efficient but rather inflexible for interactions with other enzymes. Thus, TE activity, which removes the growing fatty acids already in a premature stage of synthesis, is difficult to introduce into fungal FAS. In contrast, animal FAS are quite flexible and are less scaffolded (Leibundgut et al. 2008). Therefore, to achieve short-chain fatty acid production, a *Homo sapiens* fatty acid synthase (hFAS), from which the native TE subunit was removed, was expressed. hFAS lacks a 4′-phosphopantetheinyl transferase (PPT), which is required to activate the acyl carrier protein (ACP). Therefore, heterologous PPT, either from *E. coli* (AcpS) or *Bacillus subtilis* (Sfp), were expressed in the yeast. When rat *TEII* was expressed together with the mutated hFAS and the bacterial PPTs, short-chain fatty acids were produced. Best production was obtained in a strain expressing Sfp, where in total 20 mg/l short-chain fatty acid were produced, 17 mg/l of them being C8 (Leber and Da Silva 2014). In this background, further optimisations were investigated. Inactivating β-oxidation resulted in increased short-chain fatty acid production. However, specific inactivation of short-chain fatty acid β-oxidation increased short-chain free fatty acid production, compared to the strain with complete inactivation of β-oxidation. Specific inactivation of short-chain fatty acid β-oxidation was achieved by disrupting *FAA2*, *ANT1* and *PEX11* (Leber et al. 2016). These genes encode peroxisomal ACS (*FAA2*); adenosine nucleotide transporter (*ANT1*), which exchange peroxisomal AMP with cytoplasmic ATP; and the peroxisomal membrane protein Pex11 (*PEX11*), which is suggested to be involved in the transport of medium-chain free fatty acids (i.e. fatty acids up to a chain length of C12) into the peroxisome. All three genes have been shown to be involved in the degradation of medium-chain free fatty acids but not long-chain free fatty acids (Hiltunen et al. 2003). Apart from inactivating these genes, the hFAS gene was partially codon optimised (the first 834 bp from the N-terminus; surprisingly, codon optimising the whole protein rather decreased the amount of short-chain free fatty acids). Moreover, proteinase B, encoded by *PRB1*, was inactivated, with the aim to improve the synthase stability. The resulting final strain produced 119 mg/l short-chain free fatty acids (Leber et al. 2016).

6.7.3 Hydrocarbons

Production of hydrocarbons such as alkanes and alkenes would be especially attractive, as they are the major components of fossil gasoline and diesel, and could thus be used as drop-in fuels. Apart from plants, several microorganisms, including fungi, can naturally produce hydrocarbons. The ability of some yeast species to produce long-chain (C_{10}–C_{31}) and volatile (C_2–C_5) *n*-alkanes was already discovered during the 1960s. Among those are several *Saccharomyces* species, including *S. oviformis* and *S. ludwigii*, which accumulated long-chain *n*-alkanes up to 10.2% of their cell dry weight; *Candida tropicalis* (up to 0.031%), *R. glutinis*, *Brettanomyces bruxellensis*, *Saccharomyces octosporus* and *Schizosaccharomyces pombe*, which produced ethane in the range from 21.5 to

11.4 ml/l and h; and *Rhodotorula minuta* var. *texensis*, which produced 16.4 ml isobutene per litre and hour (Ladygina et al. 2006; Fu et al. 2015). These findings have not received much attention, probably because of the rather low amounts of hydrocarbons produced. However, recently an isolate from a mangrove ecosystem, identified as *Aureobasidium pullulans* var. *melanogenum*, produced 32.5 g/l heavy oil when grown on glucose/corn steep liquor. About 60% of this oil consisted of alkanes—mainly tetradecane (C14), tetracosane (C24), hexacosane (C26), heptacosane (C27), octacosane (C28) and tetratetracontane (C44) (Liu et al. 2014). Not much is known about the metabolic pathways for natural n-alkane production in yeasts (Fu et al. 2015). Head-to-head condensation of fatty acids has been assumed, where two fatty acids are condensed at their carboxylic groups. Another possibility is a process similar to that in *Arabidopsis thaliana*, where alkanes are produced from very long-chain acyl-CoA (VLC-acyl-CoA), which are reduced to aldehydes by VLC-acyl-CoA reductase and then converted to alkanes by VLC-aldehyde decarbonylase (summarised in Passoth 2017). Interestingly, genes similar to the plant genes encoding VLC-aldehyde decarbonylase have been found in the genome of *A. pullulans* var. *melanogenum* (Gostincar et al. 2014).

Due to the attractiveness of alkanes, there are growing activities to manipulate yeasts to produce hydrocarbons. The *A. thaliana* alkane synthesis pathway was expressed in *S. cerevisiae*. Genes encoding VLC-acyl-CoA reductase and VLC-aldehyde decarbonylase were expressed, in a strain with a modified fatty acid elongase, which produces elongated (C28 and C30) fatty acids. Additionally, long-chain acyl-CoA synthetase I and CYTB5-B, a hemeprotein that serves as electron-transporting factor, were expressed. The resulting strain produced 86 μg per gram yeast biomass (dry weight), mainly nonacosane, C29. This was the first example of engineering yeast towards alkane production (Bernard et al. 2012). However, those long-chain *n*-alkanes as produced by *A. pullulans* var. *melanogenum* or the engineered *S. cerevisiae* strain are less attractive as biofuels, due to their chain length. A synthesis pathway for shorter-chain alkanes was therefore introduced. A fatty acyl-ACP-reductase (FAR) gene and a fatty aldehyde-deformylating oxygenase (FADO) gene from the cyanobacterium *Synechococcus elongatus* were expressed in *S. cerevisiae*. FAR reduces fatty acyl-CoA to fatty aldehyde, and then FADO converts the fatty aldehydes further to alkanes or alkenes. *E. coli* ferredoxin and ferredoxin reductases were also expressed, to provide the required redox cofactors for FADO in the cytoplasm. *HFD1*, encoding a hexadecanal dehydrogenase, was disrupted, because it competed with FADO for the fatty aldehydes. The resulting strain produced 22 μg heptadecane per gram dry weight (Buijs et al. 2013). To further improve alkane production by *S. cerevisiae*, a carboxylic acid reductase from *M. marinum* was expressed instead of the FAR in a strain overproducing free fatty acids (see above). To activate this enzyme, a 4′-phosphopantetheinyl transferase from *Aspergillus nidulans* was expressed. Deletion of *POX1* further increased alkane production, and deletion of *ADH5* resulted in a lowered production of fatty alcohols and increased alkane production. Overexpressing aldehyde-deformylating oxygenases from *S. elongatus* and the cyanobacterium *Nostoc punctiforme* finally increased the

alkane production to 0.82 mg/l (Zhou et al. 2016). Higher amounts of alkanes have been obtained by manipulating *Y. lipolytica*. A soybean lipoxygenase I (encoded by a codon-optimised Gmlox1 gene) was expressed converting linoleic acid into 13-hydroperoxylinoleic acid. This acid can be converted to pentane and 13-oxo-cis-9, trans 11-tridecadienoic acid, either spontaneously or catalysed by lipoxygenase I and/or a hydroperoxide lyase. Expression of this gene resulted in the production of 3.28 mg/l pentane. Optimising the growth medium and knocking out genes involved in β-oxidation further increased the production to 4.98 mg/l (Blazeck et al. 2013). The filamentous fungus *Aspergillus carbonarius* has the natural ability to produce a variety of hydrocarbons, including dodecane, tetradecane and hexadecane (Sinha et al. 2015). Codon-optimised fatty acyl-ACP-reductase (FAR) and fatty aldehyde-deformylating oxygenase (FADO) from *S. elongatus* were also expressed in *A. carbonarius*. Shake flask cultivations in different media were tested, and on oatmeal medium, 2.7 mg/l pentadecane and 10.2 mg/l heptadecane were found after 6 days of cultivation (Sinha et al. 2017).

Production of alkenes has also been demonstrated in *S. cerevisiae*. The cytochrome P450 fatty acid decarboxylase (encoded by $OleT_{JE}$) of the gram-positive bacterium *Jeotgalicoccus* spec. ATCC 8456 directly converts fatty acids to terminal alkenes. Expression of a corresponding codon-optimised gene in *S. cerevisiae* resulted in alkene production (total final concentration 54.5 μg/l). Deletion of *FAA1* and *FAA2* further increased alkene production. To improve the cofactor supply of the cytochrome P450 enzyme, *HEM3* was overexpressed. Since the cytochrome P450 fatty acid decarboxylase is known to be most active in the presence of H_2O_2, several genes involved in its degradation were deleted. Deletion of *CTT1*, encoding cytoplasmic catalase T; *CTA1*, encoding peroxisomal catalase A; and *CCP1*, encoding a mitochondrial cytochrome c peroxidase, had a positive effect on alkene production. Fine-tuning of gene expression by choosing appropriate promoters and plasmids and optimisation of the fermentation conditions in a bioreactor enabled alkene production with a final concentration of 3.7 mg/l after 144 h of cultivation (Chen et al. 2015). Attempts have also been made to establish *S. cerevisiae* as a whole-cell catalyst to convert free fatty acids to alkanes. Expression of a fatty acid α-dioxygenase from *Oryza sativa* and an aldehyde-deformylating oxygenase from *S. elongatus* enabled conversion of free fatty acids. When the pH in the medium was increased to 7.0, hereby increasing the solubility of the added fatty acids, conversion of free fatty acids was observed: out of a mixture of 200 mg/l even chain length saturated fatty acids (C12–C18) final titres of dodecane, tetradecane and hexadecane of 28.7, 304 and 5.1 μg/l, respectively, were obtained. The two genes were also expressed in a strain overproducing free fatty acids (*Δfaa1*, *Δfaa4*), and final concentrations of 42.4 μg/l tetradecane and 31.1 μg/l hexadecane were attained from glucose (Foo et al. 2017).

6.7.4 Isoprenoids

Terpenes, also called isoprenoids or isoprenes, are built up from derivatives of the five-carbon compound isoprene ($CH_2{=}C(CH_3)CH{=}CH_2$). The precursors of all

isoprenoids in fungi are synthesized via the methylmalonate (MVA) pathway. In the first step of this pathway, two cytosolic acetyl-CoA are condensed to acetoacetyl-CoA by acetoacetyl-CoA thiolase (in *S. cerevisiae* encoded by *ERG10*). Acetoacetyl-CoA is then condensed with another acetyl-CoA to 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) by HMG-CoA synthase (encoded by *HMGS*). HMG-CoA is reduced to mevalonate by HMG-CoA reductase (*HMG1*), consuming two NADPH. Mevalonate is phosphorylated by mevalonate-5-kinase (*ERG12*) to mevalonate-5-P, which is further phosphorylated to mevalonate pyrophosphate by phosphomevalonate kinase (*ERG8*). Mevalonate pyrophosphate is then decarboxylated to isopentenyl pyrophosphate (IPP) by mevalonate pyrophosphate decarboxylase (*MVD1*), and IPP is converted to dimethylallyl pyrophosphate (DMAPP) by IPP isomerase (*IDI1*) (Lv et al. 2014; Paddon and Keasling 2014; Ye et al. 2016). All terpenoids in fungi are derived from IPP and DMAPP.

Isoprene (C_5H_8; 2-*methyl*-1,3-butadiene) is the simplest compound of the isoprene family. It has a broad range of industrial applications, including the production of synthetic rubber, adhesives and speciality elastomers. It also has a potential as fuel additive for gasoline, diesel or jet fuel. Currently it is mainly produced from mineral oil. It is produced by a variety of plants and microorganisms. Fungi also produce terpene compounds (see below); however, current knowledge suggests that they are not able to produce isoprene, since they lack the isoprene synthase, which converts DMAPP to isoprene (Ye et al. 2016). Hong and colleagues introduced a codon-optimised *IspS* gene encoding an isoprene synthase from *Pueraria montana* (kudzu vine) into the *S. cerevisiae* genome. Multicopy integration resulted in the production of about 0.5 mg isoprene and isoprene derivatives in the headspace gas of a 1 l culture (Hong et al. 2012). A variety of attempts have been made to improve isoprene production by *S. cerevisiae*, including overexpressing of the rate-limiting HMGR, downregulating or deleting of metabolic pathways competing for DMAPP or overexpressing a global transcription factor (summarised in Ye et al. 2016). In a recent study, several of the above-mentioned approaches were combined with overproduction of acetyl-CoA. An isoprene synthase gene (*ispS* from *Populus alba*) was overexpressed in *S. cerevisiae*. Then, truncated *HMG1* (*tHMG1*) was overexpressed (Lv et al. 2014). It has been demonstrated that feedback inhibition can be avoided when only the catalytic unit of *HMG1* is expressed (Donald et al. 1997). The activity of farnesyl pyrophosphate synthetase, which removes IPP from the pathway, was decreased by expressing the corresponding gene (*ERG20*) under control of a weak promoter. Attempts to enhance the supply of acetyl-CoA by increasing the activities of assimilatory alcohol dehydrogenase (*ADH2*) and cytosolic aldehyde dehydrogenase (*ALD6*) indicated that the limiting step in acetaldehyde production is the conversion of acetate to acetyl-CoA. Therefore, acetyl-CoA synthetase (*ACS2*) and acetoacetyl-CoA thiolase (*ERG10*) were overexpressed. Further fine-tuning of expression by using appropriate promoters and modifying regulation of some metabolic pathways (deletion of the repressor *GAL80*, since the *GAL1* promoter was used for the expression of some genes), optimising carbon sources (glycerol and sucrose were more conducive than glucose) and oxygen

supply, yielded a final production of 37 mg/l isoprene (Lv et al. 2014). In a subsequent study, the isoprene synthase gene was placed under control of the *GAL1* promoter; at the same time, the activator *GAL4* was overexpressed, and native *GAL1*, *GAL7* and *GAL10* promoters were deleted to diminish competition for the transcription factor. Further improvements were obtained by directly selecting enzymes with higher activity. DMAPP has a cytotoxic effect, and cells carrying a very active isoprene synthase would develop a certain resistance to DMAPP. A mutant library of isoprene synthase was generated by error-prone PCR, and, finally, enzymes with enhanced activity were isolated. These enzymes were expressed in the background described above. Cultivation of the resulting strain in a high cell-density bioreactor resulted in an isoprene production of 3.7 g/l (Wang et al. 2017). Since acetyl-CoA supply is crucial for high production of isoprene, and since cytoplasmic acetyl-CoA production is limited in *S. cerevisiae*, the MVA pathway and the isoprene synthase were expressed in mitochondria, by fusing the genes with a corresponding mitochondrial import signal sequence. Resulting strains produced more isoprene than strains with manipulated cytoplasmic isoprene synthesis; however, this strain did not achieve high cell-density in fed-batch cultivation. Best performance was obtained when strains with mitochondrial and cytoplasmic isoprene synthesis were crossed, forming a diploid strain containing the pathway both in the cytoplasm and mitochondria. The resulting strain produced 2.5 g/l isoprene in a high cell-density fed-batch cultivation, demonstrating the potential of manipulating the metabolism in different cell compartments (Lv et al. 2016).

Apart from isoprene, other, long-chain isoprenoids also have immense biotechnological potential. Synthesis of those isoprenoids is achieved by condensation of IPP and DMAPP to the prenyl pyrophosphates, geranyl pyrophosphate (GPP, C10), farnesyl pyrophosphate (FPP, C15) and geranylgeranyl pyrophosphate (GGPP, C20). From these compounds, monoterpenes (C10), sesquiterpenes (C15) or diterpenes (C20) are formed (Zhang et al. 2011; Buijs et al. 2013). A variety of terpene-derived compounds are produced by yeasts, including the membrane sterol ergosterol (Parks and Casey 1995) and carotenoids (Mata-Gomez et al. 2014). A very prominent example of an isoprenoid-derived compound is the antimalaria drug artemisinin. Artemisinin is a sesquiterpene with a lactone endoperoxide. This compound is naturally produced by a plant, *Artemisia annua*, commonly known as sweet wormwood. Artemisinin production starts with the conversion of FPP to amorphadiene by amorphadiene synthase (ADS). *A. annua* accumulates, probably by the action of cytochrome P450 monooxygenase (also called amorphadiene oxidase), two oxidation products of amorphadiene, artemisinic acid and dihydroartemisinic acid. The latter is the precursor of artemisinin, and it is thought that its conversion occurs spontaneously in the presence of sunlight (Paddon and Keasling 2014). By engineering the MVA pathway, expressing ADS and a cytochrome P450 monooxygenase from *A. annua* up to 100 mg/l artemisinic acid could be produced in *S. cerevisiae* S288C (Ro et al. 2006). Artemisinic acid can easily be converted to artemisinin by chemical methods. Further improvements were achieved by additional metabolic engineering and by using a more appropriate yeast strain. The whole MVA pathway, including *tHMG1*, and *ERG20* were

overexpressed in CEN.PK2. Three copies of *tHMG1* were integrated into the genome, as this step had previously been identified as limiting for production of artemisinic acid. As the genes were expressed under control of the *GAL1* promoter, galactose metabolism was also manipulated, by deleting the *GAL* gene cluster and the repressor *GAL80*. Strains were then constructed containing either the whole pathway for artemisinic acid (ADS, cytochrome P450 monooxygenase and its cognate reductase, *A. annua* cytochrome P450 reductase) or only ADS. Production of amorphadiene was more strongly enhanced in the latter strain than production of artemisinic acid. Therefore, the next study focused on the production of amorphadiene and its subsequent conversion to artemisinin by chemical methods. After optimising fermentation conditions, 41 g/l amorphadiene was produced, and the overall yield of converting amorphadiene to dihydroartemisinic acid was 48.4% (Westfall et al. 2012). Further investigation showed that the low ability of engineered cells to produce artemisinic acid was due to oxidative stress, which was generated due to poor coupling between the cytochrome P450 enzyme and its reductase. This was overcome by fine-tuning their expression and by expression of an *A. annua* cytochrome b5, which has been shown to improve the activity of cytochrome P450 enzymes. The resulting strain, although improved, still did not produce sufficient amounts of artemisinic acid. Moreover, artemisinic aldehyde, an oxidation intermediate, was accumulated, which is probably toxic to the cell. To overcome this, *A. annua* artemisinic aldehyde dehydrogenase was expressed, resulting in substantial increase of artemisinic acid. Expression of an NAD^+-dependent *A. annua* artemisinic alcohol dehydrogenase further increased the artemisinic acid concentration and yielded a final concentration of 25 g/l artemisinic acid, the highest obtained titre so far (Paddon et al. 2013).

Isoprenoids also have great potential as biofuels. Hydrolysis of IPP, GPP and FPP results in alcohols, which can be used as fuel additives (Zhang et al. 2011). These alcohols and especially isopentanol have been suggested as gasoline additives; they have all advantageous features of ethanol, i.e. increasing the octane number and reducing the concentration of harmful pollutants in the exhaust, but lack the negative characteristics; they do not increase the vapour pressure, have better tolerance to water and do not increase fuel consumption (Hull et al. 2006). Farnesol and farnesene have been suggested as diesel fuels (Zhang et al. 2011). *S. cerevisiae* was engineered to produce bisabolene. This was achieved in a strain with overexpressed FPP production pathway and reduced activity of a competing synthesis pathway (squalene synthesis), as well as overexpression of bisabolene synthase. The resulting strain produced more than 900 mg bisabolene per litre. Bisabolene is a sesquiterpene that can be chemically hydrogenated to bisabolene, a biosynthetic alternative to D2 diesel (D2 diesel is usually used as fuel in cars), which has similar or partially even better characteristics compared to D2 diesel (Peralta-Yahya et al. 2012). Recently, it has been discovered that bisabolene and α-farnesene provide resistance against the detergent Tween 20, which has been used to isolate mutants with higher production of these compounds (Kirby et al. 2014). Further improvements of isoprenoid production are possible by, for instance, isolating appropriate enzymes, such as terpene synthases (Tippmann et al. 2016).

Carotenoids are another group of isoprene-derived compounds. Astaxanthin is one example of a biotechnologically interesting carotenoid that is produced by a yeast. Astaxanthin is used in a variety of cosmetics and other products, and it provides the characteristic colour of salmon. It is naturally produced by marine microbes. As the fish cannot synthesise astaxanthin, it has to be added to the feed of farmed salmon and is actually one of the most expensive ingredients. Currently, most astaxanthin is produced from fossil resources; a little is produced by microbial fermentations using, for instance, the green alga *Haematococcus pluvialis* or the yeast *Phaffia rhodozyma*. This yeast was for several years designated with its teleomorph name *Xanthophyllomyces dendrorhous*, but recently, the priority of the name *P. rhodozyma* has been pointed out (Liu et al. 2015b). Only few yeast species are able to produce astaxanthin; this includes the heterobasidous yeast *P. rhodozyma* and close relatives (Colabella and Libkind 2016). In fermentation experiments, up to 420 mg/l astaxanthin were produced. However, astaxanthin produced by fermentation costs more than three times the price of synthetic astaxanthin would cost. Economic production would require production of 4 mg/g cell dry weight and a final concentration of at least 60 g/l (Rodriguez-Saiz et al. 2010; Schmidt et al. 2011). There are three specific genes responsible for astaxanthin synthesis in *P. rhodozyma* (Schmidt et al. 2011). Two GGPP are fused to form phytoene, by the protein encoded by the fusion gene crtYB. This gene encodes a bifunctional enzyme, which acts both in the condensation of two GGPP and later in the cyclisation of lycopene. Then, two double bonds are stepwise introduced into the phytoene molecule by the enzyme encoded by crtI, generating neurosporene and finally lycopene. Lycopene is, as mentioned above, converted to β-carotene by a cyclisation catalysed by crtYB (Verdoes et al. 1999a, b). In a side reaction of the pathway, lycopene can be converted by crtI to 3,4-dehydrolycopene, which is then converted by crtYB to torulene. β-carotene is then converted by insertion of 3-hydroxyl and 4-keto groups into the ionone rings of β-carotene. This multistep conversion is achieved by astaxanthin synthase, which is a cytochrome P450 monooxygenase. Cytochrome P450 enzymes usually require an electron-donating system, and this activity is performed by a specific cytochrome P450 reductase (Schmidt et al. 2011) (and references therein). Astaxanthin production by *P. rhodozyma* requires sophisticated regulation of fermentation, as the yeast, although showing a high activity of the pentose phosphate pathway and thus more similar to respiratory yeasts and filamentous fungi, exhibits a Crabtree effect, i.e. it produces ethanol under aerobic conditions, when there is a surplus of glucose (Cannizzaro et al. 2004; Liu and Wu 2008). Moreover, methods for metabolic engineering of this yeast have been recently developed, e.g. a system for multiple overexpression of genes. Simultaneous overexpression of the genes of the MVA pathway resulted in a 2.1-fold increase in astaxanthin production compared to the parental strain (Hara et al. 2014). Further improvement could be achieved by deleting both copies of *CYP61* in the diploid genome, encoding C-22 sterol desaturase, which is involved in sterol biosynthesis. Ergosterol has been shown to exert feedback inhibition on the MVA pathway (Yamamoto et al. 2016). Astaxanthin production has been shown to be under glucose repression, and it has

recently been demonstrated that a homologue of the *S. cerevisiae MIG1* is involved in this repression. A strain mutated in *P. rhodozyma MIG1* showed enhanced astaxanthin production (Alcaino et al. 2016).

6.8 Lipid Extraction

Apart from the above-mentioned examples of glycolipids and free fatty acids, neutral lipids are usually produced and stored intracellularly. Therefore, they have to be extracted from the cells. Extraction is one of the major downstream processes, which may substantially contribute to the cost of implementing lipid production as bioprocess. While a variety of studies have been performed on algae, not much is known about efficient lipid extraction from fungi (Ochsenreither et al. 2016). Analytical lipid extraction is often performed with the help of solvents, which both disintegrate the cell and extract the lipids. Chloroform, often mixed with methanol, has been shown to be very efficient for lipid extraction (Folch et al. 1957). Soxhlet extraction is another classical extraction method that has originally been used to extract lipids from milk powder. It is currently mainly used as a standard method against which other extraction methods are compared. The method involves drying and grinding the sample, which is then placed on a porous thimble in an extraction chamber. Extraction occurs in several washing steps with an organic solvent under reflux (Soxhlet 1879). A variety of automated Soxhlet systems are commercially available. However, Soxhlet extraction requires large amounts of solvents and is time-consuming. Furthermore, extensive use of chloroform and organic solvents is environmentally problematic and should be avoided for food and feed production, due to potential toxic effects. To reduce the utilisation of organic solvents, cell disruption is usually required for lipid extraction, due to the robust cell walls of fungi. One can largely distinguish between mechanical and nonmechanical cell disruption methods. Mechanical methods include bead milling, homogenisation and ultrasound treatment; for lipid extraction they are often combined with solvent extraction. Nonmechanical methods can be divided in physical methods, such as decompression or microwave treatment, or drying, or chemical methods such as the application of solvents or acid treatment. Enzyme treatment is also possible (Ochsenreither et al. 2016). In one study, several of these different methods were applied to disrupt cells of the yeast *C. curvatus* and the filamentous fungus *M. isabellina*. Lipid was then extracted using a methanol/chloroform solution. The tested cell disruption methods were autoclaving, microwave treatment, ultrasonication, bead beating, HCl digestion and Soxhlet, which included disruption of freeze-dried cells with mortar and pestle and a subsequent methanol/chloroform extraction. All these methods had been tested in other studies for lipid extraction from oleaginous algae. Results showed that for *M. isabellina*, Soxhlet gave the best results, and HCl digestion and bead beating were still acceptable, whereas ultrasonication, microwaving and autoclaving gave poor extraction results. For *C. curvatus*, HCl digestion, bead beating and ultrasonication performed best; Soxhlet enabled similar extraction efficiencies to the three other, whereas

microwaving and autoclaving gave poor yields. Differences are due to different cell structures of yeast and filamentous fungi. Nevertheless, HCl extraction performed well for both organisms; because it is relatively simple and the fatty acid composition was not impaired by the method (i.e. there was no significant decrease in the nutritionally valuable non-saturated fatty acids), HCl cell disruption may be the method of choice in the future for extracting lipids from fungi (Yu et al. 2015). High-pressure homogenisation (800 bar at 4 °C for several passages) and subsequent hexane extraction have been applied for cell disruption of *C. curvatus* and an oleaginous strain of *Pichia kudriavzevii*. The extraction yield was not directly compared to other methods; however, lipid recoveries of 20% of dry matter (*P. kudriavzevii*) and about 50% (*C. curvatus*) indicate an acceptable efficiency of the method (Thiru et al. 2011; Sankh et al. 2013). Ultrasonic treatment may be another option for cell disruption; however, large-scale cell disruption does not seem to be possible due to difficulties of energy transmission in larger volumes. It may also generate heat, which can influence the quality of the resulting lipids, and it was shown not to be the most efficient method when compared to other methods in fungi (Yu et al. 2015; Ochsenreither et al. 2016). Hydrothermal treatment was used to extract lipids from *C. curvatus*, grown in a 5 l pilot scale reactor (the lipid content of the yeast biomass was 53%). The biomass was treated at 280 °C and 500 psi for 1 h, and after hexane washing, 43% of the solids, corresponding to 70% of the initial total lipids, was recovered in the hexane fraction. The resulting lipids were free of sulphur and low in salts and nitrogen and were thus appropriate for further processing to biofuels. The energy demand of this technique is quite high; thus, it would be essential to integrate it into a process, whereby the energy is recovered via heat circulation systems (Espinosa-Gonzalez et al. 2014). Treatment with supercritical fluids, especially CO_2, is another option for lipid extraction. Supercritical CO_2 enables good recovery, has a high selectivity to non-polar substances (i.e. salt, water, proteins, polysaccharides and sugars are not extracted) and can be easily removed after extraction. It is non-toxic, non-flammable, non-polluting, inexpensive and inert. Extraction with supercritical CO_2 can be performed at relatively low temperatures due to the low critical temperature of CO_2 (31.1 °C). It also protects against oxidation, making it a gentle extraction method, e.g. for polyunsaturated fatty acids (Ochsenreither et al. 2016). When using supercritical CO_2 extraction of oil from the zygomycete *Cunninghamella echinulata*, the best performance was achieved at 30 MPa, 50 °C, an incubation time of 180 min and a mass flow of 50 kg CO_2/kg dry biomass, and when fungal particles smaller than 0.5 mm were used. Interestingly, CO_2 extraction resulted in a higher recovery of γ-linolenic acid and total lipids than all other tested methods, including Folch and Soxhlet with different solvents (Certik and Horenitzky 1999). In *S. cerevisiae*, good recovery and separation of triglycerides from phospholipids was obtained by CO_2 extraction at 2 MPa and 40 °C with 9% (w/w) ethanol as co-solvent. However, pretreatment was required, and acid hydrolysis followed by methanol maceration was found to be the best method to ensure high lipid extraction efficiency (Hegel et al. 2011). Enzyme-assisted cell disruption may also be an option for lipid extraction. *R. toruloides* was treated with β-1,3-glucomannanase, after a heat pretreatment

with microwaves. Lipids were extracted with ethyl acetate, at room temperature and without dewatering. Enzyme treatments may save energy costs, as they can often be performed at room temperature and at atmospheric pressure (Jin et al. 2012). On the other hand, enzymes may be expensive so sophisticated methods to recycle enzyme will be necessary.

Lipid extraction is one of the factors that are crucial for obtaining sustainable biolipid production from fungi (e.g. Karlsson et al. 2016). A variety of lipid extraction methods have been tested during the last years. Investigations about the energy efficiency of the different cell disruption and lipid extraction methods would probably help to identify the most appropriate methodology for isolating lipids from fungi.

6.9 Conclusions

Lipid metabolism in fungi has been studied for many years. However, huge progress has been achieved during the last decade, promoted by a growing interest for biotechnological utilisation of products from lipid metabolism and to identify novel targets for antifungal therapy. Techniques of metabolic engineering and omics analyses in oleaginous and model organisms provided novel insights into lipid metabolism, which was shown to be amazingly complex in terms of biosynthesis pathways and their regulation. Given the necessity to generate fuels, chemicals and food from non-fossil, sustainable resources, and the growing availability of molecular manipulation and analysis techniques even in non-conventional oleaginous organisms, one can easily predict that the already rapid development in this field will continue to accelerate in the coming years. Moreover, as lipid metabolism is one essential component of cellular metabolism, research in lipid metabolism in fungi will greatly contribute to understanding general eukaryotic metabolism and thus have a strong impact on fundamental biological research, with implications for human and animal medicine as well as plant production.

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References

Akpina-Bayizit A (2014) Fungal lipids: the biochemistry of lipid accumulation. Int J Chem Eng Appl 5:409–414

Alcaino J, Bravo N, Cordova P, Marcoleta AE, Contreras G, Barahona S, Sepulveda D, Fernandez-Lobato M, Baeza M, Cifuentes V (2016) The involvement of Mig1 from *Xanthophyllomyces*

dendrorhous in catabolic repression: an active mechanism contributing to the regulation of carotenoid production. PLoS One 11:e0162838

André A, Diamantopoulou P, Philippoussis A, Sarris D, Komaitis M, Papanikolaou S (2010) Biotechnological conversions of bio-diesel derived waste glycerol into added-value compounds by higher fungi: production of biomass, single cell oil and oxalic acid. Ind Crop Prod 31:407–416

Asadi SZ, Khosravi-Darani K, Nikoopour H, Bakhoda H (2015) Evaluation of the effect of process variables on the fatty acid profile of single cell oil produced by *Mortierella* using solid-state fermentation. Crit Rev Biotechnol 35:94–102

Asmer H-J, Lang S, Wagner F, Wray V (1988) Microbial production, structure elucidation and bioconversion of sophorose lipids. J Am Oil Chem Soc 65:1460–1466

Azócar L, Ciudad G, Heipieper HJ, Navia R (2010) Biotechnological processes for biodiesel production using alternative oils. Appl Microbiol Biotechnol 88:621–636

Barnett JA, Kornberg HL (1960) The utilization by yeasts of acids of the tricarboxylic acid cycle. Microbiology 23:65–82

Béligon V, Christophe G, Fontanille P, Larroche C (2016) Microbial lipids as potential source to food supplements. Curr Opin Food Sci 7:35–42

Bellou S, Triantaphyllidou IE, Aggeli D, Elazzazy AM, Baeshen MN, Aggelis G (2016) Microbial oils as food additives: recent approaches for improving microbial oil production and its polyunsaturated fatty acid content. Curr Opin Biotechnol 37:24–35

Beopoulos A, Mrozova Z, Thevenieau F, Le Dall MT, Hapala I, Papanikolaou S, Chardot T, Nicaud JM (2008) Control of lipid accumulation in the yeast *Yarrowia lipolytica*. Appl Environ Microbiol 74:7779–7789

Beopoulos A, Cescut J, Haddouche R, Uribelarrea JL, Molina-Jouve C, Nicaud JM (2009) *Yarrowia lipolytica* as a model for bio-oil production. Prog Lipid Res 48:375–387

Beopoulos A, Nicaud JM, Gaillardin C (2011) An overview of lipid metabolism in yeasts and its impact on biotechnological processes. Appl Microbiol Biotechnol 90:1193–1206

Bernard A, Domergue F, Pascal S, Jetter R, Renne C, Faure J-D, Haslam RP, Napier JA, Lessire R, Joubès J (2012) Reconstitution of plant alkane biosynthesis in yeast demonstrates that *Arabidopsis* ECERIFERUM1 and ECERIFERUM3 are core components of a very-long-chain alkane synthesis complex. Plant Cell 24:3106–3118

Biddy MJ, Davis R, Humbird D, Tao L, Dowe N, Guarnieri MT, Linger JG, Karp EM, Salvachúa D, Vardon DR, Beckham GT (2016) The techno-economic basis for coproduct manufacturing to enable hydrocarbon fuel production from lignocellulosic biomass. ACS Sustain Chem Eng 4:3196–3211

Black PN, DiRusso CC (2007) Yeast acyl-CoA synthetases at the crossroads of fatty acid metabolism and regulation. Biochim Biophys Acta 1771:286–298

Blazeck J, Liu L, Knight R, Alper HS (2013) Heterologous production of pentane in the oleaginous yeast *Yarrowia lipolytica*. J Biotechnol 165:184–194

Brandenburg J, Blomqvist J, Pickova J, Bonturi N, Sandgren M, Passoth V (2016) Lipid production from hemicellulose with *Lipomyces starkeyi* in a pH regulated fed-batch cultivation. Yeast 33:451–462

Buijs NA, Siewers V, Nielsen J (2013) Advanced biofuel production by the yeast *Saccharomyces cerevisiae*. Curr Opin Chem Biol 17:480–488

Calvey CH, Willis LB, Jeffries TW (2014) An optimized transformation protocol for *Lipomyces starkeyi*. Curr Genet 60:223–230

Cannizzaro C, Christensen B, Nielsen J, von Stockar U (2004) Metabolic network analysis on *Phaffia rhodozyma* yeast using ^{13}C-labeled glucose and gas chromatography-mass spectrometry. Metab Eng 6:340–351

Certik M, Horenitzky R (1999) Supercritical CO_2 extraction of fungal oil containing γ-linolenic acid. Biotechnol Tech 13:11–15

Certik M, Shimizu S (1999) Biosynthesis and regulation of microbial polyunsaturated fatty acid production. J Biosci Bioeng 87:1–14

Chatzifragkou A, Makri A, Belka A, Bellou S, Mavrou M, Mastoridou M, Mystrioti P, Onjaro G, Aggelis G, Papanikolaou S (2011) Biotechnological conversions of biodiesel derived waste glycerol by yeast and fungal species. Energy 36:1097–1108

Chen X, Li Z, Zhang X, Hu F, Ryu DY, Bao J (2009) Screening of oleaginous yeast strains tolerant to lignocellulose degradation compounds. Appl Biochem Biotechnol 159:591–604

Chen Y, Daviet L, Schalk M, Siewers V, Nielsen J (2013) Establishing a platform cell factory through engineering of yeast acetyl-CoA metabolism. Metab Eng 15:48–54

Chen B, Lee DY, Chang MW (2015) Combinatorial metabolic engineering of *Saccharomyces cerevisiae* for terminal alkene production. Metab Eng 31:53–61

Cheng Y, McNally DJ, Labbe C, Voyer N, Belzile F, Bélanger RR (2003) Insertional mutagenesis of a fungal biocontrol agent led to discovery of a rare cellobiose lipid with antifungal activity. Appl Environ Microbiol 69:2595–2602

Ciesielska K, Van Bogaert IN, Chevineau S, Li B, Groeneboer S, Soetaert W, Van de Peer Y, Devreese B (2014) Exoproteome analysis of *Starmerella bombicola* results in the discovery of an esterase required for lactonization of sophorolipids. J Proteome 98:159–174

Colabella F, Libkind D (2016) PCR-based method for the rapid identification of astaxanthin-accumulating yeasts (*Phaffia* spp.) Rev Argent Microbiol 48:15–20

Cui Y, Blackburn JW, Liang Y (2012) Fermentation optimization for the production of lipid by *Cryptococcus curvatus*: use of response surface methodology. Biomass Bioenergy 47:410–417

Daniel H-J, Otto RT, Binder M, Reuss M, Syldatk C (1999) Production of sophorolipids from whey: development of a two-stage process with *Cryptococcus curvatus* ATCC 20509 and *Candida bombicola* ATCC 22214 using deproteinized whey concentrates as substrates. Appl Microbiol Biotechnol 51:40–45

Davila A-M, Marchal R, Vandecasteele J-P (1992) Kinetics and balance of a fermentation free from product inhibition: sophorose lipid production by *Candida bombicola*. Appl Microbiol Biotechnol 38:6–11

Della-Bianca B, Basso T, Stambuk B, Basso L, Gombert A (2013) What do we know about the yeast strains from the Brazilian fuel ethanol industry? Appl Microbiol Biotechnol 97:979–991

Díaz De Rienzo MA, Banat IM, Dolman B, Winterburn J, Martin PJ (2015) Sophorolipid biosurfactants: possible uses as antibacterial and antibiofilm agent. New Biotechnol 32:720–726

Donald KA, Hampton RY, Fritz IB (1997) Effects of overproduction of the catalytic domain of 3-hydroxy-3-methylglutaryl coenzyme A reductase on squalene synthesis in *Saccharomyces cerevisiae*. Appl Environ Microbiol 63:3341–3344

Espinosa-Gonzalez I, Parashar A, Bressler DC (2014) Hydrothermal treatment of oleaginous yeast for the recovery of free fatty acids for use in advanced biofuel production. J Biotechnol 187:10–15

Evans CT, Ratledge C (1984) Induction of xylulose-5-phosphate phosphoketolase in a variety of yeasts grown ond-xylose: the key to efficient xylose metabolism. Arch Microbiol 139:48–52

Fakas S, Papanikolaou S, Batsos A, Galiotou-Panayotou M, Mallouchos A, Aggelis G (2009) Evaluating renewable carbon sources as substrates for single cell oil production by *Cunninghamella echinulata* and *Mortierella isabellina*. Biomass Bioenergy 33:573–580

Feng X, Lian J, Zhao H (2015) Metabolic engineering of *Saccharomyces cerevisiae* to improve 1-hexadecanol production. Metab Eng 27:10–19

Filippucci S, Tasselli G, Scardua A, Di Mauro S, Cramarossa MR, Perini D, Turchetti B, Onofri A, Forti L, Buzzini P (2016) Study of *Holtermanniella wattica*, *Leucosporidium creatinivorum*, *Naganishia adeliensis*, *Solicoccozyma aeria*, and *Solicoccozyma terricola* for their lipogenic aptitude from different carbon sources. Biotechnol Biofuels 9:259

Fillet S, Adrio JL (2016) Microbial production of fatty alcohols. World J Microbiol Biotechnol 32:152

Fillet S, Gibert J, Suárez B, Lara A, Ronchel C, Adrio JL (2015) Fatty alcohols production by oleaginous yeast. J Ind Microbiol Biotechnol 42:1463–1472

Flores-Cotera L, Martín R, Sánchez S (2001) Citrate, a possible precursor of astaxanthin in *Phaffia rhodozyma*: influence of varying levels of ammonium, phosphate and citrate in a chemically defined medium. Appl Microbiol Biotechnol 55:341–347

Folch J, Lees M, Sloane Stanley GH (1957) A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem 226:497–509

Foo JL, Susanto AV, Keasling JD, Leong SS, Chang MW (2017) Whole-cell biocatalytic and de novo production of alkanes from free fatty acids in *Saccharomyces cerevisiae*. Biotechnol Bioeng 114:232–237

Frieman MB, Cormack BP (2004) Multiple sequence signals determine the distribution of glycosylphosphatidylinositol proteins between the plasma membrane and cell wall in *Saccharomyces cerevisiae*. Microbiology 150:3105–3114

Fu WJ, Chi Z, Ma ZC, Zhou HX, Liu GL, Lee CF, Chi ZM (2015) Hydrocarbons, the advanced biofuels produced by different organisms, the evidence that alkanes in petroleum can be renewable. Appl Microbiol Biotechnol 99:7481–7494

Gao Q, Cui Z, Zhang J, Bao J (2014) Lipid fermentation of corncob residues hydrolysate by oleaginous yeast *Trichosporon cutaneum*. Bioresour Technol 152:552–556

Garay LA, Boundy-Mills KL, German JB (2014) Accumulation of high-value lipids in single-cell microorganisms: a mechanistic approach and future perspectives. J Agric Food Chem 62:2709–2727

Ghosh A, Ando D, Gin J, Runguphan W, Denby C, Wang G, Baidoo EEK, Shymansky C, Keasling JD, García Martín H (2016) ^{13}C Metabolic flux analysis for systematic metabolic engineering of *S. cerevisiae* for overproduction of fatty acids. Front Bioeng Biotechnol 4:76

Gnansounou E (2010) Production and use of lignocellulosic bioethanol in Europe: current situation and perspectives. Bioresour Technol 101:4842–4850

Golden C, Allison EH, Cheung WW, Dey M, Halpern B, McCauley DJ, Smith M, Vaitla B, Zeller D, Myers SS (2016) Fall in fish catch threatens human health. Nature 534:317–320

Gostincar C, Ohm RA, Kogej T, Sonjak S, Turk M, Zajc J, Zalar P, Grube M, Sun H, Han J, Sharma A, Chiniquy J, Ngan CY, Lipzen A, Barry K, Grigoriev IV, Gunde-Cimerman N (2014) Genome sequencing of four *Aureobasidium pullulans* varieties: biotechnological potential, stress tolerance, and description of new species. BMC Genomics 15:549

Graham-Rowe D (2011) Agriculture: beyond food versus fuel. Nature 474:S6–S8

Guo W, Sheng J, Zhao H, Feng X (2016) Metabolic engineering of *Saccharomyces cerevisiae* to produce 1-hexadecanol from xylose. Microb Cell Fact 15:24

Haas MJ, McAloon AJ, Yee WC, Foglia TA (2006) A process model to estimate biodiesel production costs. Bioresour Technol 97:671–678

Haque F, Alfatah M, Ganesan K, Bhattacharyya MS (2016) Inhibitory effect of sophorolipid on *Candida albicans* biofilm formation and hyphal growth. Sci Rep 6:23575

Hara KY, Morita T, Mochizuki M, Yamamoto K, Ogino C, Araki M, Kondo A (2014) Development of a multi-gene expression system in *Xanthophyllomyces dendrorhous*. Microb Cell Fact 13:175

Hast MA, Nichols CB, Armstrong SM, Kelly SM, Hellinga HW, Alspaugh JA, Beese LS (2011) Structures of *Cryptococcus neoformans* protein farnesyltransferase reveal strategies for developing inhibitors that target fungal pathogens. J Biol Chem 286:35149–35162

Hasunuma T, Kondo A (2012) Development of yeast cell factories for consolidated bioprocessing of lignocellulose to bioethanol through cell surface engineering. Biotechnol Adv 30:1207–1218

Hegel PE, Camy S, Destrac P, Condoret JS (2011) Influence of pretreatments for extraction of lipids from yeast by using supercritical carbon dioxide and ethanol as cosolvent. J Supercrit Fluids 58:68–78

Hewald S, Linne U, Scherer M, Marahiel MA, Kämper J, Bölker M (2006) Identification of a gene cluster for biosynthesis of mannosylerythritol lipids in the basidiomycetous fungus *Ustilago maydis*. Appl Environ Microbiol 72:5469–5477

Hiltunen JK, Mursula AM, Rottensteiner H, Wierenga RK, Kastaniotis AJ, Gurvitz A (2003) The biochemistry of peroxisomal beta-oxidation in the yeast *Saccharomyces cerevisiae*. FEMS Microbiol Rev 27:35–64

Hirano H, Kimura Y, Kimura A (2016) Biological significance of co- and post-translational modifications of the yeast 26S proteasome. J Proteome 134:37–46

Holzer H, Goedde HW (1957) Zwei Wege von Pyruvat zu Acetyl-Coenzym A in Hefe. Biochem Z 329:175–191

Hong SY, Zurbriggen AS, Melis A (2012) Isoprene hydrocarbons production upon heterologous transformation of *Saccharomyces cerevisiae*. J Appl Microbiol 113:52–65

Hu C, Zhao X, Zhao J, Wu S, Zhao ZK (2009) Effects of biomass hydrolysis by-products on oleaginous yeast *Rhodosporidium toruloides*. Bioresour Technol 100:4843–4847

Huang C, Zong M-h, Wu H, Liu Q-p (2009) Microbial oil production from rice straw hydrolysate by *Trichosporon fermentans*. Bioresour Technol 100:4535–4538

Huang C, Wu H, Li R-f, Zong M-h (2012) Improving lipid production from bagasse hydrolysate with *Trichosporon fermentans* by response surface methodology. New Biotechnol 29:372–378

Hull A, Golubkov I, Kronberg B, Marandzheva T, van Stam J (2006) An alternative fuel for spark ignition engines. Int J Engine Res 7:203–214

Hyvönen R, Olsson BA, Lundkvist H, Staaf H (2000) Decomposition and nutrient release from *Picea abies* (L.) Karst. and *Pinus sylvestris* L. logging residues. Forest Ecol Manag 126:97–112

Im JH, Yanagishita H, Ikegami T, Takeyama Y, Idemoto Y, Koura N, Kitamoto D (2003) Mannosylerythritol lipids, yeast glycolipid biosurfactants, are potential affinity ligand materials for human immunoglobulin G. J Biomed Mater Res A 65:379–385

Inoue S, Ito S (1982) Sophorolipids from *Torulopsis bombicola* as microbial surfactants in alkane fermentations. Biotechnol Lett 4:3–8

Jackson FM, Fraser TC, Smith MA, Lazarus C, Stobart AK, Griffiths G (1998) Biosynthesis of C18 polyunsaturated fatty acids in microsomal membrane preparations from the filamentous fungus *Mucor circinelloides*. Eur J Biochem 252:513–519

Jarboe L, Royce L, Liu P (2013) Understanding biocatalyst inhibition by carboxylic acids. Front Microbiol 4:272

Jin G, Yang F, Hu C, Shen H, Zhao ZK (2012) Enzyme-assisted extraction of lipids directly from the culture of the oleaginous yeast *Rhodosporidium toruloides*. Bioresour Technol 111:378–382

Jin M, Slininger PJ, Dien BS, Waghmode S, Moser BR, Orjuela A, Sousa Lda C, Balan V (2015) Microbial lipid-based lignocellulosic biorefinery: feasibility and challenges. Trends Biotechnol 33:43–54

Johnson DT, Taconi KA (2007) The glycerin glut: options for the value-added conversion of crude glycerol resulting from biodiesel production. Environ Prog 26:338–348

Jönsson LJ, Alriksson B, Nilvebrant N-O (2013) Bioconversion of lignocellulose: inhibitors and detoxification. Biotechnol Biofuels 6:16

Kamisaka Y, Noda N, Tomita N, Kimura K, Kodaki T, Hosaka K (2006) Identification of genes affecting lipid content using transposon mutagenesis in *Saccharomyces cerevisiae*. Biosci Biotechnol Biochem 70:646–653

Kamisaka Y, Tomita N, Kimura K, Kainou K, Uemura H (2007) *DGA1* (diacylglycerol acyltransferase gene) overexpression and leucine biosynthesis significantly increase lipid accumulation in the D*snf2* disruptant of *Saccharomyces cerevisiae*. Biochem J 408:61–68

Kamisaka Y, Kimura K, Uemura H, Yamaoka M (2013) Overexpression of the active diacylglycerol acyltransferase variant transforms *Saccharomyces cerevisiae* into an oleaginous yeast. Appl Microbiol Biotechnol 97:7345–7355

Kamiya Y, Sakurai A, Tamura S, Takahashi N (1978) Structure of rhodotorucine A, a novel lipopeptide, inducing mating tube formation in *Rhodosporidium toruloides*. Biochem Biophys Res Commun 83:1077–1083

Karlsson H, Ahlgren S, Sandgren M, Passoth V, Wallberg O, Hansson PA (2016) A systems analysis of biodiesel production from wheat straw using oleaginous yeast: process design, mass and energy balances. Biotechnol Biofuels 9:229

Kendrick A, Ratledge C (1992) Desaturation of polyunsaturated fatty acids in *Mucor circinelloides* and the involvement of a novel membrane-bound malic enzyme. Eur J Biochem 209:667–673

Kirby J, Nishimoto M, Chow RW, Pasumarthi VN, Chan R, Chan LJ, Petzold CJ, Keasling JD (2014) Use of nonionic surfactants for improvement of terpene production in *Saccharomyces cerevisiae*. Appl Environ Microbiol 80:6685–6693

Kitamoto D, Yanagishita H, Shinbo T, Nakane T, Kamisawa C, Nakahara T (1993) Surface active properties and antimicrobial activities of mannosylerythritol lipids as biosurfactants produced by *Candida antarctica*. J Biotechnol 29:91–96

Kitamoto D, Ghosh S, Ourisson G, Nakatani Y (2000) Formation of giant vesicles from diacylmannosylerythritols, and their binding to concanavalin A. Chem Commun 2000:861–862

Kitamoto D, Ikegami T, Suzuki GT, Sasaki A, Takeyama Y-i, Idemoto Y, Koura N, Yanagishita H (2001) Microbial conversion of n-alkanes into glycolipid biosurfactants, mannosylerythritol lipids, by *Pseudozyma* (*Candida antarctica*). Biotechnol Lett 23:1709–1714

Kulakovskaya TV, Golubev WI, Tomashevskaya MA, Kulakovskaya EV, Shashkov AS, Grachev AA, Chizhov AS, Nifantiev NE (2010) Production of antifungal cellobiose lipids by *Trichosporon porosum*. Mycopathologia 169:117–123

Kurtzman CP, Price NPJ, Ray KJ, Kuo T-M (2010) Production of sophorolipid biosurfactants by multiple species of the *Starmerella* (*Candida*) *bombicola* yeast clade. FEMS Microbiol Lett 311:140–146

Kurz M, Eder C, Isert D, Li Z, Paulus EF, Schiell M, Toti L, Vertesy L, Wink J, Seibert G (2003) Ustilipids, acylated beta-D-mannopyranosyl D-erythritols from *Ustilago maydis* and *Geotrichum candidum*. J Antibiot (Tokyo) 56:91–101

Ladygina N, Dedyukhina EG, Vainshtein MB (2006) A review on microbial synthesis of hydrocarbons. Process Biochem 41:1001–1014

Leber C, Da Silva NA (2014) Engineering of *Saccharomyces cerevisiae* for the synthesis of short chain fatty acids. Biotechnol Bioeng 111:347–358

Leber C, Polson B, Fernandez-Moya R, Da Silva NA (2015) Overproduction and secretion of free fatty acids through disrupted neutral lipid recycle in *Saccharomyces cerevisiae*. Metab Eng 28:54–62

Leber C, Choi JW, Polson B, Da Silva NA (2016) Disrupted short chain specific β-oxidation and improved synthase expression increase synthesis of short chain fatty acids in *Saccharomyces cerevisiae*. Biotechnol Bioeng 113:895–900

Ledesma-Amaro R, Nicaud J-M (2016) Metabolic engineering for expanding the substrate range of *Yarrowia lipolytica*. Trends Biotechnol 34:798–809

Ledesma-Amaro R, Dulermo R, Niehus X, Nicaud J-M (2016a) Combining metabolic engineering and process optimization to improve production and secretion of fatty acids. Metab Eng 38:38–46

Ledesma-Amaro R, Lazar Z, Rakicka M, Guo Z, Fouchard F, Coq AC, Nicaud JM (2016b) Metabolic engineering of *Yarrowia lipolytica* to produce chemicals and fuels from xylose. Metab Eng 38:115–124

Leibundgut M, Maier T, Jenni S, Ban N (2008) The multienzyme architecture of eukaryotic fatty acid synthases. Curr Opin Struct Biol 18:714–725

Lennen RM, Pfleger BF (2013) Microbial production of fatty acid-derived fuels and chemicals. Curr Opin Biotechnol 24:1044–1053

Li Y, Zhao Z, Bai F (2007) High-density cultivation of oleaginous yeast *Rhodosporidium toruloides* Y4 in fed-batch culture. Enzym Microb Technol 41:312–317

Lian J, Zhao H (2015) Recent advances in biosynthesis of fatty acids derived products in *Saccharomyces cerevisiae* via enhanced supply of precursor metabolites. J Ind Microbiol Biotechnol 42:437–451

Liang Y, Cui Y, Trushenski J, Blackburn JW (2010) Converting crude glycerol derived from yellow grease to lipids through yeast fermentation. Bioresour Technol 101:7581–7586

Liu YS, Wu JY (2008) Modeling of *Xanthophyllomyces dendrorhous* growth on glucose and overflow metabolism in batch and fed-batch cultures for astaxanthin production. Biotechnol Bioeng 101:996–1004

Liu Z, Gao Y, Chen J, Imanaka T, Bao J, Hua Q (2013) Analysis of metabolic fluxes for better understanding of mechanisms related to lipid accumulation in oleaginous yeast *Trichosporon cutaneum*. Bioresour Technol 130:144–151

Liu YY, Chi Z, Wang ZP, Liu GL, Chi ZM (2014) Heavy oils, principally long-chain n-alkanes secreted by *Aureobasidium pullulans* var. *melanogenum* strain P5 isolated from mangrove system. J Ind Microbiol Biotechnol 41:1329–1337

Liu L, Markham K, Blazeck J, Zhou N, Leon D, Otoupal P, Alper HS (2015a) Surveying the lipogenesis landscape in *Yarrowia lipolytica* through understanding the function of a Mga2p regulatory protein mutant. Metab Eng 31:102–111

Liu XZ, Wang QM, Goker M, Groenewald M, Kachalkin AV, Lumbsch HT, Millanes AM, Wedin M, Yurkov AM, Boekhout T, Bai FY (2015b) Towards an integrated phylogenetic classification of the Tremellomycetes. Stud Mycol 81:85–147

Liu Y, Yap SA, Koh CM, Ji L (2016) Developing a set of strong intronic promoters for robust metabolic engineering in oleaginous *Rhodotorula* (*Rhodosporidium*) yeast species. Microb Cell Fact 15:200

Lodge JK, Jackson-Machelski E, Higgins M, McWherter CA, Sikorski JA, Devadas B, Gordon JI (1998) Genetic and biochemical studies establish that the fungicidal effect of a fully depeptidized inhibitor of *Cryptococcus neoformans* myristoyl-CoA:protein N-myristoyltransferase (Nmt) is Nmt-dependent. J Biol Chem 273:12482–12491

Lv X, Xie W, Lu W, Guo F, Gu J, Yu H, Ye L (2014) Enhanced isoprene biosynthesis in *Saccharomyces cerevisiae* by engineering of the native acetyl-CoA and mevalonic acid pathways with a push-pull-restrain strategy. J Biotechnol 186:128–136

Lv X, Wang F, Zhou P, Ye L, Xie W, Xu H, Yu H (2016) Dual regulation of cytoplasmic and mitochondrial acetyl-CoA utilization for improved isoprene production in *Saccharomyces cerevisiae*. Nat Commun 7:12851

Mann PA, McLellan CA, Koseoglu S, Si Q, Kuzmin E, Flattery A, Harris G, Sher X, Murgolo N, Wang H, Devito K, de Pedro N, Genilloud O, Kahn JN, Jiang B, Costanzo M, Boone C, Garlisi CG, Lindquist S, Roemer T (2015) Chemical genomics-based antifungal drug discovery: targeting glycosylphosphatidylinositol (GPI) precursor biosynthesis. ACS Infect Dis 1:59–72

Mata-Gomez LC, Montanez JC, Mendez-Zavala A, Aguilar CN (2014) Biotechnological production of carotenoids by yeasts: an overview. Microb Cell Fact 13:12

McLellan CA, Whitesell L, King OD, Lancaster AK, Mazitschek R, Lindquist S (2012) Inhibiting GPI anchor biosynthesis in fungi stresses the endoplasmic reticulum and enhances immunogenicity. ACS Chem Biol 7:1520–1528

Metz JG, Roessler P, Facciotti D, Levering C, Dittrich F, Lassner M, Valentine R, Lardizabal K, Domergue F, Yamada A, Yazawa K, Knauf V, Browse J (2001) Production of polyunsaturated fatty acids by polyketide synthases in both prokaryotes and eukaryotes. Science 293:290–293

Michaelis S, Barrowman J (2012) Biogenesis of the *Saccharomyces cerevisiae* pheromone a-factor, from yeast mating to human disease. Microbiol Mol Biol Rev 76:626–651

Mnif I, Ghribi D (2016) Glycolipid biosurfactants: main properties and potential applications in agriculture and food industry. J Sci Food Agric 96:4310–4320

Morita T, Fukuoka T, Konishi M, Imura T, Yamamoto S, Kitagawa M, Sogabe A, Kitamoto D (2009) Production of a novel glycolipid biosurfactant, mannosylmannitol lipid, by *Pseudozyma parantarctica* and its interfacial properties. Appl Microbiol Biotechnol 83:1017

Morita T, Ogura Y, Takashima M, Hirose N, Fukuoka T, Imura T, Kondo Y, Kitamoto D (2011) Isolation of *Pseudozyma churashimaensis* sp. nov., a novel ustilaginomycetous yeast species as

a producer of glycolipid biosurfactants, mannosylerythritol lipids. J Biosci Bioeng 112:137–144

Morita T, Fukuoka T, Imura T, Kitamoto D (2012) Formation of the two novel glycolipid biosurfactants, mannosylribitol lipid and mannosylarabitol lipid, by *Pseudozyma parantarctica* JCM 11752T. Appl Microbiol Biotechnol 96:931–938

Morita T, Koike H, Koyama Y, Hagiwara H, Ito E, Fukuoka T, Imura T, Machida M, Kitamoto D (2013) Genome sequence of the basidiomycetous yeast *Pseudozyma antarctica* T-34, a producer of the glycolipid biosurfactants mannosylerythritol lipids. Genome Announc 1:e0006413

Mulligan CN (2005) Environmental applications for biosurfactants. Environ Pollut 133:183–198

Muñiz M, Zurzolo C (2014) Sorting of GPI-anchored proteins from yeast to mammals—common pathways at different sites? J Cell Sci 127:2793–2801

Nguyen UTT, Goody RS, Alexandrov K (2010) Understanding and exploiting protein prenyltransferases. Chembiochem 11:1194–1201

Nichols CB, Ost KS, Grogan DP, Pianalto K, Hasan S, Alspaugh JA (2015) Impact of protein palmitoylation on the virulence potential of *Cryptococcus neoformans*. Eukaryot Cell 14:626–635

Nitschke M, Costa SGVAO (2007) Biosurfactants in food industry. Trends Food Sci Technol 18:252–259

Ochsenreither K, Glück C, Stressler T, Fischer L, Syldatk C (2016) Production strategies and applications of microbial single cell oils. Front Microbiol 7:1539

Olstorpe M, Pickova J, Kiessling A, Passoth V (2014) Strain- and temperature-dependent changes of fatty acid composition in *Wickerhamomyces anomalus* and *Blastobotrys adeninivorans*. Biotechnol Appl Biochem 61:45–50

Omer CA, Gibbs JB (1994) Protein prenylation in eukaryotic microorganisms: genetics, biology and biochemistry. Mol Microbiol 11:219–225

Orlean P, Menon AK (2007) Thematic review series: lipid posttranslational modifications. GPI anchoring of protein in yeast and mammalian cells, or: how we learned to stop worrying and love glycophospholipids. J Lipid Res 48:993–1011

Paddon CJ, Keasling JD (2014) Semi-synthetic artemisinin: a model for the use of synthetic biology in pharmaceutical development. Nat Rev Microbiol 12:355–367

Paddon CJ, Westfall PJ, Pitera DJ, Benjamin K, Fisher K, McPhee D, Leavell MD, Tai A, Main A, Eng D, Polichuk DR, Teoh KH, Reed DW, Treynor T, Lenihan J, Jiang H, Fleck M, Bajad S, Dang G, Dengrove D, Diola D, Dorin G, Ellens KW, Fickes S, Galazzo J, Gaucher SP, Geistlinger T, Henry R, Hepp M, Horning T, Iqbal T, Kizer L, Lieu B, Melis D, Moss N, Regentin R, Secrest S, Tsuruta H, Vazquez R, Westblade LF, Xu L, Yu M, Zhang Y, Zhao L, Lievense J, Covello PS, Keasling JD, Reiling KK, Renninger NS, Newman JD (2013) High-level semi-synthetic production of the potent antimalarial artemisinin. Nature 496:528–532

Papanikolaou S, Aggelis G (2011) Lipids of oleaginous yeasts. Part I: biochemistry of single cell oil production. Eur J Lipid Sci Technol 113:1031–1051

Parks LW, Casey WM (1995) Physiological implications of sterol biosynthesis in yeast. Annu Rev Microbiol 49:95–116

Passoth V (2014) Molecular mechanisms in yeast carbon metabolism: bioethanol and other biofuels. In: Piškur J, Compagno C (eds) Molecular mechanisms in yeast carbon metabolism. Springer, Berlin, pp 217–259

Passoth V (2017) Conventional and non-conventional yeasts for the production of biofuels. In: Satyanarayana T, Kunze G (eds) Yeast diversity in human welfare. Springer Science + Business Media, Singapore (in press)

Passoth V, Schnürer J (2003) Non-conventional yeasts in antifungal application. In: de Winde JH (ed) Functional genetics of industrial yeasts. Springer, Berlin, pp 297–329

Paulino BN, Pessôa MG, Mano MCR, Molina G, Neri-Numa IA, Pastore GM (2016) Current status in biotechnological production and applications of glycolipid biosurfactants. Appl Microbiol Biotechnol 100:10265–10293

Peralta-Yahya PP, Zhang F, del Cardayre SB, Keasling JD (2012) Microbial engineering for the production of advanced biofuels. Nature 488:320–328

Pronk JT, Steensma HY, van Dijken JP (1996) Pyruvate metabolism in *Saccharomyces cerevisiae*. Yeast 12:1607–1633

Pugh EL, Kates M (1979) Membrane-bound phospholipid desaturases. Lipids 14:159–165

Qiao K, Imam Abidi SH, Liu H, Zhang H, Chakraborty S, Watson N, Kumaran Ajikumar P, Stephanopoulos G (2015) Engineering lipid overproduction in the oleaginous yeast *Yarrowia lipolytica*. Metab Eng 29:56–65

Qiao K, Wasylenko TM, Zhou K, Xu P, Stephanopoulos G (2017) Lipid production in *Yarrowia lipolytica* is maximized by engineering cytosolic redox metabolism. Nat Biotechnol 35:173–177. Advance online publication

Ratledge C (2004) Fatty acid biosynthesis in microorganisms being used for single cell oil production. Biochimie 86:807–815

Ratledge C (2012) Omega-3 biotechnology: errors and omissions. Biotechnol Adv 30:1746–1747

Ratledge C (2013) Microbial oils: an introductory overview of current status and future prospects. OCL 20:D602

Ratledge C (2014) The role of malic enzyme as the provider of NADPH in oleaginous microorganisms: a reappraisal and unsolved problems. Biotechnol Lett 36:1557–1568

Ratledge C, Wynn JP (2002) The biochemistry and molecular biology of lipid accumulation in oleaginous microorganisms. Adv Appl Microbiol 51:1–51

Rau U, Nguyen LA, Roeper H, Koch H, Lang S (2005) Fed-batch bioreactor production of mannosylerythritol lipids secreted by *Pseudozyma aphidis*. Appl Microbiol Biotechnol 68:607–613

Reinertsen SA, Elliot LF, Cochran VL, Campbell GS (1984) Role of available carbon and nitrogen in determining the rate of wheat straw decomposition. Soil Biol Biochem 16:459–464

Ro DK, Paradise EM, Ouellet M, Fisher KJ, Newman KL, Ndungu JM, Ho KA, Eachus RA, Ham TS, Kirby J, Chang MC, Withers ST, Shiba Y, Sarpong R, Keasling JD (2006) Production of the antimalarial drug precursor artemisinic acid in engineered yeast. Nature 440:940–943

Rodriguez GM, Hussain MS, Gambill L, Gao D, Yaguchi A, Blenner M (2016) Engineering xylose utilization in *Yarrowia lipolytica* by understanding its cryptic xylose pathway. Biotechnol Biofuels 9:149

Rodríguez-Frómeta RA, Gutiérrez A, Torres-Martínez S, Garre V (2013) Malic enzyme activity is not the only bottleneck for lipid accumulation in the oleaginous fungus *Mucor circinelloides*. Appl Microbiol Biotechnol 97:3063–3072

Rodriguez-Saiz M, de la Fuente JL, Barredo JL (2010) *Xanthophyllomyces dendrorhous* for the industrial production of astaxanthin. Appl Microbiol Biotechnol 88:645–658

Roelants SL, Saerens KM, Derycke T, Li B, Lin YC, Van de Peer Y, De Maeseneire SL, Van Bogaert IN, Soetaert W (2013) *Candida bombicola* as a platform organism for the production of tailor-made biomolecules. Biotechnol Bioeng 110:2494–2503

Roelants SLKW, De Maeseneire SL, Ciesielska K, Van Bogaert INA, Soetaert W (2014) Biosurfactant gene clusters in eukaryotes: regulation and biotechnological potential. Appl Microbiol Biotechnol 98:3449–3461

Runguphan W, Keasling JD (2014) Metabolic engineering of *Saccharomyces cerevisiae* for production of fatty acid-derived biofuels and chemicals. Metab Eng 21:103–113

Saenge C, Cheirsilp B, Suksaroge TT, Bourtoom T (2011) Potential use of oleaginous red yeast *Rhodotorula glutinis* for the bioconversion of crude glycerol from biodiesel plant to lipids and carotenoids. Process Biochem 46:210–218

Saerens KMJ, Roelants SLKW, Van Bogaert INA, Soetaert W (2011a) Identification of the UDP-glucosyltransferase gene *UGTA1*, responsible for the first glucosylation step in the sophorolipid biosynthetic pathway of *Candida bombicola* ATCC 22214. FEMS Yeast Res 11:123–132

Saerens KMJ, Saey L, Soetaert W (2011b) One-step production of unacetylated sophorolipids by an acetyltransferase negative *Candida bombicola*. Biotechnol Bioeng 108:2923–2931

Saerens KMJ, Zhang J, Saey L, Van Bogaert INA, Soetaert W (2011c) Cloning and functional characterization of the UDP-glucosyltransferase UgtB1 involved in sophorolipid production by *Candida bombicola* and creation of a glucolipid-producing yeast strain. Yeast 28:279–292

Sakuradani E, Ando A, Shimizu S, Ogawa J (2013) Metabolic engineering for the production of polyunsaturated fatty acids by oleaginous fungus *Mortierella alpina* 1S-4. J Biosci Bioeng 116:417–422

Sankh S, Thiru M, Saran S, Rangaswamy V (2013) Biodiesel production from a newly isolated *Pichia kudriavzevii* strain. Fuel 106:690–696

Santiago-Tirado FH, Doering TL (2016) All about that fat: lipid modification of proteins in *Cryptococcus neoformans*. J Microbiol 54:212–222

Scharnewski M, Pongdontri P, Mora G, Hoppert M, Fulda M (2008) Mutants of *Saccharomyces cerevisiae* deficient in acyl-CoA synthetases secrete fatty acids due to interrupted fatty acid recycling. FEBS J 275:2765–2778

Schmidt I, Schewe H, Gassel S, Jin C, Buckingham J, Hümbelin M, Sandmann G, Schrader J (2011) Biotechnological production of astaxanthin with *Phaffia rhodozyma*/*Xanthophyllomyces dendrorhous*. Appl Microbiol Biotechnol 89:555–571

Schwartz C, Shabbir-Hussain M, Frogue K, Blenner M, Wheeldon I (2016) Standardized markerless gene integration for pathway engineering in *Yarrowia lipolytica*. ACS Synth Biol 6(3):402–409

Shah V, Doncel GF, Seyoum T, Eaton KM, Zalenskaya I, Hagver R, Azim A, Gross R (2005) Sophorolipids, microbial glycolipids with anti-human immunodeficiency virus and sperm-immobilizing activities. Antimicrob Agents Chemother 49:4093–4100

Shi S, Valle-Rodríguez JO, Siewers V, Nielsen J (2014) Engineering of chromosomal wax ester synthase integrated *Saccharomyces cerevisiae* mutants for improved biosynthesis of fatty acid ethyl esters. Biotechnol Bioeng 111:1740–1747

Shi S, Ji H, Siewers V, Nielsen J (2016) Improved production of fatty acids by *Saccharomyces cerevisiae* through screening a cDNA library from the oleaginous yeast *Yarrowia lipolytica*. FEMS Yeast Res 16:fov108

Shiba Y, Paradise EM, Kirby J, Ro D-K, Keasling JD (2007) Engineering of the pyruvate dehydrogenase bypass in *Saccharomyces cerevisiae* for high-level production of isoprenoids. Metab Eng 9:160–168

Shimizu S, Shinmen Y, Kawashima H, Akimoto K, Yamada H (1988) Fungal mycelia as a novel source of eicosapentaenoic acid. Biochem Biophys Res Commun 150:335–341

Shimizu S, Kawashima H, Akimoto K, Shinmen Y, Yamada H (1989) Conversion of linseed oil to an eicosapentaenoic acid-containing oil by *Mortierella alpina* 1S-4 at low temperature. Appl Microbiol Biotechnol 32:1–4

Simopoulos AP (2006) Evolutionary aspects of diet, the omega-6/omega-3 ratio and genetic variation: nutritional implications for chronic diseases. Biomed Pharmacother 60:502–507

Sinha M, Sørensen A, Ahamed A, Ahring BK (2015) Production of hydrocarbons by *Aspergillus carbonarius* ITEM 5010. Fungal Biol 119:274–282

Sinha M, Weyda I, Sørensen A, Bruno KS, Ahring BK (2017) Alkane biosynthesis by *Aspergillus carbonarius* ITEM 5010 through heterologous expression of *Synechococcus elongatus* acyl-ACP/CoA reductase and aldehyde deformylating oxygenase genes. AMB Express 7:18

Sitepu IR, Garay LA, Sestric R, Levin D, Block DE, German JB, Boundy-Mills KL (2014) Oleaginous yeasts for biodiesel: current and future trends in biology and production. Biotechnol Adv 32:1336–1360

Slininger PJ, Dien BS, Kurtzman CP, Moser BR, Bakota EL, Thompson SR, O'Bryan PJ, Cotta MA, Balan V, Jin M, Sousa Lda C, Dale BE (2016) Comparative lipid production by oleaginous yeasts in hydrolyzates of lignocellulosic biomass and process strategy for high titers. Biotechnol Bioeng 113:1676–1690

Soccol CR, Dalmas Neto CJ, Soccol VT, Sydney EB, da Costa ES, Medeiros AB, Vandenberghe LP (2016) Pilot scale biodiesel production from microbial oil of *Rhodosporidium toruloides*

DEBB 5533 using sugarcane juice: performance in diesel engine and preliminary economic study. Bioresour Technol 223:259–268
Sorger D, Daum G (2003) Triacylglycerol biosynthesis in yeast. Appl Microbiol Biotechnol 61:289–299
Soxhlet F (1879) Die gewichtsanalytische Bestimmung des Milchfettes. Dinglers Polytech J 232:461–465
Tai M, Stephanopoulos G (2013) Engineering the push and pull of lipid biosynthesis in oleaginous yeast *Yarrowia lipolytica* for biofuel production. Metab Eng 15:1–9
Takeno S, Sakuradani E, Murata S, Inohara-Ochiai M, Kawashima H, Ashikari T, Shimizu S (2005a) Molecular evidence that the rate-limiting step for the biosynthesis of arachidonic acid in *Mortierella alpina* is at the level of an elongase. Lipids 40:25–30
Takeno S, Sakuradani E, Tomi A, Inohara-Ochiai M, Kawashima H, Ashikari T, Shimizu S (2005b) Improvement of the fatty acid composition of an oil-producing filamentous fungus, *Mortierella alpina* 1S-4, through RNA interference with delta12-desaturase gene expression. Appl Environ Microbiol 71:5124–5128
Tang W, Zhang S, Tan H, Zhao Z (2010) Molecular cloning and characterization of a malic enzyme gene from the oleaginous yeast *Lipomyces starkeyi*. Mol Biotechnol 45:121–128
Tang X, Feng H, Chen WN (2013) Metabolic engineering for enhanced fatty acids synthesis in *Saccharomyces cerevisiae*. Metab Eng 16:95–102
Tang X, Chen H, Chen YQ, Chen W, Garre V, Song Y, Ratledge C (2015) Comparison of biochemical activities between high and low lpid-producing strains of *Mucor circinelloides*: an explanation for the high oleaginicity of strain WJ11. PLoS One 10:e0128396
Tanimura A, Takashima M, Sugita T, Endoh R, Kikukawa M, Yamaguchi S, Sakuradani E, Ogawa J, Ohkuma M, Shima J (2014) *Cryptococcus terricola* is a promising oleaginous yeast for biodiesel production from starch through consolidated bioprocessing. Sci Rep 4:4776
Tchakouteu SS, Kalantzi O, Gardeli C, Koutinas AA, Aggelis G, Papanikolaou S (2015) Lipid production by yeasts growing on biodiesel-derived crude glycerol: strain selection and impact of substrate concentration on the fermentation efficiency. J Appl Microbiol 118:911–927
Tehlivets O, Scheuringer K, Kohlwein SD (2007) Fatty acid synthesis and elongation in yeast. Biochim Biophys Acta 1771:255–270
Teichmann B, Linne U, Hewald S, Marahiel MA, Bölker M (2007) A biosynthetic gene cluster for a secreted cellobiose lipid with antifungal activity from *Ustilago maydis*. Mol Microbiol 66:525–533
Teichmann B, Labbé C, Lefebvre F, Bölker M, Linne U, Bélanger RR (2011) Identification of a biosynthesis gene cluster for flocculosin a cellobiose lipid produced by the biocontrol agent *Pseudozyma flocculosa*. Mol Microbiol 79:1483–1495
Thiru M, Sankh S, Rangaswamy V (2011) Process for biodiesel production from *Cryptococcus curvatus*. Bioresour Technol 102:10436–10440
Tippmann S, Scalcinati G, Siewers V, Nielsen J (2016) Production of farnesene and santalene by *Saccharomyces cerevisiae* using fed-batch cultivations with RQ-controlled feed. Biotechnol Bioeng 113:72–81
Tocher DR (2015) Omega-3 long-chain polyunsaturated fatty acids and aquaculture in perspective. Aquaculture 449:94–107
Uçkun Kiran E, Trzcinski A, Webb C (2013) Microbial oil produced from biodiesel by-products could enhance overall production. Bioresour Technol 129:650–654
Uprety BK, Dalli SS, Rakshit SK (2017) Bioconversion of crude glycerol to microbial lipid using a robust oleaginous yeast *Rhodosporidium toruloides* ATCC 10788 capable of growing in the presence of impurities. Energy Convers Manag 135:117–128
Van Bogaert IN, De Maeseneire SL, Develter D, Soetaert W, Vandamme EJ (2008a) Cloning and characterisation of the glyceraldehyde 3-phosphate dehydrogenase gene of *Candida bombicola* and use of its promoter. J Ind Microbiol Biotechnol 35:1085–1092

Van Bogaert IN, De Maeseneire SL, Develter D, Soetaert W, Vandamme EJ (2008b) Development of a transformation and selection system for the glycolipid-producing yeast *Candida bombicola*. Yeast 25:273–278

Van Bogaert INA, Zhang J, Soetaert W (2011) Microbial synthesis of sophorolipids. Process Biochem 46:821–833

Van Bogaert INA, Holvoet K, Roelants SLKW, Li B, Lin Y-C, Van de Peer Y, Soetaert W (2013) The biosynthetic gene cluster for sophorolipids: a biotechnological interesting biosurfactant produced by *Starmerella bombicola*. Mol Microbiol 88:501–509

Van Bogaert IN, Buyst D, Martins JC, Roelants SL, Soetaert WK (2016) Synthesis of bolaform biosurfactants by an engineered *Starmerella bombicola* yeast. Biotechnol Bioeng 113:2644–2651

Verdoes JC, Krubasik KP, Sandmann G, van Ooyen AJ (1999a) Isolation and functional characterisation of a novel type of carotenoid biosynthetic gene from *Xanthophyllomyces dendrorhous*. Mol Gen Genet 262:453–461

Verdoes JC, Misawa N, van Ooyen AJ (1999b) Cloning and characterization of the astaxanthin biosynthetic gene encoding phytoene desaturase of *Xanthophyllomyces dendrorhous*. Biotechnol Bioeng 63:750–755

Vongsangnak W, Ruenwai R, Tang X, Hu X, Zhang H, Shen B, Song Y, Laoteng K (2013) Genome-scale analysis of the metabolic networks of oleaginous Zygomycete fungi. Gene 521:180–190

Vorapreeda T, Thammarongtham C, Cheevadhanarak S, Laoteng K (2013) Repertoire of malic enzymes in yeast and fungi: insight into their evolutionary functional and structural significance. Microbiology 159:2548–2557

Wang CW (2015) Lipid droplet dynamics in budding yeast. Cell Mol Life Sci 72:2677–2695

Wang W, Wei H, Knoshaug E, Van Wychen S, Xu Q, Himmel ME, Zhang M (2016) Fatty alcohol production in *Lipomyces starkeyi* and *Yarrowia lipolytica*. Biotechnol Biofuels 9:227

Wang F, Lv X, Xie W, Zhou P, Zhu Y, Yao Z, Yang C, Yang X, Ye L, Yu H (2017) Combining Gal4p-mediated expression enhancement and directed evolution of isoprene synthase to improve isoprene production in *Saccharomyces cerevisiae*. Metab Eng 39:257–266

Watanabe NA, Miyazaki M, Horii T, Sagane K, Tsukahara K, Hata K (2012) E1210, a new broad-spectrum antifungal, suppresses *Candida albicans* hyphal growth through inhibition of glycosylphosphatidylinositol biosynthesis. Antimicrob Agents Chemother 56:960–971

Westfall PJ, Pitera DJ, Lenihan JR, Eng D, Woolard FX, Regentin R, Horning T, Tsuruta H, Melis DJ, Owens A, Fickes S, Diola D, Benjamin KR, Keasling JD, Leavell MD, McPhee DJ, Renninger NS, Newman JD, Paddon CJ (2012) Production of amorphadiene in yeast, and its conversion to dihydroartemisinic acid, precursor to the antimalarial agent artemisinin. Proc Natl Acad Sci U S A 109:E111–E118

Wu S, Hu C, Jin G, Zhao X, Zhao ZK (2010) Phosphate-limitation mediated lipid production by *Rhodosporidium toruloides*. Bioresour Technol 101:6124–6129

Wu S, Zhao X, Shen H, Wang Q, Zhao ZK (2011) Microbial lipid production by *Rhodosporidium toruloides* under sulfate-limited conditions. Bioresour Technol 102:1803–1807

Wynn JP, Ratledge C (2000) Evidence that the rate-limiting step for the biosynthesis of arachidonic acid in *Mortierella alpina* is at the level of the 18:3 to 20:3 elongase. Microbiology 146(Pt 9):2325–2331

Wynn JP, Hamid AA, Li Y, Ratledge C (2001) Biochemical events leading to the diversion of carbon into storage lipids in the oleaginous fungi *Mucor circinelloides* and *Mortierella alpina*. Microbiology 147:2857–2864

Xu P, Qiao K, Ahn WS, Stephanopoulos G (2016) Engineering *Yarrowia lipolytica* as a platform for synthesis of drop-in transportation fuels and oleochemicals. Proc Natl Acad Sci U S A 113:10848–10853

Xue Z, Sharpe PL, Hong S-P, Yadav NS, Xie D, Short DR, Damude HG, Rupert RA, Seip JE, Wang J, Pollak DW, Bostick MW, Bosak MD, Macool DJ, Hollerbach DH, Zhang H, Arcilla DM, Bledsoe SA, Croker K, McCord EF, Tyreus BD, Jackson EN, Zhu Q (2013) Production of

omega-3 eicosapentaenoic acid by metabolic engineering of *Yarrowia lipolytica*. Nat Biotechnol 31:734–740

Yamamoto K, Hara KY, Morita T, Nishimura A, Sasaki D, Ishii J, Ogino C, Kizaki N, Kondo A (2016) Enhancement of astaxanthin production in *Xanthophyllomyces dendrorhous* by efficient method for the complete deletion of genes. Microb Cell Fact 15:155

Ye L, Lv X, Yu H (2016) Engineering microbes for isoprene production. Metab Eng 38:125–138

Yen H-W, Yang Y-C, Yu Y-H (2012) Using crude glycerol and thin stillage for the production of microbial lipids through the cultivation of *Rhodotorula glutinis*. J Biosci Bioeng 114:453–456

Yu KO, Jung J, Kim SW, Park CH, Han SO (2012) Synthesis of FAEEs from glycerol in engineered *Saccharomyces cerevisiae* using endogenously produced ethanol by heterologous expression of an unspecific bacterial acyltransferase. Biotechnol Bioeng 109:110–115

Yu X, Dong T, Zheng Y, Miao C, Chen S (2015) Investigations on cell disruption of oleaginous microorganisms: hydrochloric acid digestion is an effective method for lipid extraction. Eur J Lipid Sci Technol 117:730–737

Zhang Y, Adams IP, Ratledge C (2007) Malic enzyme: the controlling activity for lipid production? Overexpression of malic enzyme in *Mucor circinelloides* leads to a 2.5-fold increase in lipid accumulation. Microbiology 153:2013–2025

Zhang F, Rodriguez S, Keasling JD (2011) Metabolic engineering of microbial pathways for advanced biofuels production. Curr Opin Biotechnol 22:775–783

Zhang H, Zhang L, Chen H, Chen YQ, Ratledge C, Song Y, Chen W (2013) Regulatory properties of malic enzyme in the oleaginous yeast, *Yarrowia lipolytica*, and its non-involvement in lipid accumulation. Biotechnol Lett 35:2091–2098

Zhang H, Wu C, Wu Q, Dai J, Song Y (2016a) Metabolic flux analysis of lipid biosynthesis in the yeast *Yarrowia lipolytica* using ^{13}C-Labled glucose and gas chromatography-mass spectrometry. PLoS One 11:e0159187

Zhang S, Ito M, Skerker JM, Arkin AP, Rao CV (2016b) Metabolic engineering of the oleaginous yeast *Rhodosporidium toruloides* IFO0880 for lipid overproduction during high-density fermentation. Appl Microbiol Biotechnol 100:9393–9405

Zhang S, Skerker JM, Rutter CD, Maurer MJ, Arkin AP, Rao CV (2016c) Engineering *Rhodosporidium toruloides* for increased lipid production. Biotechnol Bioeng 113:1056–1066

Zhao L, Zhang H, Wang L, Chen H, Chen YQ, Chen W, Song Y (2015) ^{13}C-metabolic flux analysis of lipid accumulation in the oleaginous fungus *Mucor circinelloides*. Bioresour Technol 197:23–29

Zhao L, Cánovas-Márquez JT, Tang X, Chen H, Chen YQ, Chen W, Garre V, Song Y, Ratledge C (2016) Role of malate transporter in lipid accumulation of oleaginous fungus *Mucor circinelloides*. Appl Microbiol Biotechnol 100:1297–1305

Zhou YJ, Buijs NA, Siewers V, Nielsen J (2014) Fatty acid-derived biofuels and chemicals production in *Saccharomyces cerevisiae*. Front Bioeng Biotechnol 2:32

Zhou YJ, Buijs NA, Zhu Z, Qin J, Siewers V, Nielsen J (2016) Production of fatty acid-derived oleochemicals and biofuels by synthetic yeast cell factories. Nat Commun 7:11709

Zhu Z, Ding Y, Gong Z, Yang L, Zhang S, Zhang C, Lin X, Shen H, Zou H, Xie Z, Yang F, Zhao X, Liu P, Zhao ZK (2015) Dynamics of the lipid droplet proteome of the oleaginous yeast *Rhodosporidium toruloides*. Eukaryot Cell 14:252–264

Production of Organic Acids by Yeasts and Filamentous Fungi

7

Danilo Porro and Paola Branduardi

Abstract

Organic acids are broadly distributed in nature, and humans used them in their natural sources since the early ages, essentially for food and drink preparations. Organic acids are low-molecular-weight organic compounds with one or more acidic groups such as carboxyl, sulfonic, alcohol and thiol groups. These functional groups make organic acids key building block chemicals, which have huge markets and are still commonly refined from petroleum. Indeed, during the first half of the twentieth century, advances in chemical synthesis offered competitive manufacturing processes. This scenario changed starting from the 1990s when development of the industrial biotechnology field, environmental issues and the integration of biomasses processing industries, fermentation and downstream procedures, yielded improved economics for the biological pathways.

In this chapter, we mainly review the aspects of current or piloting large-scale successful industrial processes for yeast or fungal organic acid productions.

Perspectives are also reported.

Contents

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7.1 Introduction

Building block chemicals as molecules with multiple functional groups having the potential to be transformed into new families of useful high-value bio-based chemicals or biomaterials. Organic acids are low-molecular-weight organic compounds with one or more acidic groups and are considered key building block chemicals, which have huge markets and are commonly refined from petroleum.

On 2004, the US Department of Energy (DOE) (Werpy et al. 2004) identified 12 building block chemicals that can be produced from sugars via biological or chemical conversions (Box 7.1). Since this first report, many studies have been carried out to develop microbial cell factories and accompanying biorefineries for their industrial productions. Biorefinery development has two strategic goals: the displacement of petroleum in favour of renewable raw materials (i.e. mainly an energy goal) and the establishment of a robust bio-based industry (an environmental and economic goal).

Box 7.1: The 2004 DOE's and Revised 2010 Top Sugar-Derived Building Block Chemicals

2004 (Werpy et al. 2004)
1,4 Diacids (Succinic acid, Fumaric acid and Malic acid), 2,5 Furan dicarboxylic acid, 3 Hydroxy propionic acid, Aspartic acid, Glucaric acid, Glutamic acid, Itaconic acid, Levulinic acid, 3-Hydroxybutyrolactone, Glycerol, Sorbitol, Aylitol/Arabinitol.

2010 (Bozell and Petersen 2010)
Ethanol, Furans, Glycerol and derivatives, Biohydrocarbons, Lactic acid, Succinic acid, 3Hydroxypropionic acid/aldehyde, Levulinic acid, Sorbitol, Xylitol.

About 70% of the first top 12 building blocks suggested on 2004 are organic acids. All of them, including **succinic acid**, **2,5-furan dicarboxylicacid**,

3-hydroxypropionic acid (3-HPA), **aspartic acid**, **glutamic acid**, **itaconic acid** and **levulinic acid**, have been already or are being commercialized. Bio-based production of **glucaric acid** (included in the top 12 list) has been actively pursued by Rivertop Renewables, and it is awaiting for commercial announcement like recently declared by BIO (Biotechnology Innovation Organization 2016). Furthermore, among the top ten chemicals revisited in the 2010 report (Bozell and Petersen 2010), see Box 7.1, also **lactic acid** has been already commercialized. In addition to these organic acids, several companies are currently piloting production of **adipic acid** from glucaric acid, which is among the top building blocks. Adipic acid is a precursor to nylon and can be used in coatings and detergents. In this respect, Rennovia is currently operating a pilot project at the Johnson Matthey Process Technologies R&D Center in Stockton, England, converting bio-based glucaric acid to adipic acid. Verdezyne is also operating a pilot production facility in Carlsbad, California, and BioAmber has formed a partnership with Celexion to produce adipic acid from succinic acid. Genomatica, California, began efforts in 2014 to commercialize bio-based production of adipic acid and other nylon intermediates (reviewed by Biotechnology Innovation Organization 2016). Even if not included in the top building block chemicals, also **acrylic** and the aliphatic diacid **1,18-octadecanedioic acids** (ODDA) deserve attention. Acrylic acid is polymerized and used as an absorbent in diapers and hygiene products as well as in coatings, adhesives, carpets and fabrics. Cargill is operating a pilot scale production plant fermenting 3-HPA and converting it to acrylic acid. Cargill acquired Colorado-based OPX Biotechnologies and its proprietary of a fermentation-based process for 3-HPA. The long chain dicarboxylic acid (ODDA) is also a building block for polyurethanes and polyamides. Elevance Renewable Sciences is producing Inherent™ C18 diacid at a biorefinery in Gresik, Indonesia, using the company's proprietary olefin metathesis chemical technology (Biotechnology Innovation Organization 2016) (Elevance Renewable Sciences claimed a microbial pathway too, see Conclusions and Perspectives).

About **36%** (succinic, fumaric, itaconic and lactic acids) of the 2004-DOE's and 2010 derived top organic chemicals are produced by yeast or fungi, while the remaining are essentially still produced by chemical synthesis (about 45%; malic, 2,5 furan dicarboxylic, aspartic, glucaric and levulinic acids) or from bacteria cells (about 18%; 3 hydroxypropionic acid and glutamic acids) Table 7.1 and Fig. 7.1.

Table 7.1 Production of the 2004 DOE's and 2010 revised top organic acids: overview of the commercialization or demonstration-pilot scale successes

–2,5 Furan dicarboxylic acid
$C_6H_4O_5$, 156.09 g mol^{-1}, pK_a 2.28
ADM (USA), Ava Biochem (CH), Avantium (NL), Corbion (NL)
–Chemical (dehydration and oxydation)
–3 Hydroxy propionic acid
$C_3H_6O_3$, 90.08 g mol^{-1}, pK_a 4.87
Cargill (USA) and Novozymes (DK), Perstorp (S)
–Fermentation (*Escherichia coli)*
–Aspartic acid
$C_4H_7NO_4$, 133.10 g mol^{-1}, pK_a 3.9
Ajinomoto (J), Grace Fine Chemical Manufacturing (USA)
–Enzymatic conversion from Fumaric acid
–Fumaric acid
$C_4H_4O_4$, 116.07 g mol^{-1}, pK_{a1} 3.03, pK_{a2} 4.44
MBI international (USA), Myriant corporation (USA)
–Chemical (oxydation and isomerization) and Fermentation (*Rhizopus oryzae*)
–Glucaric acid
$C_6H_{10}O_8$, 210.138 g mol^{-1}, pK_a 2.83
Cargill (USA), Rennovia Inc. (USA), Rivertop renewables (USA)
–Chemical (oxidation)
–Glutamic acid
$C_5H_9NO_4$, 147.13 g mol^{-1}, pK_{a1} 2.10, pK_{a2} 4.07
Many companies
–Fermentation (*Corynebacterium glutammicum*)
–Itaconic acid
$C_5H_6O_4$, 130.10 g mol^{-1}, pK_{a1} 3.85, pK_{a2} 5.45
Cargill (USA), Itaconix corporation (USA), Qingdao kehai biochemistry Co., Ltd (CHN)
–Fermentation (*Aspergillus terreus*)
–Lactic acid
$C_3H_6O_3$, 90.07948 g mol^{-1}, pK_a 3.86
Blue Marble Biomaterials (USA), Calysta (USA), Cargill (USA), Corbion (NL), Galactic (USA), Glycosbio (USA), Myriant Corporation (USA), Natureworks (PLA) (USA), Plaxica (UK), PTT Global Chemical (also PLA) (Thailand), Solegear (PLA) (CAN)
–Fermentation (yeast)
–Levulinic acid
$C_5H_8O_3$, 116.11 g mol^{-1}, pK_a 4.78
Arzeda (USA), Avantium (NL), Biofine Technology LLC (USA), GF Biochemicals (IT), Segetis (USA)
–Chemical (acid treatment—dehydration, hydration)
–Malic acid
$C_4H_6O_5$, 134.09 g mol^{-1}, pK_{a1} 3.40, pK_{a2} 5.20
Different companies

(continued)

Table 7.1 (continued)

–Chemical and Fermentation (malic acid is currently produced from fumaric or maleic acid, both derived from maleic anhydride. The conversion from fumaric to malic is done using fermentation)
–*Succinic acid*
$C_4H_6O_4$, 118.09 g mol^{-1}, pK_{a1} 4.2, pK_{a2} 5.6
Bioamber (USA), Myriant (USA), Reverdia (NL), Succinity (D)
–Fermentation (yeast)

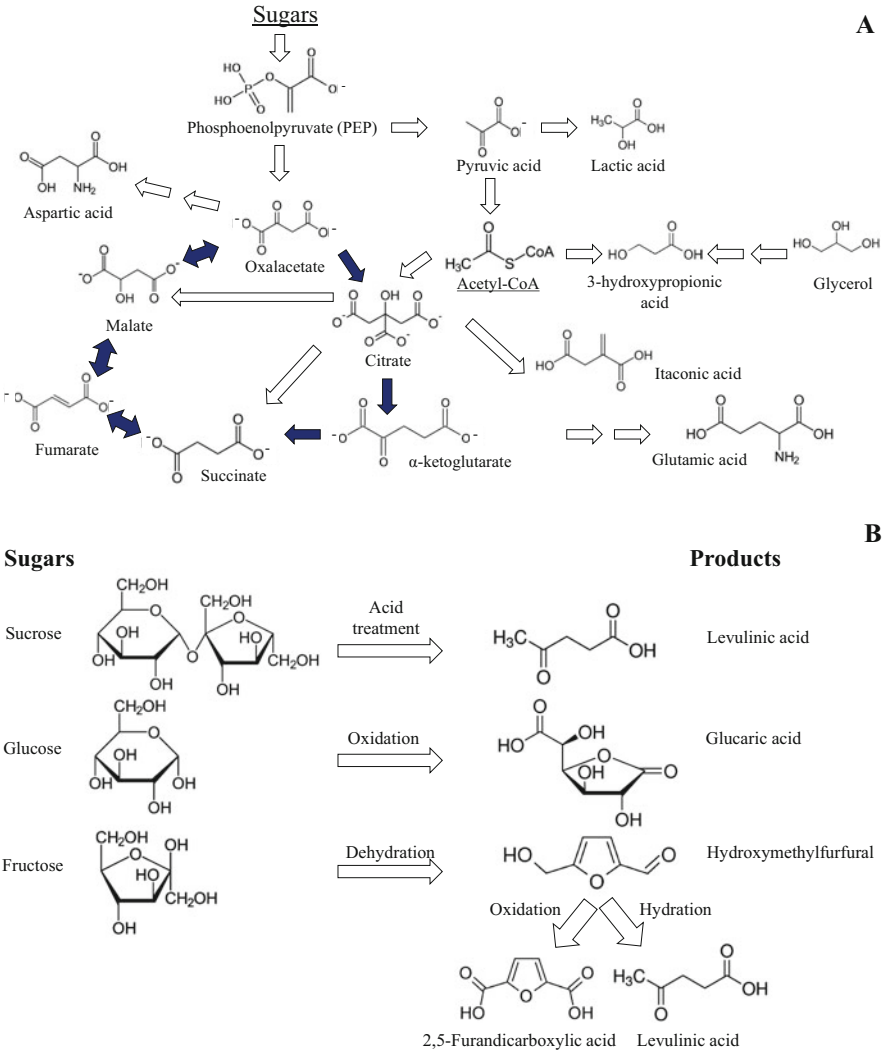

Fig. 7.1 Metabolic and chemical pathways for the production of the 2004 DOE's and top ten revised organic acids from biomass [redrawn from Choi et al. (2015)]

7.2 Renewable Yeast and Fungal Organic Acids Commercialization or Demonstration-Pilot Scale Successes

From a chronological point of view, citric acid has been the first organic acid industrially produced by wild type/recombinant yeast/fungi followed by lactic acid, fumaric acid, succinic acid and itaconic acid.

7.2.1 Citric Acid (2 Million Tons/Year)

Citric acid (2-hydroxy-propane-1,2,3-tricarboxylic acid) ($C_6H_8O_7$, 192.12 g mol^{-1}, $pK_{a1} = 3.13$, $pK_{a2} = 4.76$, $pK_{a3} = 6.39$) is a key intermediate metabolite in the TCA cycle and derives its name from the Latin word citrus, the citrus tree, the fruit of which resembles a lemon. Citric acid is a universal intermediate product of metabolism, and its traces are found in virtually all plants and animals. The acid was first isolated from lemon juice in 1784 by Carl Scheele, a Swedish chemist (1742–1786). Citric acid was first produced commercially in England around 1826 from imported Italian lemons (Papagianni 2007). At around 1880, the firm Charles Pfizer developed a chemical synthesis route. Different synthetic routes using different starting materials have since been published.

In the early twentieth century, surface culture methods using *Aspergillus niger* were implemented and in the middle of the century further developed towards a submerged process. Global citric acid production has reached 2 million tons/year (Sauer et al. 2014). The vast majority of this is produced via fermentation using *A. niger* (Papagianni 2007) although many microorganisms can be used to produce citric acid (*A. wentii*, *A. awamori*, *A. foetidus*, *A. fenicis*, *A. fonsecalus*, *A. fumaricus*, *A. luchensis*, *A. saitoi* and *A. usumii*, *Candida lipolytica*, *C. tropicalis*, *C. guilliermondii*, *C. intermedia*, *C. parapsilosis*, *C. zeylanoides*, *C. fibriae*, *C. subtropicalix*, *C. oleophila* from the genus *Candida*, among others).

Specific strains that are capable of overproducing citric acid have been developed for various types of fermentation processes. The yield of citric acid from these strains often exceeds 70% of the theoretical yield on the carbon source (Papagianni 2007). The main companies producing citric acid are ADM (USA), Cargill (USA), Tate and Lyle (UK) and Vogelbusch Biocommodities GmbH (Austria). Citric acid is a well-known organic acid that is extensively used in the food and beverage industry as it combines a pleasant taste with low toxicity and palatability. It serves several functions in the food formulation, like sterilization, flavour fixation and enhancement, bacterial stabilization and standardization of acid levels. Furthermore, it can be used as a chelating agent as it efficiently complexes metal ions.

Despite a long and successful history of production of citric acid, there is still no straight explanation for the biochemical basis of the process to consistently explain the observed behaviour(s) of this fermentation. The critical parameters, which must be addressed to get an efficient production, include at least (1) high substrate concentration, (2) low and finite content of nitrogen and (3) certain trace metals,

thorough maintenance of (4) high dissolved oxygen and low (5) pH fermentation broth values. Many of the biochemical and physiological mechanisms underlying the process remain unknown. As simple sake of example of the complexity of the biochemical production, in *A. niger* citric acid itself, is a potent inhibitor of glycolysis. Citric acid effectively inhibits phosphofructokinase I (Pfk). Until recently it was generally accepted that a certain, relatively high, intracellular ammonium concentration would relieve this inhibition (Papagianni 2007; Sauer et al. 2008). However, Papagianni showed that the intracellular ammonium concentration is low throughout the citric acid production process (Papagianni et al. 2005). Legisa and Mattey described post-translational modifications of Pfk I triggered by a drop of the intracellular pH and a cAMP peak before the onset of citric acid accumulation (2007). The enzyme is cleaved and phosphorylated leading to a fragment that is highly active and highly inducible by ammonium, but less susceptible to citrate inhibition, which explains why glycolysis is not repressed. The findings described so far relate directly to metabolic pathways and enzymatic activities. However, the correct morphological appearance of the cells is also a crucial prerequisite for efficient organic acid production (Dai et al. 2004; Magnuson and Lasure 2004; Grimm et al. 2005; Berovic and Legisa 2007).

Growth in the form of pellets of <1 mm in diameter is associated with high production rates and yields (Magnuson and Lasure 2004). To date the conditions for obtaining such pellets have been determined empirically (Liu et al. 2008; Liao et al. 2007). The regulating signals involved remain unclear, but investigations are necessary and still ongoing. Because of all this and considering the ever-increasing demand for citric acid, alternative fermentation processes using high-yield yeast strains like *Yarrowia lipolytica* and different *Candida* species are investigated for its production (Sauer et al. 2014). Many other yeast species have been proved to accumulate citric acid. Among these are *Hansenula*, *Pichia*, *Debaromyces*, *Torula*, *Torulopsis*, *Kloekera*, *Saccharomyces* and *Zygosaccharomyces* species (Papagianni 2007).

In contrast with all the organic acid productions analysed in this section, citric acid production from *A. niger* is carried out in Air-Lift bioreactor (Vs Stirred Tank).

7.2.2 Lactic Acid (400,000 Tons/Year)

Fermentation processes that involve the production of lactic acid have been used in food preparation since centuries.

Lactic acid has been included in the promising platform chemicals in 2010, and it is commercially available and produced by microorganisms (Sauer et al. 2008, 2010). The market size of the lactic acid is about 400,000 tons/year (NNFCC 2010, NNFCC Renewable Chemicals Fact sheet: LacticAcid, available at: http://www.nnfcc.co.uk/publications/nnfcc-renewable-chemicals-factsheet-lactic-acid; Choi et al. 2015).

Many companies have already commercialized bio-based lactic acid. First productions have been obtained using bacterial cells. Lactic acid has versatile

properties as a precursor for various chemicals and materials, and about 40% of produced lactic acid has been used in manufacturing polylactic acid (PLA) (Bozell and Petersen 2010). Figure 7.2 shows the main compounds that can be obtained starting from lactic acid. Indeed, one of the earliest renewable chemicals to be commercialized has been polylactic acid (PLA), which was truly a tipping point for renewable chemicals. PLA is commonly used in food wrap and utensils and can be made into textile fibers. Since 2003 NatureWorks has produced PLA at a facility in Blair, Nebraska, with a capacity of 140,000 metric tons. NatureWorks markets the product as Ingeo® biopolymer. Corbion more recently announced that it will build a bio-based PLA plant with an annual capacity of 75 kilotons and expand by 25 kilotons/year its existing lactide plant in Rayong Province, Thailand (Biotechnology Innovation Organization 2016). PLA is made through sequential chemical reactions of lactide formation followed by polymerization, but it has been very elegantly proved that it can be also produced at intracellular level (Jung and Lee 2011). The costs for the required high degree of lactic acid purification still contribute significantly to the overall production costs, and this relates directly to the choice of the production organism. Lactic acid bacteria are natural producers of the desired acid. Processes based on lactic acid bacteria have been established long time ago. High concentrations of lactate (well over 100 g/L) and high volumetric productivities (10–23 g/L h) can be reached. Metabolic engineering allows the heterologous production of lactic acid with bacteria and yeasts. However, only the production from yeasts appears well suited for this task due to their tolerance to low pH conditions and the possibility to grow them on mineral media. Nevertheless, volumetric productivities are significantly lower compared to bacterial values under optimal conditions.

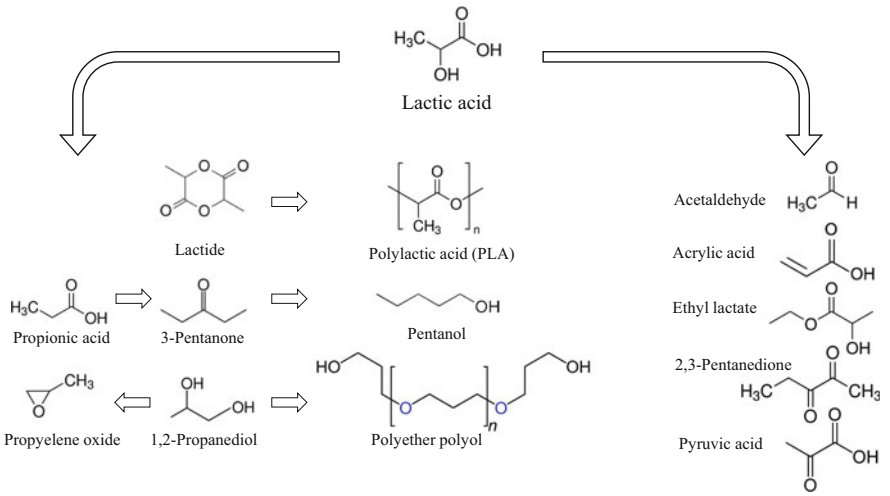

Fig. 7.2 Lactic acid and derivatives

For *Saccharomyces cerevisiae*, production rates of about 1 g/L h, reaching a pH of about 2.5 (well below the pK_a value, Table 7.1), have been published. While data for industrially achieved productivities are not available, the order of magnitude can be estimated from the values above.

Information about the properties of the engineered yeast is not available too. However, high yield and productions definitely require a strong limitation of the production of ethanol and the development of robust cell factories (Sauer et al. 2008, 2010).

They remain well behind the values of bacterial production hosts. Nevertheless, the advantage of the easier downstream processing outweighs this fact, so industrial production of lactic acid is taking place with recombinant yeasts as production host (Sauer et al. 2010; Porro and Branduardi 2012).

7.2.3 Fumaric Acid (90,000 Tons/Year)

Fumaric acid is a key intermediate metabolite in the TCA cycle. It was first isolated from the plant *Fumaria officinalis*, from which it derives its name.

The 2008 world's production of fumaric acid, about 90,000 tons/year, is derived overwhelmingly from petroleum (Roa Engel et al. 2008; MBI International 2011).

The majority of global fumaric acid production, approximately 60%, is used for the production of resins and unsaturated polyesters (UPRs). A more modest, though still significant (>30% of global production), portion of global fumaric acid production is used in food and beverage applications (Roa Engel et al. 2008). The use of fumaric acid in food applications is based upon its acidulent, preservative, flavour enhancing and dough improvement characteristics, which differentiate fumaric acid from other more general purpose acidulates, such as citric acid and phosphoric acid. The food and beverage application is an attractive initial entry point for a bio-based fumaric acid as it leads a higher price point and is an application where the bio-based manufacture can give a natural product a marketable differentiation over a petrochemical-derived product.

Although the current predominant route to fumaric acid is petrochemical, via maleic anhydride (using either benzene or butane as a feedstock) at high temperatures (90–100 °C), with high product yield (% w/w) 112 g/g (Lohbeck et al. 1990; Roa Engel et al. 2008), fermentation processes for the production of bio-based fumaric acid has been developed and reported in the literature, mainly using *Rhizopus* species (Roa Engel et al. 2008). Generally speaking, these first processes were limited in terms of commercial utility due to undesirable characteristics associated with the requirement for $CaCO_3$ as the neutralizing base (the process is conducted at close to neutral pH), being the calcium fumarate salt insoluble. As the insoluble product accumulates during the fermentation, the broth becomes thixotropic, increasing the viscosity, limiting oxygen mass transfer and increasing the power input required to keep the culture broth well mixed. Formation of an insoluble product also complicates the product recovery. A broth heating step is required to solubilize the calcium fumarate and allows it to be separated from the

biomass and other fermentation broth solids. The use of $CaCO_3$ as the base also results in the generation of gypsum ($CaSO_4 \cdot H_2O$) during the separation/purification of the fumaric acid. The gypsum by-product constitutes an industrial waste, and the associated cost of disposal negatively impacts the overall process economics (Gangl et al. 1990).

More recently, with the desire to replace petrochemical-derived chemicals and fuels with bio-based, renewable sources (due to economic, energetic and environmental concerns), the possibility of producing fumaric acid via fermentation became more attractive. This would be especially true if the major detrimental characteristics of the "traditional" fermentation process could be overcome. Roa Engel et al. 2011 studied the influence of the pH, working volume and shaking frequency on cell cultivation (*Rhizopus oryzae*) that helped to identify the best conditions to obtain appropriate pellet morphologies and interesting productions. Using these pellets, the effects of pH and CO_2 addition were studied to determine the best conditions to produce fumaric acid in batch fermentations under nitrogen-limited conditions with glucose as carbon source. CO_2 was supplied to achieve low pH instead of supplying carbonate salt. CO_2 is also used by the cells for pyruvate carboxylation. However, it has been shown that the productivity of the fermentation is not negatively affected when the inlet air is not blended with CO_2, suggesting that the CO_2 produced by the cells is sufficient to fulfil the CO_2 requirements for pyruvate carboxylation.

Decreasing either the fermentation pH below 5 or increasing the CO_2 content of the inlet air above 10% was unfavourable for the cell-specific productivity, fumaric acid yield (g/g) and fumaric acid titer (g/L). However, switching off the pH control late in the batch phase did not affect these performance parameters and allowed achieving pH of 3.6. Consequently, relatively modest amounts of inorganic base were required for pH control, while recovery of the acid is relatively easier at pH 3.6.

Concluding, a novel fermentation and one-step recovery process were developed that allowed produced titers of >80 g/L with a productivity of 1.8 g/L h and 0.57 (g/g glucose) yield. The process was scaled to 2000 L pilot scale. The economic analysis projected interesting production costs for an appropriate use in the food and beverage application.

Recycling and reuse of the base were demonstrated and incorporated into the process. The ability of the organism to produce fumaric acid from other carbon sources and biomass hydrolysate was also demonstrated (MBI International 2011).

7.2.4 Succinic Acid (270,000 Tons/Year)

Succinic acid is a chemical building block that replaces petroleum-based maleic anhydride in polyesters, alkyd resins, polyurethanes, plasticizers and solvents, also used in the manufacture of food and pharmaceuticals among other products, Fig. 7.3. The demand is predicted to expand to commodity chemical status with 270,000 tons/year (Choi et al. 2015). Succinic acid is a key intermediate metabolite in the TCA cycle and can be produced via the reductive or oxidative

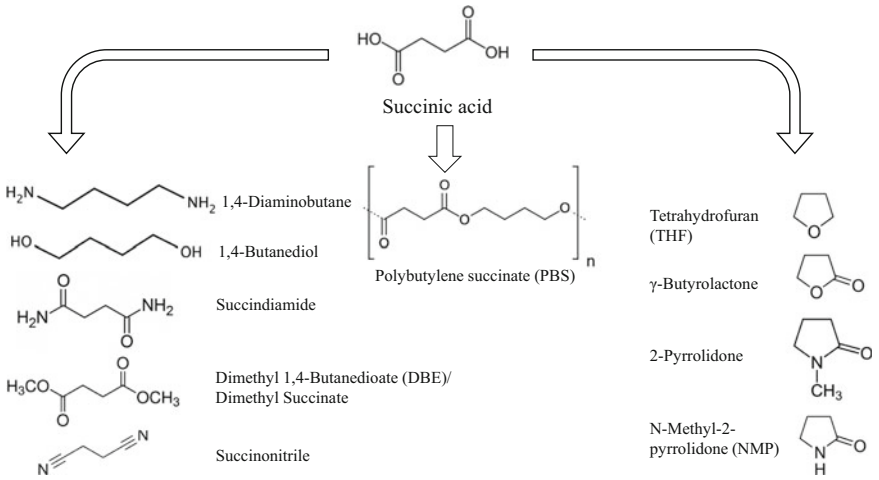

Fig. 7.3 Succinic acid and derivatives

pathway of TCA cycle or via the glyoxylate shunt (Fig. 7.1). Microbial production of succinic acid has been actively studied using diverse microorganisms including rumen bacteria such as *Actinobacillus succinogenes*, *Mannheimia succiniciproducens*, *Basfia succiniciproducens* and other microorganisms such as *Escherichia coli*, *Anaerobiospirillum succiniciproducens*, *Corynebacterium glutamicum* and *Saccharomyces cerevisiae* (Choi et al. 2015).

Several companies have very recently commercialized bio-based routes to succinic acid. Companies that are currently producing succinic acid include Myriant, which is operating a 13,600 metric ton per year facility in Lake Providence, Louisiana. BioAmber piloted its bio-based process for succinic acid at a 3000 metric ton facility in Pomacle, France, and is now producing 17,000 metric tons per year in Sarnia, Ontario, Canada. Reverdia, a joint venture between DSM and Roquette, has built a 10,000 metric ton per year facility in Cassano Spinola, Italy. And Succinity, a joint venture between BASF and Corbion, is due to start up a 25,000 metric ton per year facility in Barcelona, Spain (Biotechnology Innovation Organization 2016).

The company Reverdia recently announced that they have produced 100 g/L of succinic acid by fermentation of engineered *S. cerevisiae*. The proprietary yeast-based fermentation process, which operates at a much lower pH than competing processes, allows succinic acid to be produced with significantly higher energy efficiencies compared to the bacteria or chemical traditional method.

Data for industrially achieved productivities are not available; however the engineering strategy of the *S. cerevisiae* cell factory includes a combination of the reductive and oxidative TCA cycle (Raab and Lang 2011). In brief, a yeast was constructed with deletions for the genes of succinate DH and isocitrate DH, which redirected carbon flux into the glyoxylate cycle in the cytosol, thus bypassing two oxidative decarboxylation steps in the TCA cycle; at the same time, this redirection

circumvents problems of mitochondrial transport of succinate. The pathways leading to the accumulation of secondary metabolites like glycerol and ethanol have been deleted. In the cell factory, the overexpression of the transporter leading to the accumulation of succinic acid in the culture medium has also been considered. Remarkably, it should be underlined that the production of succinic acid from engineered *S. cerevisiae* cells is also one of the first bio-based processes sequestering carbon dioxide in the production process. The new production facility will, for the time being, use starch derivatives as feedstock. Of course, in the longer term, the aspiration is to switch to second-generation feedstocks (lignocellulosic biomass) (Porro and Branduardi 2012; Sauer et al. 2014).

7.2.5 Itaconic Acid (41,400 Tons/Year)

In contrast with all the organic acids analysed on this section, itaconic acid is used exclusively in non-food applications. Itaconic acid is not a primary metabolite, so both the anabolism and catabolism of this acid are relatively rare metabolic attributes.

This acid was selected as a top building block chemical in the 2004 report, but it was excluded in the 2010 report because the market size was not expanded as expected. However, itaconic acid is a building block for adhesives and sealants, finishing agents, paint and coating additives, detergents and cleaners, absorbents and dispersants (Fig. 7.4). Itaconic acid can replace banned chemical phosphates in detergents, and recently, one company, Itaconix Corporation, has commercialized the fermentation technology, using *Aspergillus terreus* for the production of this organic acid from carbohydrates. This company operates a large-scale production facility in Stratham, New Hampshire (USA), marketing a growing line of itaconic

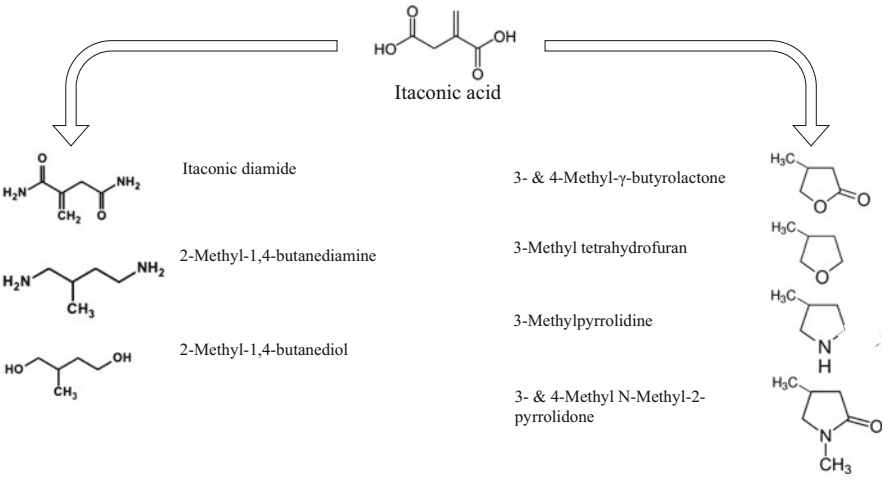

Fig. 7.4 Itaconic acid and derivatives

acid applications and polyitaconic acid. Itaconix recently announced its acquisition through merger by UK-based Revolymer plc, a specialty chemical company.

The market size is relatively small (41,400 tons/year in 2011—Choi et al. 2015), but it is projected to more than 410,000 tons/year in 2020 through its applications in producing diverse value-added products. The most promising market is a superabsorbent polymer such as poly(acrylamide-*co*-itaconic acid). This super absorbent polymer has been used as an absorbent for water and aqueous solutions including diapers and feminine hygiene products. While these superabsorbent polymers have been conventionally produced from poly(acrylic acid), the polymerized bio-based itaconic acid can be a good alternative due to its similar properties (Choi et al. 2015; see also Weastra sro).

In addition, itaconic acid can be converted to the methylmethacrylate (MMA), which is the monomer of poly(MMA), commercially known as Plexiglass. The current market size of MMA is over than 3.2 million tons/year, and thus the market size of itaconic acid can be potentially increased (Choi et al. 2015; see also Weastra sro).

7.3 Renewable Yeast and Fungal Organic Acids

In the previous section, we reviewed the commercial aspects of current or piloting large-scale industrial processes for yeast or fungal organic acid productions. In this respect, yeast and fungi are workhorses for the production of organic acids by fermentation. Like already underlined, one of the main reasons of this is the relatively robustness of these cell factories to low pH values. Yeast and fungi also found applications in the production of yeast-fungi biomasses, foods, feeds, enzymes, pharmaceuticals and fine and bulk chemicals (Porro and Branduardi 2012).

Organic acids are broadly distributed in nature and have attracted considerable attention for their role in natural ecology (Dutton and Evans 1996; Jones 1998; Liaud et al. 2014) and their potential industrial applications in any sector of a modern society. They could be produced at industrial level using wild-type or engineered cells. Yeast and fungi are already producers, even if not at economic level, of important organic acids like acetic acid, ascorbic acid, butyric acid, epoxysuccinic acid, formic acid, gallic acid, gluconic acid, isobutyric acid, isocitric acid, kojic acid, malic acid, oxalic acid, oxaloacetic acid, propionic acid, pyruvic acid, tartaric acid and α-ketoglutaric acid, to mention the most relevant.

Even prokaryotic cell factories can be used for the production of organic acids. However, these prokaryotic cells require neutral pH growth conditions and consequently secrete the salt forms of organic acids, which require cost-intensive acidification and precipitation for conversion to the desired products.

Advances and improvements in metabolic engineering and bioprocess development will make yeasts and fungi the best candidates for environmentally responsible and economically feasible fermentations processes for the production of organic acids.

7.4 Conclusions and Perspectives

Analysts predict a rapid expansion of renewable chemical production in the near future based on planned capacity expansion or new construction. McKinsey & Co. estimates that there were 204 billion euros in sales of bio-based products in 2012, with biofuels and plant extracts comprising more than half. Sales of renewable chemicals represented 9% of the 2281 billion euros in worldwide chemical sales in 2012. By 2020, McKinsey expects bio-based products to make up 11% of the 3130 billion euros global chemical market. Sales of bio-based products would reach 345–406 billion euros by 2020. While biofuels and plant extracts continue to comprise half of the projected sales of bio-based products in 2020, McKinsey expects the highest growth rates in sales of new biopolymers and renewable chemicals, biocatalysts for industrial processes and biologic medicines, as well as biofuels (Biotechnology Innovation Organization 2016).

In April 2016, the US White House Office of Science and Technology Policy released a report on Advanced Manufacturing. This roadmap emphasizes that growth of the bio-based economy is dependent on advanced bio-based manufacturing and engineering biology. According to the roadmap, synthetic biology (Glieder et al. 2016) will promote the commercial development of new renewable chemicals via faster and cheaper methodologies that use appropriate design of microorganisms.

In this context, the constraints for creating a viable bio-business with the production of commodities with a low selling price starting from renewable resources are still tight. In order to establish an economically viable industrial process, the classical parameters to fulfil are titer, yield and productivity (Porro et al. 2014). Titer (product concentration in the fermentation broth) is important to keep the purification costs as low as possible. Yield (product per substrate ratio) is important to gain sufficient revenue from the invested substrate costs. Productivity (product per bioreactor volume and time) relates to the size of the bioreactors and therefore to the investment costs. Traditionally, to achieve useful values of these parameters, the focus for strain development was strictly on the metabolic pathway responsible for the production (Bailey 1991).

Remarkable microbial successful commercial examples outside the yeast and fungi world are the production of octadecanedioic acid (ODDA) (this C18 diacid is now commercially available from two pathways, one using microbial fermentation—no more data are available—and one using metathesis of natural oils, Elevance Renewable Sciences, USA), acetone (Green Biologics, USA), artemisinin (Amyris, USA), biofuels (many companies in USA, EU, ASIA), bioisoprene (DuPont Ind. Biosciences, Goodyear, USA and Ajinomoto, J), hydrocortisone (Sanofi, F), isoprene (GlycosBio, USA), polyhydroxyalkanoate (PHA, Metabolix's, USA), polytetrahydrofuran (from renewable 1,4-BDO, BASF, D), resveratrol (Fluxome, DK), 1,2-propanediol (ADM, USA), 1,3 propanediol (1,3-PDO™, DuPont Tate & Lyle, USA) and 1,4-butanediol (1,4-BDO) (BASF, D) (Novamont, It), with many other new compounds at the pilot plant phase (Biotechnology Innovation Organization 2016; Choi et al. 2015; Chen and Nielsen 2016).

Analysing all these bioprocesses, it is clear that the rational engineering was first focused on overexpressing genes for enzymes involved in the pathway and knocking out genes responsible for competing reactions (to abolish by-product formation). However, in most cases, this is not sufficient to come even close to industrial relevance. Aspects related to the metabolic balance including (1) stoichiometry, (2) redox and (3) energy household need to be balanced. Furthermore, (4) flux coupling, (5) irreversible reactions and (6) process and cell factory robustness add to the efficiency of the pathway when the balances are fulfilled (Table 7.2).

This last property (i.e. cell robustness) deserves a deeper consideration. It is quite obvious that a functional proteome is a prerequisite for a stable phenotype of production when a cell factory is challenged during an industrial process. To cite Prof. Radman: "*the proteome sustains and maintains life, whereas the genome ensures the perpetuation of life by renewing the proteome, a process contingent on a preexisting proteome that repairs, replicates, and expresses the genome*" (Krisko and Radman 2013). However, very often exactly the conditions of an industrial process elicit damages to the proteome and of course to the metabolome. In most cases, oxidative stress plays an important detrimental role. More generally, the occurrence of free radical is quite common in industrial process conditions, triggering a cascade of negative effects on different cellular networks. There are a growing number of examples where engineering cellular components belonging to the general mechanisms of scavenging activities result in an improved phenotype.

Table 7.2 Metabolic features constituting successful microbial production processes (rewritten from Porro et al. 2014)

Metabolic feature	Significance
Thermodynamic feasibility	Thermodynamics is ultimately decisive if a chemical reaction or metabolic pathway is possible
Stoichiometric balance	The theoretical stoichiometric yield—especially of carbon atoms—defines the potential economical feasibility of a production process
Redox balance	If substrate and product have a different degree of reduction re-equilibration of the electron balance leads either to by-product formation or energy overflow
Energy balance	If production requires metabolic energy, part of the substrate is lost for its supply
Flux coupling	Coupling the product flux to the central carbon flux by a shared reaction may create a strong driving force towards product formation
Irreversible reactions	Including an irreversible step, like decarboxylation, creates a metabolic driving force pulling flux towards the product
Transport	High production must be associated to an efficient accumulation of the final compound at extracellular level. Uptake of the substrate and transport among different organelles and the cytoplasm are included
Microbial and bioprocess robustness	Including tolerance to the final product and to adverse bioprocess conditions (i.e. low pH, high T, low RT, low OTR, high substrate concentration, etc.)

This is the case of *S. cerevisiae* strains engineered for ascorbic acid production, resulting in cells more robust against a series of stressors, among which organic acids (Martani et al. 2013) or overexpressing gene(s) of the glutathione pathway resulting in a less sensitive phenotype to toxic compounds released from lignocellulose pretreatment (Ask et al. 2013). Such modifications determine a complete reorganization of the cells, influencing many pathways, not only directly related to the operated modification or to the desired productive pathway. Indeed, altering master regulators of general cellular rewiring represents a possible strategy to evoke shaded potential that may accomplish the desirable features. The *S. cerevisiae* poly(A) binding protein Pab1, as stress granules component, was selected in our laboratory as the target for obtaining widespread alterations in mRNA metabolism, resulting in stress-tolerant yeast phenotypes. Firstly, we demonstrated that the modulation of Pab1 levels improves robustness against different stressors, including acetic acid. Secondly, the mutagenesis of *PAB1* and the application of a specific screening protocol on acetic acid-enriched medium allowed the isolation of the further ameliorated mutant *pab1* A60-9. These findings pave the way for a novel approach to unlock industrially promising phenotypes through the modulation of a post-transcriptional regulatory element (Martani et al. 2015).

A last and important consideration should be dedicated to the transport of the final product. Organic acids in their salt-charged status cannot diffuse across the cellular membranes, while the lipophilic undissociated form of the acid (RCOOH) may permeate the plasma membrane by simple diffusion, even if with a very small efficiency (this is the key base principle of a weak organic acid as preservatives). Consequently, high organic acids productions must be associated to the secretion and accumulation of the final compound at extracellular level, see Table 7.2 and Fig. 7.5. Surprisingly, of all the industrial organic acid productions described in the Sect. 7.2, only for succinic acid the transport of the final compound at extracellular level has been taken into consideration during the rational design of the cell factory. Diametrically opposed is the scenario for the production of lactic acid from recombinant yeast cells, where secretion of the organic acid is still based on the activity of one or more endogenous, probably non-specific, organic acid transporter(s).

For all the other organic acids, the accumulation at extracellular level is obtained throughout the endogenous transporter(s), even if in most cases it is still unknown/uncharacterised. It can be easily anticipated that engineering of the plasma membranes, as well as the development of synthetic membranes, for improving the efficiency of the transport and reducing the passive diffusion of the undissociated form back inside the cells, will lead to great developments.

Finally, it might be worthwhile to rethink the organic acid chemistry and to consider which compounds can be produced efficiently by microbial activity (and which not) and how to use them reasonably. Petrochemistry essentially developed by the quest to find applications for abundant substrates. In most cases, the involved chemistry is focused on the introduction of functional groups. Today biotechnology is dedicated to the replacement of current platform chemicals, developed and used by petrochemistry. Starting from biomass, this generally involves reduction of the

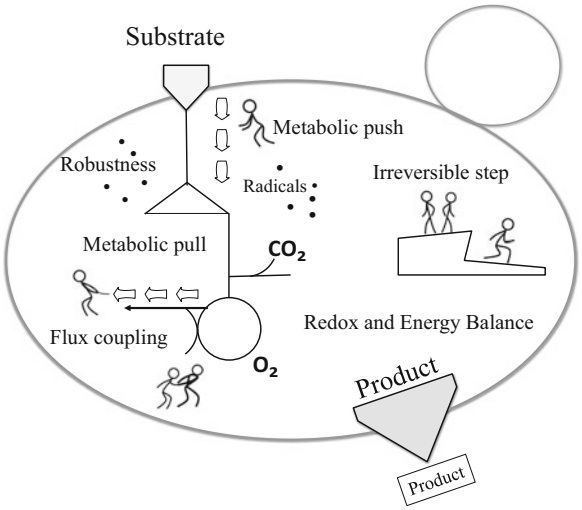

Fig. 7.5 General concepts leading to high yield (g/g), productions (g/L) and productivities (g/L/h) (see also Table 7.2). High yield are depending by the **thermodynamic** and **stoichiometric balances.** Instead, **uptake** of the substrate and **transport** of the final product in the medium could strongly limit the productivity of the process. **Metabolic push** utilizes enhancement of the main pathway as a driving force, while **metabolic pull** drives a pathway by depleting intermediate products towards the end of the pathway. **Flux coupling** enforces an engineered reaction by coupling it metabolically to a strong essential pathway. Including an **irreversible step** into an engineered pathway creates a strong driving force towards the product. The accumulation of the product is strongly dependent by the **energy** and **redox balances.** High productions and productivities also require high **cellular** and **bioprocess robustness**

number of functional groups. In other words, the starting material is completely different, and the required chemistry is different. Furthermore, most of the classical building block molecules are not efficiently produced naturally. We already suggest a paradigm shift to produce the right chemicals. This implies looking at chemistry with an open mind: in many cases the aim is not to substitute existing processes or molecules but more radically to reimagine them and their possible use (Porro et al. 2014).

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References

Ask M, Mapelli V, Höck H, Olsson L, Bettiga M (2013) Engineering glutathione biosynthesis of *Saccharomyces cerevisiae* increases robustness to inhibitors in pretreated lignocellulosic materials. Microb Cell Fact 12:87

Bailey JE (1991) Toward a science of metabolic engineering. Science 252:1668–1675

Berovic M, Legisa M (2007) Citric acid production. Biotechnol Annu Rev 13:303–343
Biotechnology Innovation Organization (BIO) (2016) Advancing the biobased economy: renewable chemical biorefinery, commercialization, progress, and market opportunities, 2016 and beyond. © 2016 Biotechnology Innovation Organization (BIO)
Bozell JJ, Petersen GR (2010) Technology development for the production of biobased products from biorefinery carbohydrates-the US Department of Energy's "Top10" revisited. Green Chem 12:539–554
Chen Y, Nielsen J (2016) Biobased organic acids production by metabolically engineered microorganisms. Curr Opin Biotechnol 37:165–172
Choi S, Song CW, Shin JH, Lee SY (2015) Biorefineries for the production of top building block chemicals and their derivatives. Metab Eng 28:223–239
Dai Z, Mao X, Magnuson JK, Lasure LL (2004) Identification of genes associated with morphology in Aspergillus niger by using suppression subtractive hybridization. Appl Environ Microbiol 70:2474–2485
Dutton MV, Evans CS (1996) Oxalate production by fungi: its role in pathogenicity and ecology in the soil environment. Can J Microbiol 42:881–895
Gangl IC, Weigand WA, Keller FA (1990) Economic comparison of calcium fumarate and sodium fumarate production by *Rhizopus arrhizus*. Appl Biochem Biotechnol 24–25:663–677
Glieder A, Kubicek CP, Mattanovich D, Wiltschi B, Sauer M (eds) (2016) Synthetic biology. Springer, New York
Grimm LH, Kelly S, Krull R, Hempel DC (2005) Morphology and productivity of filamentous fungi. Appl Microbiol Biotechnol 69:375–384. https://www.bio.org/advancing-biobased-economy-renewable-chemical-biorefinery-commercialization-progress-and-market
Jones DL (1998) Organic acids in the rhizosphere – a critical review. Plant Soil 205:25–44
Jung YK, Lee SY (2011) Efficient production of polylactic acid and its copolymers by metabolically engineered *Escherichia coli*. J Biotechnol 151(1):94–101
Krisko A, Radman M (2013) Phenotypic and genetic consequences of protein damage. PLoS Genet 9(9):e1003810. doi:10.1371/journal.pgen.1003810
Legisa M, Mattey M (2007) Changes in primary metabolism leading to citric acid overflow in *Aspergillus niger*. Biotechnol Lett 29:181–190
Liao W, Liu Y, Frear C, Chen S (2007) A new approach of pellet formation of a filamentous fungus Rhizopus oryzae. Bioresour Technol 98:3415–3423
Liaud N, Giniés C, Navarro D, Fabre N, Crapart S, Herpoël-Gimbert I, Levasseur A, Raouche S, Sigoillot JC (2014) Exploring fungal biodiversity: organic acid production by 66 strains of filamentous fungi. Fungal Biol Biotechnol 1:1–10
Liu Y, Liao W, Chen S (2008) Study of pellet formation of filamentous fungi *Rhizopus oryzae* using a multiple logistic regression model. Biotechnol Bioeng 99:117–128
Lohbeck K, Haferkorn H, Fuhrmann W, Fedtke N (1990) Maleic and fumaric acids. In: Ullmann's encyclopedia of industrial chemistry, vol A16. Wiley-VCH, Weinheim, Germany, pp 53–62
Magnuson JK, Lasure LL (2004) Organic acid production by filamentous fungi. In: Tkacz JS, Lange L (eds) Advances in fungal biotechnology for industry, agriculture, and medicine. Kluwer Academic/Plenum Publishers, New York, pp 307–340
Martani F, Fossati T, Posteri R, Signori L, Porro D, Branduardi P (2013) Different response to acetic acid stress in *Saccharomyces cerevisiae* wild-type and l-ascorbic acid-producing strains. Yeast 30:365–378
Martani F, Marano F, Bertacchi S, Porro D, Branduardi P (2015) The *Saccharomyces cerevisiae* poly(A) binding protein Pab1 as a target for eliciting stress tolerant phenotypes. Sci Rep 5:18318
MBI International, Lansing, MI (2011) Bioeconomy initiative at MBI International (final report). Department of Energy, Washington, DC. Office of Basic Energy Sciences. Sponsored by Department of Energy, Washington, DC. https://ntrl.ntis.gov/NTRL/dashboard/searchResults/titleDetail/DE20121034906.xhtml

NNFCC (2010) NNFCC renewable chemicals fact sheet: LacticAcid. http://www.nnfcc.co.uk/publications/nnfcc-renewable-chemicals-factsheet-lactic-acid

Papagianni M (2007) Advances in citric acid fermentation by *Aspergillus niger*: biochemical aspects, membrane transport and modeling. Biotechnol Adv 25:244–263

Papagianni M, Wayman F, Mattey M (2005) Fate and role of ammonium ions during fermentation of citric acid by *Aspergillus niger*. Appl Environ Microbiol 71:7178–7186

Porro D, Branduardi P (2012) Chapter 14: Yeast biotechnology. In: Yeast, 2nd edn. Wiley-VCH, Weinheim, Germany

Porro D, Branduardi P, Sauer M, Mattanovich D (2014) Old obstacles and new horizons for microbial chemical production. Curr Opin Biotechnol 30C:101–106

Raab AM, Lang C (2011) Oxidative versus reductive succinic acid production in the yeast *Saccharomyces cerevisiae*. Bioeng Bugs 2:120–123

Roa Engel CA, Straathof AJJ, Zijlmans TW, van Gulik WM, van der Wielen LAM (2008) Fumaric acid production by fermentation. Appl Microbiol Biotechnol 78:379–389

Roa Engel CA, van Gulik WM, Marang L, van der Wielen LA, Straathof AJ (2011) Development of a low pH fermentation strategy for fumaric acid production by *Rhizopus oryzae*. Enzym Microb Technol 48(1):39–47

Sauer M, Porro D, Mattanovich D, Branduardi P (2008) Microbial production of organic acids: expanding the markets. Trends Biotechnol 26:100–108

Sauer M, Porro D, Mattanovich D, Branduardi P (2010) 16 years research on lactic acid production with yeast - ready for the market? Biotechnol Genet Eng Rev 27:229–256

Sauer M, Branduardi P, Rußmayer H, Marx H, Porro D, Mattanovich D (2014) Production of metabolites and heterologous proteins. In: Piškur J, Compagno C (eds) Molecular mechanisms in yeast carbon metabolism. Springer, Berlin, pp 299–326

Weastra sro, Determination of market potential for selected platform chemicals. Itaconic acid, Succinic acid, 2,5-furandicarboxylic acid. http://www.bioconsept.eu/wp-content/uploads/BioConSepT_Market-potential-for-selected-platform chemicals_report1.pdf

Werpy T, Petersen G, Aden A, Bozel J, Holladay J, White J, Manheim A, Eliot D, Lasure L, Jones S (2004) Top value added chemicals from biomass, Vol. 1: Results of screening for potential candidates from sugars and synthesis gas. Pacific Northwest National Laboratory, National Renewable Energy Laboratory and Department of Energy, Washington, DC, p 76

Carotenoid Production by Filamentous Fungi and Yeasts

8

Javier Avalos, Steffen Nordzieke, Obdulia Parra, Javier Pardo-Medina, and M. Carmen Limón

Abstract

Carotenoids are widespread pigments in nature, obtained by direct synthesis or by ingestion in all taxonomic groups, and playing a large diversity of biological functions. Carotenoid biosynthesis is frequently found in fungi, and the amenability of some producing species to research studies has made them ideal models to investigate the genes and enzymes involved in the biosynthesis and its regulation. Best known examples are those for the production of β-carotene by the mucorales *Phycomyces blakesleeanus*, *Mucor circinelloides*, and *Blakeslea trispora*, neurosporaxanthin by the ascomycetes *Neurospora crassa* and *Fusarium fujikuroi*, and astaxanthin by the basidiomycete yeast *Xanthophyllomyces dendrorhous*, formerly *Phaffia rhodozyma*. Because of their coloring and health-promoting properties, some carotenoids have biotechnological applications, usually as food or feed additives. In the case of the fungi, the biotechnological studies have been mostly centered on the productions of β-carotene or its red precursor lycopene by *B. trispora* and astaxanthin by *X. dendrorhous*, extended to the heterologous expression of the relevant genes in non-carotenogenic yeasts as potentially favorable industrial producers. Less attention has been addressed to the synthesis of other carotenoids, with the only exception of torularhodin, produced by *Rhodotorula* and other related basidiomycete yeasts, but the genetics of its biosynthesis has not been investigated.

Contents

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8.1 Introduction

Carotenoids are natural fat-soluble pigments widely represented in nature, where they have very diverse functions (Britton et al. 1998, 2004). Carotenoids are universally present in photosynthetic species, where they play essential roles associated to photosynthesis. Their functions are better known in higher plants, where they are responsible of the yellow, orange or reddish colors of many fruits and flowers. However, their most important roles are related with light harvesting and photoprotection of the photosynthetic machinery (Domonkos et al. 2013), and they are present in considerable amounts in green tissues, where their colors are masked by chlorophyll. These pigments are also found in some animals to provide their characteristic colors, as some birds and fishes. Irrespective of their sporadic coloring functions, carotenoids are ubiquitous in animals, which get them through their food to produce retinoids, as the visual chromophore retinal (vitamin A) and the signaling molecule retinoic acid (Blomhoff and Blomhoff 2006). However, with some outstanding exception represented by certain aphids (Moran and Jarvik 2010), animals lack the genes required for their synthesis and get carotenoids through the diet. The carotenoids are also produced by non-photosynthetic microorganisms and are found in many archaea, bacteria, and fungi (Sieiro et al. 2003; Avalos and Cerdá-Olmedo 2004; Avalos et al. 2014a; Sandmann and Misawa 2002).

From another point of view, several carotenoids are valuable biotechnological compounds, with diverse commercial applications in cosmetics and food industry, and some fungi are used as industrial carotenoid producers (Avalos and Cerdá-Olmedo 2004). Carotenoids are used, e.g., to provide color to egg yolks in poultry, to flesh of some fish, or to crustaceans' shells in aquaculture. Different epidemiological and clinical studies have revealed counteracting effects of the consumption of carotenoids on several chronic illnesses, resulting in reduced risks of heart disease, cancer, eye disease, and other health disorders (Krinsky and Johnson 2005; Stahl and Sies 2005; Rao and Rao 2007), with a positive impact specially in older people (Woodside et al. 2015). Their health-promoting properties,

attributed to a large extent to their antioxidant properties (Stahl and Sies 2003; Tapiero et al. 2004), are leading to an expanding interest in the biotechnological production of carotenoids, which are increasingly used as food and feed additives or as vitamin dietary supplements.

The carotenoids are terpenoid compounds containing an aliphatic polyene chain usually composed of eight isoprene units. Terpenoids, also known as isoprenoids, are a vast family of organic chemicals that derive from the sequential condensation of 5-carbon (C_5) isoprene units. Their heterogeneity comes from variations in the number of units and in subsequent chemical modifications, which gives rise to a large structural diversification. Thus, terpenoids cover from small volatile compounds, as geraniol or limonene, to essential cell components as sterols, dolichols, or ubiquinone. The C_5 precursor in the condensation steps is isopentenyl pyrophosphate (IPP). Depending on the organism or the cell compartment, IPP may be synthesized through two different biochemical pathways. The first one proceeds from acetyl-CoA through hydroxymethylglutaryl coenzyme A (HMG-CoA) and mevalonate (Fig. 8.1), and it is known as mevalonate pathway. The second one,

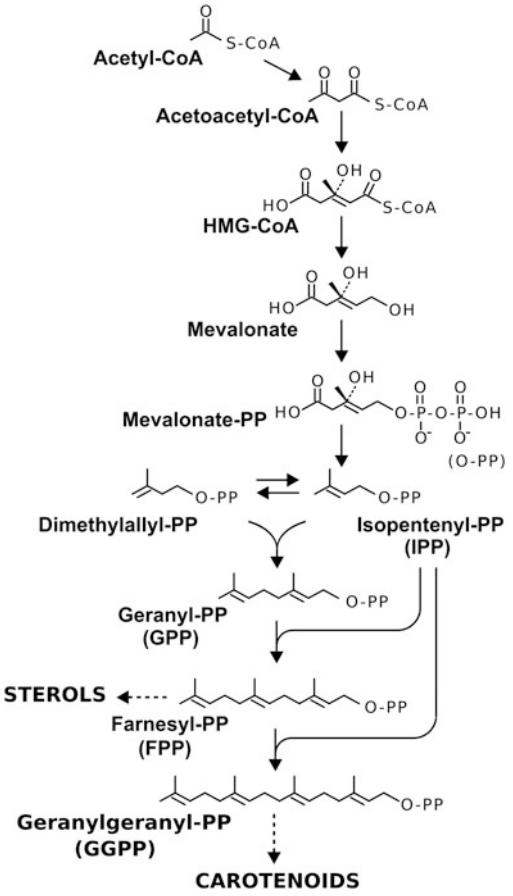

Fig. 8.1 First steps of the mevalonate terpenoid pathway. The branches for sterol and carotenoids biosynthesis are indicated

known as methylerythritol pathway, was discovered later in bacteria (Rohmer et al. 1993) and involves the condensation of hydroxyethyl-thiamin and glyceraldehyde 3-phosphate, via D-1-deoxyxylulose 1-phosphate. The plants have both biosynthetic pathways, but the available information in fungi, based on labeling of carotenoids upon addition of radioactive mevalonic acid in different species, indicate the origin of fungal carotenoids through the mevalonate pathway.

The early steps of terpenoid biosynthesis are shown in Fig. 8.1. The first condensation step, between IPP and its isomer dimethylallyl diphosphate (DMAPP), results in the production of the C_{10} monoterpene geranyl pyrophosphate (GPP). Subsequent IPP additions give rise to the C_{15} sesquiterpene C_{15} farnesyl pyrophosphate (FPP) and the C_{20} diterpene geranylgeranyl pyrophosphate (GGPP). FPP is the source of sterols, whose connection with cholesterol biosynthesis has attracted considerably the attention of researchers (DeBose-Boyd 2008). Similarly, this early part of the pathway is particularly relevant because of the use of FPP and GGPP in posttranslational modifications of proteins associated to cancer (Zahra Bathaie et al. 2016). Carotenoids are tetraterpenoids derived from phytoene, a colorless precursor generated by a head-to-head condensation of two GGPP molecules (Fig. 8.2), a

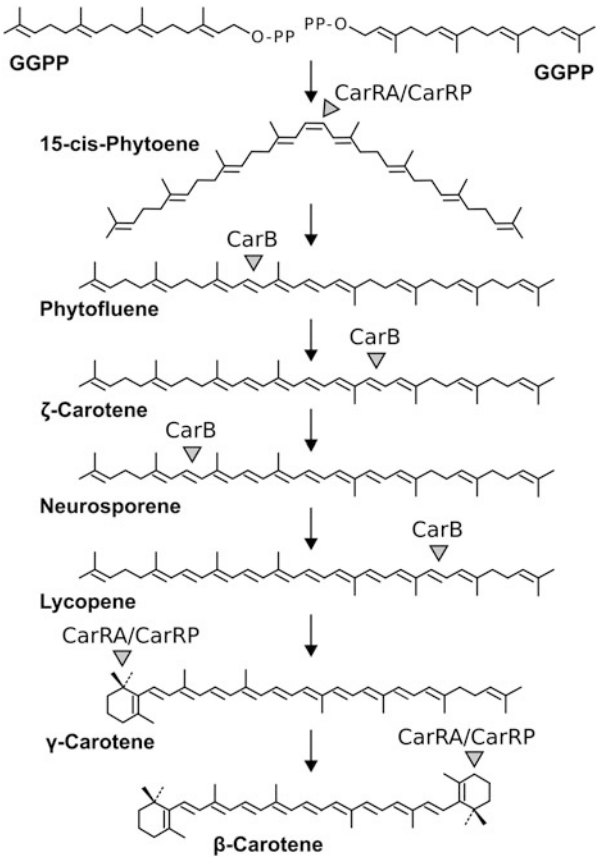

Fig. 8.2 β-carotene biosynthesis from GGPP. Enzymes responsible for each reaction in *Mucorales* are indicated close to the chemical modification from each enzymatic step (*gray arrowhead*)

reaction achieved by the enzyme phytoene synthase. The reaction is very similar to the condensation of two FPP units to produce squalene, the precursor of sterols.

In all the carotenoid pathways, the phytoene hydrocarbon backbone is object of several desaturations, which generate a chain of conjugated double bonds that confers the ability to absorb visible light, typically in the blue region of the spectrum. This chromophore provides the typical yellow, orange, or reddish pigmentations to the different carotenoids, depending on their specific absorption spectra. In the fungus *Phycomyces blakesleeanus*, phytoene is synthesized in a *cis* configuration, which is isomerized to its *trans* isomer in the first desaturation step (Fraser and Bramley 1994). Typically, carotenoids contain one or two cycled ends, known as ionone rings, resulting from a cyclase enzymatic activity. The rings may be α or β depending on the position of a C=C double bond, with the β-ionone ring being predominant in fungi. Additionally, the carotenoids may suffer other enzymatic reactions, explaining their wide structural diversity in nature (Britton et al. 2004). In the absence of oxygen in the molecule, the carotenoids are termed carotenes. However, the carotenoid biosynthetic pathways frequently include oxidative steps, which result in the production of xanthophylls.

Carotenoid biosynthesis is frequent but not universal in fungi. Thus, two well-known fungal models, the yeast *Saccharomyces cerevisiae* and the mold *Aspergillus nidulans*, do not produce carotenoids. However, carotenoid production is present in many fungi, either unicellular of filamentous, and the genetics and biochemistry of their biosynthetic pathways have been subjected to detailed attention in some model systems, which are described in the next sections.

8.2 Carotenoid Biosynthesis in Filamentous Fungi

Carotenoid biosynthesis has been investigated in different filamentous fungi, but some of them stand out for the amount of available information. These are the mucorales (a phylum of *Mucoromycotina*, formerly known as zygomycetes) *Phycomyces blakesleeanus*, *Mucor circinelloides*, and *Blakeslea trispora* for β-carotene biosynthesis and the ascomycetes *Neurospora crassa* and *Fusarium fujikuroi* for neurosporaxanthin biosynthesis. In the next sections, we update the available information on the production of these carotenoids, with special attention to the biotechnological production in the case of β-carotene.

8.2.1 β-Carotene Biosynthesis in Mucorales

The yellowish β-carotene, one of the most ubiquitous carotenes in nature, is the one usually found in mucorales fungi, as shown in *P. blakesleeanus* (Cerdá-Olmedo 1987), *M. circinelloides* (Navarro et al. 1995; Fraser et al. 1996), and *B. trispora* (Lampila et al. 1985). β-carotene production has been also found in filamentous fungi from other taxonomic groups, as the basidiomycetes *Sclerotium rolfsii* (Georgiou et al. 2001b) and *Sclerotinia sclerotiorum* (Georgiou et al. 2001a), the

ascomycetes *Aspergillus giganteus* (El-Jack et al. 1988), *Cercospora nicotianae* (Daub and Payne 1989), and *Penicillium* sp. (Han et al. 2005), as well as in the "imperfect fungus" *Aschersonia aleyroides* (van Eijk et al. 1979). As cited in later sections, β-carotene is also present in variable amounts as a secondary product or as an intermediary molecule of the carotenoid pathway in other fungi.

β-carotene biosynthesis requires four desaturations on the phytoene backbone, resulting in the reddish intermediate lycopene, and the β-cyclization of both ends of the molecule (Fig. 8.2). Because of the symmetry of phytoene, the four desaturations are actually two pairs, one corresponding to internal positions and another to external positions. In photosynthetic organisms two different enzymes, typically known as phytoene and ζ-carotene desaturases, achieve each couple of desaturations (Domonkos et al. 2013). However, a single enzyme carries out the four desaturations in fungi. Similarly, separate genes encode the phytoene synthase and the cyclase enzymes in photosynthetic species and non-photosynthetic bacteria, but both enzymatic activities reside in a single polypeptide in fungi. Therefore, only two fungal genes are needed to make β-carotene from GGPP, one encoding a bifunctional phytoene synthase/lycopene cyclase and another encoding a desaturase. The desaturase gene has been described in *C. nicotianae* (Ehrenshaft and Daub 1994) and in the mucorales *P. blakesleeanus* (Ruiz-Hidalgo et al. 1997a), *M. circinelloides* (Velayos et al. 2000a), and *B. trispora* (Rodríguez-Saiz et al. 2004), where it is known as gene *carB*. In the three species of the mucorales group investigated, the phytoene synthase/lycopene cyclase gene, called *carRA* or *carRP*, is linked in the genome with the gene *carB* and divergently transcribed from a common upstream region (Velayos et al. 2000b; Arrach et al. 2001; Rodríguez-Saiz et al. 2004), forming a single regulatory unit (Fig. 8.3).

The functions of these genes have been supported by extensive genetic and biochemical analyses that started with the genetic characterization of their mutant phenotypes, resulting in the alteration of the wild-type yellow pigmentation. Thus, the mutants of the gene *carB* are albino and accumulate phytoene, while the mutants of the gene *carRA* are either albino and without phytoene, in the case of the alteration of the phytoene synthase domain, or reddish because of the accumulation of lycopene in the case of the loss of the lycopene cyclase domain. Such mutants have been described in *P. blakesleeanus* (Cerdá-Olmedo 1985, 1987), *M. circinelloides* (Navarro et al. 1995; Velayos et al. 1997), and *B. trispora* (Mehta and Cerdá-Olmedo 1995; Mehta et al. 2003). The first substrate of the desaturase is 15-*cis*-phytoene, as shows the accumulation of this isomeric form in the *carB* mutants of *P. blakesleeanus* (Goodwin 1980), and purified desaturase enzyme converts it to all-*trans* desaturated carotene products (Fraser and Bramley 1994). In *P. blakesleeanus*, all the phytoene-accumulating mutants belong to a single complementation group (Ootaki et al. 1973), which defines genetically the gene *carB*, and the heterokaryons carrying wild-type and *carB* mutant nuclei accumulate partially desaturated intermediates, as phytofluene, ζ-carotene, and neurosporene. Their amounts, in relation to the proportion of wild-type and *carB* mutant nuclei, are consistent with the operation of the enzyme as a four-unit complex (De la Guardia et al. 1971). The analysis of the carotenes produced by

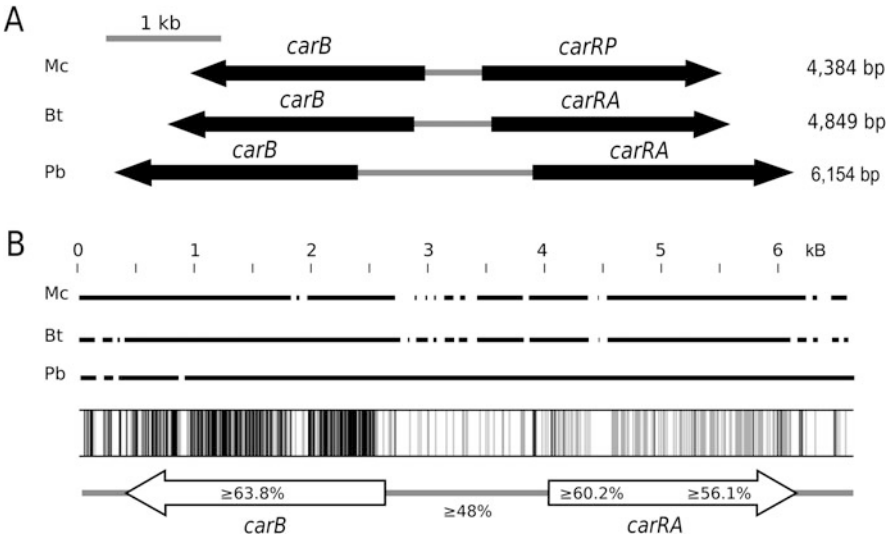

Fig. 8.3 (**a**) Genomic organization of the *carRA* and *carB* genes in *M. circinelloides* (Mc, data from strain CBS 277.49, NCBI reference AMYB01000001), *B. trispora* (Bt, strain NRRL2457, NCBI reference AY176663), and *P. blakesleeanus* (Pb, NRRL 1555, NCBI reference NW_017265138). (**b**) Schematic representation of the alignment of their sequences with MUSCLE (Edgar 2004) and visualization of alignment's conservation with the CLC sequence viewer software (Version 7.7.1; CLC bio Inc., Massachusetts, USA). *Darker lines* indicate most conserved sequences. Percentages in the genes under the conservation graphic correspond to the lowest value of the identity matrix between the three sequences of the alignment. In the case of *carRA*, the percentages for the *carR* (*left*) and *carA* (*right*) domains are indicated

leaky mutants of the *carB* gene with different catalytic efficiencies for the different steps, in one case leading to a significant overaccumulation of ζ-carotene, provided further evidence to the participation of a single desaturase in the four reactions (Bejarano et al. 1987).

In relation to *carRA*/*carRP*, the bifunctional role of this gene was confirmed in *M. circinelloides* through the phenotypic effects of partial *carRP* deletions (Velayos et al. 2000b) and by expression in *E. coli* strains engineered to produce carotene but missing phytoene synthase or lycopene cyclase activities (Sanz et al. 2011). Similar conclusions were reached in *P. blakesleeanus* with the correspondence between albino and lycopene-accumulating mutants of the *carRA* gene, called *carA* and *carR*, with mutations affecting the phytoene synthase or the lycopene cyclase in the same gene, respectively (Arrach et al. 2001). These molecular data corroborated former genetic interpretations of mutations in the *carR* and *carA* domains of the *carRA* gene, which led to propose the cleavage of the CarRA polypeptide in separate CarR and CarA enzymes (Torres-Martínez et al. 1980). This hypothesis was later supported by the finding of a conserved proteolytic cleavage site between both protein domains in *P. blakesleeanus* (Arrach et al. 2001)

and independent 40-kDa CarR and 30-kDa CarA polypeptides by western blot analyses in *B. trispora* (Breitenbach et al. 2012).

The *carB* or *carR* mutant phenotypes may be reproduced by specific chemical block of the enzymatic activities. In *P. blakesleeanus*, cinnamic alcohol, thymol, and diphenylamine inhibit the phytoene desaturase (Bejarano and Cerdá-Olmedo 1989), while CPTA (2-[4-chlorophenylthio]-triethylamine), imidazole, nicotine, and other nitrogenated compounds obstruct the lycopene cyclase (Elahi et al. 1973a, b). Different lines of evidence, based on quantitative analysis of heterokaryons between *carRA* and *carB* mutants (De la Guardia et al. 1971; Aragón et al. 1976), the effect of specific enzyme inhibitors on carotene proportions (Candau et al. 1991b), and complementation analysis between null and leaky *carB* mutants (Sanz et al. 2002), strongly support the organization of the enzymes as a complex consisting of four desaturases and two cyclases. In the complex, the 4 + 2 monomers carry out sequentially the six reactions, and all the desaturase and cyclase units are able to act on any of their corresponding substrates, which are transferred internally. However, this scenario may differ between different mucorales, as indicates the finding in *B. trispora* of two different lycopene cyclases, one similar to the CarR enzyme of *P. blakesleeanus*, and a second one insensitive to inhibitors and involved only in the cyclization of lycopene into γ-carotene (Mehta and Cerdá-Olmedo 1999).

There is very limited information on the physical location of carotene synthesis in fungal cells. In *P. blakesleeanus*, it occurs in a distinct cell compartment than sterol biosynthesis despite the coincidence in the first enzymatic steps. This is evidenced by the different specific radioactivity found in ergosterol and β-carotene in the wild type and in a *carS* β-carotene-overproducing mutant, mentioned in the next section, when ^{14}C-labeled mevalonate was added to the culture medium (Bejarano and Cerdá-Olmedo 1992). Therefore, both pathways are physically separated and use independent precursor pools. A similar experimental approach also identified independent syntheses for ubiquinone, triacylglycerols, and β-carotene in *P. blakesleeanus* and *B. trispora* (Kuzina et al. 2006). Taking together, the available data are consistent with control mechanisms to locate the enzymes for the synthesis of different terpenoids, including those of carotenogenesis, in appropriate cell compartments.

In different zygomycetes it is well established that β-carotene is not an end product but the source of a large array of apocarotenoid derivatives, which comprise at least C_{18} trisporoids, C_{15} cyclofarnesoids, and C_7 methylhexanoids (Barrero et al. 2011). C_{18} trisporoids include a family of chemicals, the trisporic acids (Austin et al. 1969, 1970), which play the role of sexual hormones in the life cycle of these fungi (see next sections). β-carotene degradation starts with the activity of carotenoid oxygenases (CCDs), a family of enzymes catalyzing the oxidative cleavage of specific carotenoid substrates to produce apocarotenoids (Ahrazem et al. 2016). The first CCD enzymes in this fungal group where identified in *Rhizopus oryzae* (Tsp3 and Tsp4) and *B. trispora* (Tsp3), their names coming from their presumed participation in the production of trisporic acids (Burmester et al. 2007). The apocarotenoids found in *P. blakesleeanus* are consistent with the

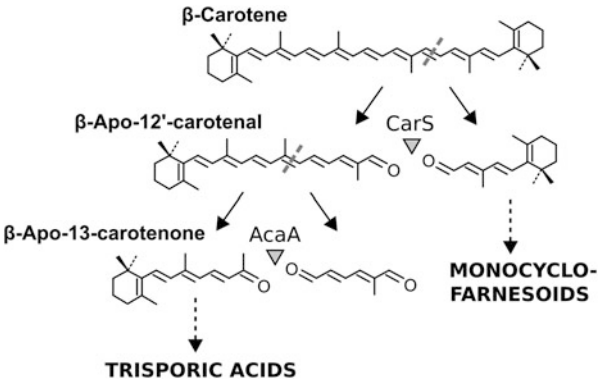

Fig. 8.4 Cleavage reactions of β-carotene in *P. blakesleeanus*. *Dotted gray lines* in β-carotene and β-apo-12′-carotenal indicate cleavage sites for the CCDs CarS and AcaA, respectively

sequential cleavage of β-carotene at two different internal positions (Polaino et al. 2010) (Fig. 8.4), the first cleavage carried out by the CCD enzyme CarS, ortholog of Tsp3 (Medina et al. 2011; Tagua et al. 2012). The gene *carS* was formerly interpreted as regulatory (see next sections) because of the accumulation of large amounts of β-carotene resulting from its mutation. The second cleavage reaction, achieved on the CarS product β-apo-12′-carotenal, is carried out in *P. blakesleeanus* by the CCD AcaA (Medina et al. 2011). The genome of this fungus contains a third predicted CCD enzyme, but its function has not yet been elucidated. Other genes and enzymes for trisporoid metabolism are currently under investigation (Avalos et al. 2014a), but they are out of the scope of this review.

8.2.2 Regulation of β-Carotene Biosynthesis in Mucorales

In the three mucorales that have received more attention, *P. blakesleeanus*, *M. circinelloides*, and *B. trispora*, the synthesis of β-carotene is stimulated by light and, depending on the strains, by the interaction with a strain of the opposite sex. As described above, the three species have the same β-carotene biosynthetic pathway mediated by the products of a similar set of divergently transcribed genes. However, the coding sequences of the *carB* or *carRA*/*carRP* genes are more similar in the three species than their regulatory intergenic sequences (Fig. 8.3), and there are differences in their responses to external signals.

Surface cultures of wild-type *P. blakesleeanus* grown in the light contain about tenfold more β-carotene than those grown in the dark (Bergman et al. 1973; Bejarano et al. 1991; Salgado et al. 1991), and a comparable induction is found in *M. circinelloides* (Navarro et al. 1995; Velayos et al. 1997). Such stimulation was not initially described for *B. trispora* (Sutter 1970), but when a dark-grown culture of this fungus is illuminated, its β-carotene content increases (Quiles-Rosillo et al. 2005). The regulation by light in the mucorales is also associated to the different developmental stages exhibited by their surface cultures, which include the formation of asexual fruiting bodies called sporangiophores. This process has been

investigated in special detail in *P. blakesleeanus*, which is able to form two classes of sporangiophores. Light represses the formation of small ones, called microphores, and stimulates the formation of large ones, macrophores, in a process known as photomorphogenesis (Corrochano and Cerdá-Olmedo 1992). Illumination of this fungus during 12-h periods at different growth times showed that carotenogenesis is able to respond to light only during a specific developmental stage, coinciding with the cessation of growth and the start of sporangiophore formation (Bejarano et al. 1991). These authors also found two levels of light sensitivity of photocarotenogenesis in this fungus, with a minor increase of β-carotene content after a weak illumination and a much stronger response observed only with a more intense illumination.

Regulation by light of the genes *carB* and *carRA*/*carRP* is achieved at transcription level. The magnitude of the photoinduction, referred to the mRNA content in the dark, is particularly high in *M. circinelloides*, where 10 s of blue light are sufficient to produce about a 100-fold increase of the transcript levels during the first 20 min after light exposure (Velayos et al. 2000a, b). Photoinduction is also exhibited in *M. circinelloides* in the gene *carG*, coding for the GGPP synthase (Velayos et al. 2003) providing the substrate for CarRA phytoene synthase activity. A similar *carRA*/*carB* response is exhibited by *B. trispora*, but in this case the photoinduction is ephemeral, and the mRNA content returns to dark levels just 40 min after the light pulse. This feature may explain the lack of increase in carotenoid content under continuous illumination in this species. In contrast, the photoinduction rate is not so strong in *P. blakesleeanus*, probably because of the presence of higher mRNA amounts in the dark. Thus, the exposure to light in this fungus results in less than a tenfold increase in *carB* (Ruiz-Hidalgo et al. 1997b; Blasco et al. 2001) and *carRA* mRNA levels (Almeida and Cerdá-Olmedo 2008; Sanz et al. 2010). The time course of mRNA accumulation shows a clear biphasic kinetics for both genes in *P. blakesleeanus* (Sanz et al. 2010), which could be related with the two different levels of light sensitivity formerly found for photocarotenogenesis in this fungus (Bejarano et al. 1991).

The analysis of mutants affected in photoinduction has been a valuable tool to identify the regulatory proteins responsible for the control by light. Mutagenic searches in *P. blakesleeanus* led to identify several mutants with reduced photocarotenogenesis: *madA*, *madB*, *picA*, *picB* (López-Díaz and Cerdá-Olmedo 1980), *carC* (Revuelta and Eslava 1983), and *pimA* (Flores et al. 1998). The *madA*, *madB*, and *pimA* mutants are also affected in photomorphogenesis, and they were actually identified because of this developmental alteration. Photocarotenogenesis is also modified by the presence of inhibitors of protein phosphatases and kinases (Tsolakis et al. 1999), suggesting that at least some of these genes could participate in a signal transduction mechanism involving phosphorylation events. Mutants with reduced carotene content in the light have also been described in *M. circinelloides* (Navarro et al. 1995; Velayos et al. 1997).

While the mutants of the genes *madA* and *madB* are only partially affected in photocarotenogenesis, the double mutant *madA madB* is mostly insensitive (Jayaram et al. 1980; López-Díaz and Cerdá-Olmedo 1980). A combination of

genetic and genomic data led to identify the gene *madA* in the *P. blakesleeanus* genome (Idnurm et al. 2006), encoding a protein similar to the flavin photoreceptor White Collar-1 (WC-1) of *N. crassa*, responsible of photocarotenogenesis in this fungus (described in a later section). Accordingly, the action spectrum of carotenogenesis in *P. blakesleeanus* is consistent with the mediation of flavin photoreceptors (Bejarano et al. 1991). In *N. crassa*, WC-1 interacts with a smaller protein, WC-2, to form a photoactive complex. In a similar way, MadB is orthologous of WC-2 and it interacts with MadA (Sanz et al. 2009). Unexpectedly, the analysis of the genome of *P. blakesleeanus* revealed two additional WC-1-like proteins, named WcoA and WcoB, and three WC-2-like proteins, WctB, WctC, and WctD (Corrochano and Garre 2010), pointing to a remarkable complexity of the photosensory systems in this fungus. The action spectra for the two sensitivity levels of photocarotenogenesis, mentioned above, are consistent with the participation of flavin photoreceptors, but are not totally coincident (Bejarano et al. 1991), indicating differences in their respective photoreception systems. This could be explained by the participation of other WC-1-like photoreceptor or the combination of MadA with a different WC-2-like protein. Regrettably, at present there is no methodology available to obtain knockout transformants in *P. blakesleeanus*, and therefore the functions of the proteins WcoA, WcoB, WctB, WctC, and WctD remain to be elucidated. Yet, such methodology is available in *M. circinelloides* (Torres-Martínez et al. 2012), which contains a similar set of White Collar-1 genes, called *mcwc-1a*, *mcwc-1b*, and *mcwc-1c*. In contrast to *P. blakesleeanus*, the absence of the MadA counterpart, MCWC-1a, does not affect photocarotenogenesis but prevents sporangiophore phototropism. On the other hand, photocarotenogenesis is lost in the *mcwc-1c* null mutant while no phenotypic alteration is apparent in the *mcwc-1b* null strain (Silva et al. 2006). The functions of the MCWC-1a and MCWC-1c proteins are interconnected: unlike the *mcwc-1a* and *mcwc-1b* genes, expression of *mcwc-1c* is strongly induced by light, but this induction requires a functional *mcwc-1a* gene. Moreover, *carB* and *carRP* photoinduction is severely reduced in the absence of *mcwc-1a* (Silva et al. 2006).

8.2.3 β-Carotene Overproduction in Mucorales

The mutagenesis screenings in mucorales allow the isolation of carotenoid-overproducing mutants, which stand out because of their deeper pigmentation. In *P. blakesleeanus* such mutants are affected in three genes: *carS* (Murillo and Cerdá-Olmedo 1976), already mentioned because of its role in β-carotene cleavage (Medina et al. 2011; Tagua et al. 2012), *carD* (Salgado et al. 1989), and *carF* (Mehta et al. 1997). Depending on the mutated gene and the allele, the carotene content in the dark raises from 10- to 100-fold that of the wild type. This increase is not accompanied by concomitant increments in *carRA* and *carB* transcripts (Almeida and Cerdá-Olmedo 2008), but at least in the case of the *carS* and *carD* mutants, they exhibit higher capacities of incorporation of mevalonic acid into carotene in their cell extracts (Salgado et al. 1991), suggesting posttranscriptional

regulatory effects. The carotene increase is less pronounced, about three- to five-fold, in carotene-overproducing mutants of *B. trispora* (Mehta and Cerdá-Olmedo 1995; Mehta et al. 2003) and in some mutants of *M. circinelloides* (Navarro et al. 1995; Fraser et al. 1996). However, the null mutants of the gene *crgA* of this species reach levels as high as 80-fold the content of the wild type (Navarro et al. 2001), reminding the phenotype of the *carS* mutants of *P. blakesleeanus*.

Despite their similarities, the molecular basis for carotene overproduction is very different in the mutants of the gene *carS* of *P. blakesleeanus* and those of *crgA* of *M. circinelloides*. As already indicated, CarS is a β-carotene-cleaving oxygenase leading to the formation of apocarotenoid derivatives that are not visually detected because of their lack of pigmentation. Therefore, β-carotene overaccumulation in the *carS* mutants is explained by a block of its CarS-mediated degradation. This leads to reconsider a former regulatory hypothesis, according to which the *carS* mutants were affected in the detection of β-carotene in a feedback control mechanism (Bejarano et al. 1988). However, the occurrence of a feedback regulation through the detection of an apocarotenoid product is not yet discarded. Moreover, a mutagenesis screening from a *carS* mutant led to identify albino mutants with a second mutation in the *carS* gene (Salgado and Cerdá-Olmedo 1992), whose molecular basis remains to be explained.

The CrgA protein of *M. circinelloides*, which has no sequence relation with CarS, consists of two amino-terminal RING finger (RF) domains, two glutamine-rich regions, a LON protease domain, and a carboxy-terminal isoprenylation site (Navarro et al. 2000). The RF domains have been described to interact with E3 ligase-type proteins that mediate ubiquitylation of target proteins, usually labeling them for their degradation. At least one of the RF domains is essential for its regulatory function in carotenogenesis, suggesting that CrgA may function as an E3 ubiquitin ligase (Lorca-Pascual et al. 2004). β-carotene overproduction in the *crgA* mutants is due to a strong increase in the *carRA* and *carB* transcript levels, pointing to CrgA as a negative regulator (Navarro et al. 2001; Lorca-Pascual et al. 2004), a function in which it seems to play a role in the ubiquitylation-independent degradation of MCWC-1b (Silva et al. 2008). CrgA also regulates vegetative growth and sporulation in *M. circinelloides*, suggesting a wider regulatory function for this protein (Quiles-Rosillo et al. 2003; Murcia-Flores et al. 2007). However, despite the carotene overaccumulation, the *crgA* mutants maintain the ability to respond to light with a further increase in the carotene content and in the transcription of at least the *carB* gene (Navarro et al. 2001). The lack of efficient transformation procedures hinders the study of the function of *crgA* orthologs in *P. blakesleeanus* and *B. trispora*, but the *crgA* gene of *B. trispora* is able to complement the *crgA* mutation in *M. circinelloides*, indicating conserved functions for this protein in both species (Quiles-Rosillo et al. 2005).

As found with the *carS* mutants, the loss of either phytoene desaturase in *carB* mutants or of lycopene cyclase in *carR* mutants (R domain of gene *carRA*) leads to the accumulation of large amounts of phytoene or lycopene (Ootaki et al. 1973), respectively, and similar results are obtained by their chemical inactivation

(Bejarano and Cerdá-Olmedo 1989; Candau et al. 1991b). This high carotene production was interpreted as a clue for the occurrence of feedback or end product regulation in this species. The increases are more variable, depending on the strain or culture conditions, in similar mutants of *M. circinelloides* (Navarro et al. 1995; Velayos et al. 1997) and only minor in those of *B. trispora* (Mehta and Cerdá-Olmedo 1995). Despite their large carotene content, light is still able to stimulate phytoene accumulation in the *carB* mutants of *P. blakesleeanus* as well as that of β-carotene in some *carS* mutants (Bejarano et al. 1991). However, light is mostly ineffective on *carA* or *carR* mutants, some of them carrying leaky mutations, suggesting a regulatory role for the CarRA polypeptide before its proteolytic cleavage. No photoinduction could be detected either in double *carA carS* mutants, with a carotene content halfway between those of the two separate mutants (Bejarano et al. 1991). Other observations point to further functions for the CarA protein, facilitating substrate transfer between enzymatic aggregates in the cyclization reactions (Murillo et al. 1981) or participating in the light sensitivity of photomorphogenesis (Corrochano and Cerdá-Olmedo 1990).

β-carotene accumulation in *P. blakesleeanus* is stimulated by the presence of different chemicals, distributed in at least two families of compounds acting through independent mechanisms (Bejarano et al. 1988). The first family consists of apocarotenoids with a terminal β-ring in the molecule, such as retinol (vitamin A) or β-ionone (Eslava et al. 1974), which predictably exert their action competing with β-carotene in the binding to an enzyme or regulatory protein. In the presence of vitamin A, β-carotene concentration of the wild type rises to levels comparable to those of phytoene or lycopene in the *carB* or *carR* mutants in the absence of the inducer, but it is still able to increase significantly the carotene content of these mutants (Eslava et al. 1974). As already mentioned, the *carA* mutants are insensitive to light, but they exhibit a patent β-carotene increase in the presence of vitamin A. Such increase is similar to that exhibited by the *carA carS* double mutant, which hardly responds to vitamin A. According to the available information, it was proposed a regulatory model in which β-carotene interacts with a *carA*/*carS* complex to downregulate the pathway (Bejarano et al. 1988), but such regulatory mechanism has not been demonstrated experimentally.

The second family of chemicals consists of a series of aromatic compounds (Cerdá-Olmedo and Hüttermann 1986), with veratrol and dimethyl phthalate among the most efficient inducers. Their mechanism of action is independent from that of vitamin A, and therefore their effects are additive (Bejarano et al. 1988). The desaturase inhibitor diphenylamine is a biphenolic compound, and, not surprisingly, some of the phenolic activators, e.g., cinnamic alcohol, also interfere with the desaturase activity (Bejarano and Cerdá-Olmedo 1989). The mechanism of action of the phenolic activators in *P. blakesleeanus* has not been unraveled.

When two strains of *P. blakesleeanus* of opposite sex are grown together, the cultures (known as mated cultures) exhibit a fivefold increase in their β-carotene levels compared to the respective single cultures (Govind and Cerdá-Olmedo 1986). This stimulation is visualized as a pigmented band when the mycelia of the two strains meet on an agar surface (see, e.g., Tagua et al. 2012) and does not

require a physical contact (Burgeff 1924), indicating the mediation of diffusible products. The stimulations of the synthesis of β-carotene and the formation of sexual structures in mated cultures are probably independent, as suggest the opposite effect of acetate on both processes (Kuzina and Cerdá-Olmedo 2006). The sexual stimulation of carotene biosynthesis is the result of a strong induction in the expression of the *carRA* and *carB* genes (Almeida and Cerdá-Olmedo 2008) and in the corresponding enzymatic activities (Salgado et al. 1991).

The upregulation of carotenogenesis by mating is due to the production of trisporic acids, sexual hormones already mentioned in Sect. 8.2.1 whose synthesis requires the collaboration of the two partners (Schachtschabel et al. 2008) and triggers their sexual differentiation (Sutter 1987). At least in *B. trispora*, either the sexual interaction or the addition of trisporic acids stimulates the expression of the *tsp3* gene, needed for the production of these hormones (Burmester et al. 2007). Actually, addition of trisporic acids to single cultures reproduces the carotenoid stimulation in *P. blakesleeanus* (Govind and Cerdá-Olmedo 1986) or in other related species (Sahadevan et al. 2013), and in *P. blakesleeanus* it is additive to the inducing effects of dimethyl phthalate, light, or *carS* mutation (Govind and Cerdá-Olmedo 1986). The sexual stimulation is more efficient in sexual heterokaryons, i.e., those holding nuclei of the strains of the opposite sex in the same cytoplasm. Such heterokaryons are unstable because of random variations in the nuclear proportions but may be stabilized through the introduction of recessive lethal mutations in the partner nuclei (Murillo et al. 1978). The β-carotene content in surface cultures of these heterokaryons increases considerably if the participating nuclei hold *carS* mutations, reaching up to 2.5% of the total dry mass in media containing cheap industrial subproducts (Cerdá-Olmedo 1989). The carotene levels are not far from this value in partial sexual diploids with a *carF* mutation (Mehta and Cerdá-Olmedo 2001) and may reach about 3% of mycelial dry mass in a *carS carF* double mutant (Mehta et al. 1997).

Production of β-carotene has not reached so high levels in *M. circinelloides*, but in contrast to *P. blakesleeanus* or *B. trispora*, this species is amenable to genetic transformation, making possible gene-engineered manipulations (Torres-Martínez et al. 2012). This methodology allowed the generation of strains with increased copy numbers of genes for early steps of the terpenoid pathway, resulting in improvements of up to fivefold in β-carotene production (Csernetics et al. 2011) or with modifications of the carotenoid pathway through the expression of heterologous genes for β-carotene oxidizing enzymes, leading to the accumulation of variable amounts of xanthophylls as β-cryptoxanthin, zeaxanthin, echinenone, canthaxanthin, or astaxanthin (Papp et al. 2006, 2013; Csernetics et al. 2011, 2015). The molecular procedures for these engineered modifications in *M. circinelloides* have been recently reviewed (Barredo 2012).

8.2.4 *B. trispora* as an Industrial Carotene Source

The global carotenoid market has kept growing in the recent years, and β-carotene is the most commercialized product (Kirti et al. 2014). β-carotene may be obtained

by chemical synthesis or by biotechnological production, with *B. trispora* and the alga *Dunaliella salina* as the preferred microbial sources (Ribeiro et al. 2011). In the case of *B. trispora*, the basis of its biotechnological application is the high β-carotene yields resulting from the sexual interaction in submerged mated cultures, consistent with increased *carRA*/*carB* expression (Schmidt et al. 2005) and CarRA/CarB enzymatic levels (Breitenbach et al. 2012). The sexual stimulation is accompanied by many other physiological and developmental changes and affects the expression of many other genes (Kuzina et al. 2008). As a different approach, addition of trisporic acids to single cultures reveals that these hormones act as global metabolic regulators, producing changes in total protein patterns and in metabolism of fatty acids, amino acids, and carbohydrates (Sun et al. 2012). At industrial level, β-carotene production by *B. trispora* is based on fermentation technology, which implies large-scale growth of strains of opposite sex in submerged conditions and feasible subsequent industrial steps, such as adequate biomass separation and carotenoid extraction and purifications procedures.

A major point in the fermentation procedure is the use of suitable mating partners. Since they may differ in their germination and growth capabilities, the use of adequate proportions of the spores used to start the cultures is an important aspect in the process (Böhme et al. 2006). The production has been improved trough different experimental approaches. Thus, the comparison of the β-carotene levels after growth with different carbon and nitrogen sources, trace elements, pH, or inoculum sizes, as well as the result of combining varying concentrations of different media components, allowed a 42% yield increase (Choudhari and Singhal 2008). The production increases considerably through the use of β-carotene-overproducing mutants. *B. trispora* spores are multinucleate, which hinders the isolation of recessive mutants, but efficient mutagenesis protocols are available based on exposure to the potent chemical mutagen *N-methyl-N′*-nitro-*N*-nitrosoguanidine (Cerdá-Olmedo and Mehta 2012). A color screening of *B. trispora* colonies derived from spores surviving to this mutagenic agent allowed the identification of "superyellow" mutants, with β-carotene content reaching up to sixfold higher levels than those of the original strain (Mehta and Cerdá-Olmedo 1995). This yield may be improved further in a new round of mutagenesis from a superyellow mutant (example in Mehta et al. 2003), a typical strategy in industrial strain improvements that may be repeated many times. The β-carotene level increases in the superyellow mutants are not as high as those produced by sexual stimulation between wild-type strains, but the use of such mutants in mated cultures results in very high β-carotene productions (Mehta et al. 2003). Recently, alternative screening protocols and novel mutagenesis methods, as N+ ion implantation (Wang et al. 2013) and atmospheric and room temperature plasma (Qiang et al. 2014), proved also efficient to obtain carotene-overproducing strains.

Because of the industrial use of *B. trispora*, chemical activation of its β-carotene production has received intense attention. The aromatic chemicals that stimulate carotenogenesis in *P. blakesleeanus* are not effective in this fungus, and those of the retinoid group are less effective. Thus, addition of retinol to the medium results in only a twofold to threefold increase of β-carotene production (Choudhari et al.

2008), compared to a 20-fold increase in *P. blakesleeanus* (Eslava et al. 1974; Bejarano et al. 1988). Earlier studies found minor stimulatory effects with a large diversity of chemicals, including certain monoterpenes, as α-pinene (Cederberg and Neujahr 1969), and different nitrogenated compounds, as succinimide, pyridazyne, isonicotinoylhydrazine, or some pyridine derivatives (Ninet et al. 1969). More recent reports described new activators at appropriate concentrations and proposed putative inducing mechanisms. Addition of sorbitan monolaurate surfactant span-20 duplicates β-carotene production presumably through the shortening of the hyphal length and improvement of dispersed growth (Choudhari et al. 2008). The presence of ketoconazole, an inhibitor of the enzyme converting lanosterol to ergosterol, results in a threefold increase of β-carotene content, possibly because of compensatory effects in early steps of terpenoid biosynthesis (Tang et al. 2008). Addition of arachidonic acid at the appropriate moment during the fermentation process results in a 1.7-fold rise in the β-carotene content, which correlates with comparable increases in the mRNA levels of the *hmgR*, *carRA*, and *carB* genes (Hu et al. 2012) and with metabolic changes leading to increased glycolysis and fatty acid biosyntheses (Hu et al. 2013a). More predictable stimulatory effects are those produced by addition of terpenoid precursors, as mevalonic acid, isopentenyl alcohol, dimethylallyl alcohol, or geraniol (Shi et al. 2012).

Many observations point to a positive correlation between oxidative stress and β-carotene production in the *B. trispora* cultures. The fungus protects itself against reactive oxygen species with specific detoxifying enzymes, among which play a major role the catalases and superoxide dismutases (SOD). Addition of H_2O_2 results in a rise in β-carotene accumulation (Jeong et al. 1999), which is accompanied by a higher catalase activity (Wang et al. 2014). On the other hand, chemical inhibition of SOD activity increases the β-carotene content (Gessler et al. 2002). A similar effect is obtained increasing the oxidative stress by raising the dissolved oxygen concentrations in the medium, either directly (Nanou and Roukas 2011) or with the presence of *n*-hexadecane (Liu and Wu 2006a), *n*-hexane, *n*-dodecane (Xu et al. 2007), or butylated hydroxytoluene (Nanou and Roukas 2010). In the cases investigated, SOD and catalase activities are simultaneously increased in parallel to β-carotene concentrations. These enzyme activities also raise upon addition of liquid paraffin to the medium, which enhance oxygen concentration and β-carotene production (Hu et al. 2013b). However, not all agents producing oxidative stress induce β-carotene accumulation in this fungus, as showed the lack of induction upon addition of iron ions (Nanou and Roukas 2013). The endogenous and exogenous factors producing oxidative stress in *B. trispora* and the effects on the fungus under fermentative conditions have been recently reviewed (Roukas 2016).

Considerable efforts have been dedicated to develop novel medium compositions with aim of reducing costs and increasing β-carotene yields. A detailed scrutiny on the effect of a diversity of media components showed improved productions in the presence of corn steep liquor, oleic and linoleic acids, kerosene, and the already mentioned butylated hydroxytoluene (Mantzouridou et al. 2002). The positive impact of oils in the production has been confirmed in other reports,

such as those based on the use of crude olive or soybean oils (Mantzouridou et al. 2006) or waste cooking oil (Nanou and Roukas 2016). Supplementation of the medium with industrial glycerol, obtained either from soap manufacturing or biodiesel production industries, allows up to a tenfold increase in β-carotene levels (Mantzouridou et al. 2008). Very promising results have been also obtained using agro-food wastes rich in carbohydrates and mineral salts, as beet molasses (Goksungur et al. 2004), cheese whey (Varzakakou and Roukas 2010), cabbage, and peach peels or watermelon husks (Papaioannou and Liakopoulou-Kyriakides 2012). The studies on the use of cheese whey provide a good example of a complete survey on the effect of different variables in the search of the optimal production conditions, either in flasks (Varzakakou and Roukas 2010) or in fermentation reactors (Varzakakou et al. 2011; Roukas et al. 2015). These and other reports are based on the behavior of wild-type strains, but they might be efficient also with β-carotene-overproducing mutants.

The conditions developed for the production of β-carotene are also applicable for the production of lycopene through the block of the cyclase activity, which is usually achieved by chemical inhibition (Mantzouridou and Tsimidou 2008). Thus, lycopene is produced in high yields by mated cultures in large-scale fermentors in the presence of imidazole or pyridine (López-Nieto et al. 2004). Examples on the use of other inhibitors from this group are frequent in the literature, as reported, e.g., for 2-methyl imidazole (Pegklidou et al. 2008), piperidine, creatinine (Liu et al. 2012), or nicotine (Shi et al. 2012). Other chemicals used to block the cyclase activity are some amines, as CFTA (Hsu et al. 1972) and other amine-derived compounds (Hsu et al. 1974; Wang et al. 2016). A more efficient approach consists in the use of mutants devoid of cyclase activity. Such mutants are independent of chemical treatments and allow very high lycopene productions, especially in the form of heterokaryons with nuclei of both sexes carrying additional overproducing mutations (Mehta et al. 2003).

8.2.5 Neurosporaxanthin Biosynthesis in *Neurospora* and *Fusarium*

Neurosporaxanthin is a C_{35} carboxylic apocarotenoid initially discovered in the fungus *N. crassa*. Early biochemical analyses of cultures from this fungus revealed a complex carotenoid mixture, consisting of neutral carotenes and xanthophylls (Zalokar 1954). This included an acidic carotenoid that was subsequently isolated, characterized, and named neurosporaxanthin (Zalokar 1957), whose chemical structure was determined as β-apo-4′-carotenoic acid (Aasen and Jensen 1965). This acidic xanthophyll was later found also in other fungi, as *Arthrobotrys oligospora* (Valadon and Cooke 1963), a mutant of *Verticillium albo-atrum* (Valadon and Mummery 1969), *Podospora anserina* (Strobel et al. 2009), and different *Fusarium* species (Avalos and Estrada 2010). Analyses of the carotenoid mixtures in *N. crassa* (Harding et al. 1969; Mitzka and Rau 1977), *F. aquaeductuum* (Bindl et al. 1970), and *F. fujikuroi* (Avalos and Cerdá-Olmedo

1987) are consistent with a biosynthetic pathway that shares the first steps with that of β-carotene but differs in the occurrence of an additional desaturation, a single cyclization, and two final oxidative steps (Fig. 8.5). The substrate of the cyclization may differ between different species. The identification of β-zeacarotene, γ-carotene, lycopene, and 3,4-didehydrolycopene in *N. crassa* indicates that the cyclization may occur on substrates with either three, four, or five desaturations, while 3,4-didehydrolycopene has not been described in *F. fujikuroi* or *F. aquaeductuum*. In some cases, neurosporaxanthin is subject of esterification reactions in the terminal carboxylic group; thus, it is detected as a methyl ester in *Verticillium agaricinum* (Valadon and Mummery 1977) and as a glycosyl ester in a marine *Fusarium* species (Sakaki et al. 2002a).

The genes and enzymatic activities responsible for all the steps needed for neurosporaxanthin biosynthesis are known in *N. crassa* (Avalos and Corrochano 2013) and *F. fujikuroi* (Avalos et al. 2014b). The first genes of the pathway were identified in *N. crassa* by means of the genetic analysis of albino mutants, called in order of identification *al-1*, *al-2*, and *al-3* (Huang 1964). The gene *al-3* encodes the prenyl transferase responsible for the synthesis of the phytoene precursor GGPP from FPP (Nelson et al. 1989; Carattoli et al. 1991), as demonstrated the lack of this activity in *al-3* mutants (Spurgeon et al. 1979) or its capacity to replace a bacterial GGPP synthase in *E. coli* (Sandmann et al. 1993). This reaction is needed also for the synthesis of other essential terpenoids derived from GGPP, e.g., ubiquinone,

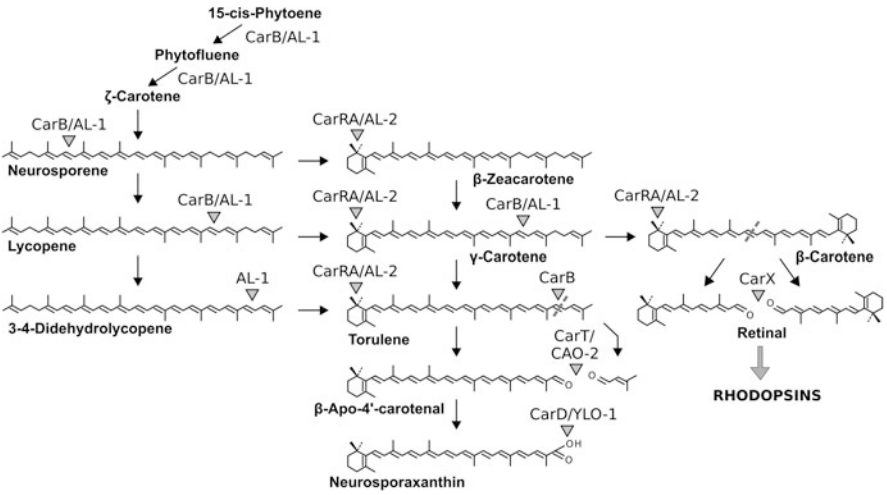

Fig. 8.5 Neurosporaxanthin and β-carotene biosynthesis in *N. crassa* and *Fusarium* sp. Enzymes responsible for each reaction (AL-1, AL-2, CAO-2, and YLO-1 for *N. crassa*, and CarRA, CarB, CarT, CarD, and CarX for *Fusarium* sp.) are indicated close to the chemical modification produced in each enzymatic step (*gray arrowhead*). *Dotted gray lines* in torulene and β-carotene indicate cleavage sites for the corresponding CCD enzymes. Cleavage of β-carotene has been only found in *Fusarium* sp. Reactions and chemical structures up to ζ-carotene are as shown in Fig. 8.2

dolichols or prenylated proteins, and therefore only leaky mutations are isolated for this gene (Barbato et al. 1996).

The synthesis of phytoene from GGPP is achieved in *N. crassa* by AL-2, whose gene was identified by its capacity to complement the *al-2* mutation (Schmidhauser et al. 1994). The activity of AL-2 was inferred from the albino phenotype of the *al-2* mutant and the sequence similarity of its carboxy-end region with phytoene synthase from bacteria or plants. A similar approach led to clone *al-1*, encoding the phytoene desaturase (Schmidhauser et al. 1990), with sequence similarity to bacterial desaturases and able to complement a mutant of *Rhodobacter capsulatus* lacking the desaturase activity (Bartley et al. 1990). As found for CarB in mucorales, the carotenoids produced by AL-1 in *R. capsulatus* indicated that this enzyme is able to catalyze four desaturations. Later in vitro and in vivo studies demonstrated that AL-1 acts as homomultimeric complex able to catalyze the five desaturations needed to convert phytoene to 3,4-didehydrolycopene or torulene in the neurosporaxanthin biosynthetic pathway (Hausmann and Sandmann 2000).

Likewise, as described for the CarRA/CarRP counterparts in mucorales, AL-2 is a bifunctional protein whose cyclase activity generates the β-ionone ring of torulene. The cyclase function of AL-2, initially disregarded because of the lack of mutants specifically affected in this enzymatic activity, was later confirmed by complementation when these mutants became available (Arrach et al. 2002). Moreover, enzymatic assays expressing AL-2 in appropriate *E. coli* strains not only demonstrated the cyclase function of the protein but also its preference to produce a single cyclization (Sandmann et al. 2006). The knowledge of the *al-1* and *al-2* sequences facilitated the cloning of its orthologs in other fungi, including *carB* and *carRA* in *F. fujikuroi* (Fernández-Martín et al. 2000; Linnemannstöns et al. 2002). The ability of CarB to carry out the five desaturations was evidenced by the isolation of a *carB* mutant specifically affected in the fifth desaturation (Prado-Cabrero et al. 2009). In the case of AL-2/CarRA, in contrast to what was observed in mucorales, the bifunctional protein is probably not separated in two independent polypeptides, as suggests the difficult identification of mutants affected in the cyclase activity and the finding in *N. crassa* of a mutant of the phytoene synthase domain that is also affected in the cyclase activity (Díaz-Sánchez et al. 2011a).

Based on the assumption that a CCD enzyme achieves the torulene cleavage step in neurosporaxanthin biosynthesis, the genomes of *F. fujikuroi* and *N. crassa* were searched for CCD-encoding genes, leading to the identification of two candidates in each species. Torulene-accumulating mutants formerly identified in *F. fujikuroi* (Avalos and Cerdá-Olmedo 1987) facilitated the assignation of torulene cleavage activity to one of them, that was called *carT* (Prado-Cabrero et al. 2007). Similar mutants were also characterized in *N. crassa*, allowing the identification of the *carT* ortholog, *cao-2* (Saelices et al. 2007). In both species, the complementation of torulene-accumulating mutants and the analysis of their enzymatic activity in vivo and in vitro established the function of the CarT/CAO-2 proteins as CCD enzymes cleaving torulene to produce C_{35} β-apo-4′-carotenal. In the case of CAO-2, the enzyme was not active on γ-carotene, indicating the need of all the previous desaturations. The function of *carT*, together to those of *carRA* and *carB*, was

also confirmed by targeted deletion experiments in *Fusarium graminearum*, also known as *Gibberella zeae* (Jin et al. 2010).

The last reaction of the pathway, the oxidation of the terminal aldehyde group to a carboxylic group, is achieved by the aldehyde dehydrogenase YLO-1 in *N. crassa* (Estrada et al. 2008b) and its ortholog CarD in *F. fujikuroi* (Díaz-Sánchez et al. 2011b). The name of the *ylo-1* mutant comes from its characteristic yellow pigmentation resulting from the accumulation of a complex mixture of carotenoids, in which neurosporaxanthin is missing (Goldie and Subden 1973). Later chemical analyses showed the presence of unusual carotenoids (Estrada et al. 2008b; Sandmann et al. 2008), possibly derived from the β-apo-4′-carotenal predictably accumulated in the absence of YLO-1 activity. However, the young colonies of the *carD* mutant of *F. fujikuroi* exhibit an orange pigmentation, as expected from β-apo-4′-carotenal accumulation, but they turn progressively to yellow as they get older due to its conversion to β-apo-4′-carotenol and, possibly, also to derived fatty acid esters (Díaz-Sánchez et al. 2011b).

Despite the predominance of neurosporaxanthin in *N. crassa* and *F. fujikuroi*, presumably due to the preference of AL-2 and CarRA to introduce a single cyclization in the pathway, the carotenoid analyses in both species detect minor amounts of β-carotene (Mitzka and Rau 1977), indicating that these enzymes are able to carry out a second cyclization. In the case of *N. crassa*, in vitro studies showed that γ-carotene is not accepted as a substrate by AL-1 (Hausmann and Sandmann 2000), and therefore if the cyclization is produced on lycopene instead of on 3,4-didehydrolycopene, the resulting γ-carotene may be only converted to β-carotene (Fig. 8.5). The occurrence of a second cyclization might be more frequent than expected in *F. fujikuroi* and other *Fusarium* species, where the genes *carRA* and *carB* are linked in a gene cluster with gene *carX*, encoding a β-carotene-cleaving CCD enzyme (Thewes et al. 2005), and with *carO*, encoding a rhodopsin (Prado et al. 2004). CarO is a photoactive proton pump that plays a role in conidia germination (García-Martínez et al. 2015). The product of β-carotene cleavage is retinal, used as chromophore by the CarO rhodopsin and presumably by a second rhodopsin in these fungi, called OpsA (Estrada and Avalos 2009). Retinal has its maximal absorption in the UV-A region of the spectrum and therefore does not provide pigmentation. There are no reports on retinal levels in these fungi, hindering any estimation of the proportion of carotene substrates deviated to the β-carotene branch. *N. crassa* has also a gene for a photoactive rhodopsin (Bieszke et al. 1999a, b), but despite having a second CCD gene, no retinal-forming activity has been found so far in this fungus (Díaz-Sánchez et al. 2013).

Information on the physical location of neurosporaxanthin biosynthesis in the fungal cells is very scarce. *F. fujikuroi* is well known for the production of gibberellins, a group of growth promoting plant hormones (Tudzynski 2005). Gibberellins are terpenoids that share with carotenoids their origin from GGPP, but meanwhile the carotenoids are accumulated in the cell, the gibberellins are excreted to the medium. Experiments with *F. fujikuroi* cultures in the presence of ^{14}C-labeled mevalonate, as mentioned before for *P. blakesleeanus* (Bejarano and

Cerdá-Olmedo 1992), indicated different cellular locations and independent substrate pools for the synthesis of carotenoids, gibberellins, and sterols (Domenech et al. 1996). In the case of carotenoids, the majority of the enzymes are associated to membranes, making difficult their purification for in vitro studies (Bramley 1985). Consequently, the carotenogenic enzymes of *N. crassa* are partially solubilized by detergent treatments, and their activities are improved by lipid addition (Mitzka-Schnabel 1985). In the same fungus, carotenoids have been found associated to the external membrane of mitochondria (Neupert and Ludwig 1971), suggesting that they are synthesized in this organelle, but they have been found also in lipid globules and in membranes of the endoplasmic reticulum (Mitzka-Schnabel and Rau 1980). Regarding the association of the carotenogenic enzymatic machinery to membranes, it is particularly relevant that the aldehyde dehydrogenases YLO-1 and CarD include a terminal transmembrane domain (Estrada et al. 2008b; Díaz-Sánchez et al. 2011b), very infrequent in this protein family.

8.2.6 Regulation of Neurosporaxanthin Biosynthesis

As already mentioned for β-carotene production by *P. blakesleeanus* and *M. circinelloides*, the synthesis of neurosporaxanthin is stimulated by light in *N. crassa* (Avalos and Corrochano 2013) and *Fusarium* sp. (Avalos and Estrada 2010). This photoresponse has been investigated in especial detail in *N. crassa*, currently a leading model in fungal photobiology (Chen et al. 2010). When grown in the dark, the submerged cultures of this fungus are albino and contain phytoene, but its exposure to light results in the rapid accumulation of colored carotenoids, reaching a maximum in about 6 h (Rau et al. 1968) or 12 h (Zalokar 1954), depending on the culture conditions. Light dosage experiments showed that 1 min of light (Zalokar 1955) or even less (Zalokar 1955; Schrott 1980) is sufficient to cause a detectable response, but after the light pulse, the mycelia become temporarily insensitive to a second light exposure (Schrott 1981). Fluence response experiments showed a biphasic curve, with a first step resulting from just some seconds of exposure at the highest light intensity and a second one needing at least 16 min of illumination (Zalokar 1955; Schrott 1980). In contrast, at least 1 h is needed for a significant response in *F. aquaeductuum* (Bindl et al. 1970) and *F. fujikuroi* (Avalos and Schrott 1990).

N. crassa photocarotenogenesis is very sensitivity to temperature. The carotenoids accumulate in much higher amounts when dark-grown mycelia are illuminated and incubated at low temperature (Harding 1974) than at high temperature, with an optimal response between 6 and 12 °C. The effect of low temperature is not only on total carotenoid production but also on enzymatic efficiency, as indicates the accumulation of a higher proportion of neurosporaxanthin, a feature that facilitated the characterization of mutants affected in the last reactions of the pathway (Saelices et al. 2007; Estrada et al. 2008b). Additionally, the analysis of intermediary carotenoids showed an unexpected change in the order of the enzymatic steps depending on the temperature of illumination (Fig. 8.6), with the

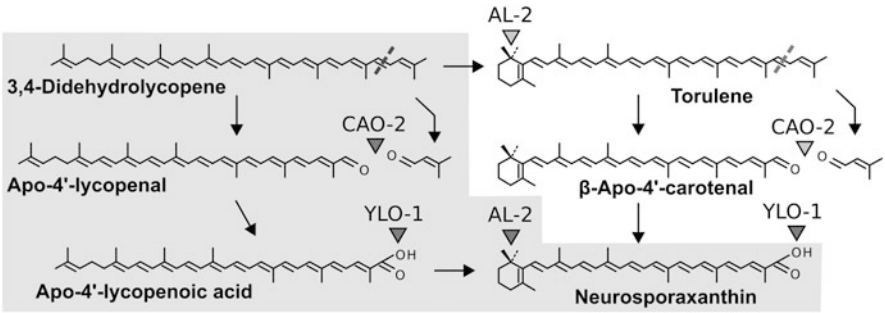

Fig. 8.6 Alternative pathways for late steps of neurosporaxanthin biosynthesis in *N. crassa*. The reactions shaded in *gray* are predominant under cold conditions. The *dotted gray lines* in 3,4-didehydrolycopene and torulene indicate cleavage sites for the CAO-2 CCD enzyme

cyclization of apo-4′-lycopenoic acid as the last reaction at low temperature (Estrada et al. 2008a). Therefore, 3,4-didehydrolycopene may be recognized as a substrate not only by the cyclase activity of AL-2 but also by the cleaving oxygenase CAO-2, with the latter prevailing under cold conditions. Such effect has not been described in *Fusarium*, and, at least in *F. aquaeductuum*, the synthesis of carotenoids is less efficient at lower temperatures (Rau 1962).

Action spectrum of photocarotenogenesis in *N. crassa* reveals an optimal efficiency at light wavelengths ranging from 440 to 490 nm (Zalokar 1955), with peaks at about 450 and 480 nm (de Fabo et al. 1976). As already mentioned for *P. blakesleeanus*, the spectrum shape is consistent with the participation of flavin photoreceptors. This hypothesis was corroborated by the reduced photoinduction exhibited by flavin deficient mutants (Paietta and Sargent 1981), although such deficiency was not restored by external supplementation of riboflavin or some analogs (Paietta and Sargent 1983). Conclusive confirmation was attained with the identification of the responsible photoreceptor, which turned out to be a heterodimeric complex called White Collar (WC), consisting of the flavin photoreceptor WC-1 and its partner WC-2. Thus, the *wc-1* and *wc-2* mutants are unable to photoinduce carotenogenesis, a defect that was confirmed at the level of phytoene synthase activity (Harding and Turner 1981), carotenoid accumulation (Degli-Innocenti and Russo 1984), and mRNA levels for structural genes (Saelices et al. 2007). Upon its activation by light, the WC complex acts directly on the promoters of the target genes (He and Liu 2005), presumably binding to upstream regulatory elements as those found in the *al-3* promoter (Carattoli et al. 1994). As a result, there is an increase in the mRNA levels of the structural genes *al-1* (Schmidhauser et al. 1990), *al-2* (Schmidhauser et al. 1994), *al-3* (Baima et al. 1991), and *cao-2* (Saelices et al. 2007). The response is very fast, reaching top levels after 15–30 min of illumination and decreasing afterward. However, the gene for the last step of the pathway, *ylo-1*, is not affected by light (Estrada et al. 2008b).

The regulation by light is basically conserved in *Fusarium*, as indicates the similar action spectrum described for *F. aquaeductuum* (Rau 1967) and the

identification of the orthologous *wc-1* genes *wcoA* in *F. fujikuroi* (Estrada and Avalos 2008) and *wc1* in *F. oxysporum* (Ruiz-Roldán et al. 2008). However, the WC heterodimer is not the only photoreceptor involved in photocarotenogenesis in *Fusarium*, since the mutants deprived of either WcoA or Wc1 are still able to accumulate carotenoids in response to light. Recently, it was shown that photocarotenogenesis in *F. fujikuroi* is carried out in two stages, a fast one mediated by WcoA and a slower one in which participates another flavin photoreceptor, the DASH cryptochrome CryD (Castrillo and Avalos 2015). The significant carotenoid accumulation in the WcoA mutants under constant illumination may be attributed to CryD, whose photoactivity has been experimentally demonstrated (Castrillo et al. 2015). Interestingly, the mutation of either *wcoA* or *cryD* also affects the production of other secondary metabolites (Estrada and Avalos 2008; Castrillo et al. 2013), including in the dark in the case of *wcoA*, indicating other functions for these photoreceptors in addition to the control of carotenogenesis.

The photoinduction of carotenogenesis in *Fusarium* reminds that of *N. crassa*, but the maximal levels are reached in this case approximately after 1 h of light, as observed in *F. fujikuroi* (Prado et al. 2004; Prado-Cabrero et al. 2007), *F. oxysporum* (Rodríguez-Ortiz et al. 2012), and *F. verticillioides* (Ádám et al. 2011). In the same way that *ylo-1* of *N. crassa*, *carD* is not affected by light (Díaz-Sánchez et al. 2011b), as seems also essentially unaffected the GGP synthase gene *ggs-1* (Mende et al. 1997), in this case in contrast to its *N. crassa* ortholog *al-3* (Baima et al. 1991). Photoinduction of carotenogenesis is also affected by other regulatory mechanisms, as indicates the lower photoresponse exhibited by the mutants of the adenylate cyclase gene *acyA* in *F. fujikuroi* (García-Martínez et al. 2012) or the mating-type *MAT1-2-1* gene in *F. verticillioides* (Ádám et al. 2011).

The decrease in the mRNA levels for the structural genes that follows the photoinduction peak even if illumination persists, a process known as photoadaptation, has been investigated in detail in *N. crassa*, where it is achieved though the deactivation of the WC complex by hyperphosphorylation (He and Liu 2005). In this process it plays a central role a small flavin photoreceptor, known as VIVID or VVD (Shrode et al. 2001; Schwerdtfeger and Linden 2003). In the *vvd* mutants, the decrease in the mRNA levels of the *al* genes after their rapid light induction is less pronounced, leading to a higher carotenoid accumulation (Youssar et al. 2005; Navarro-Sampedro et al. 2008). Other proteins presumably collaborate with VVD in photoadaptation, but although new mutants affected in this process have been identified (Navarro-Sampedro et al. 2008), the molecular functions of the affected genes remain to be elucidated.

In addition to light, other regulatory factors control neurosporaxanthin biosynthesis in *N. crassa* and *Fusarium*. In *N. crassa* the conidia formed by aerial mycelia are orange because of the accumulation of carotenoids, and the synthesis is coupled to the process of conidiation in a light–independent manner. Thus, when conidiation is induced in the wild type in the dark by transferring submerged-grown mycelia to air, there is an increase in *al-1* and *al-2* transcript levels about 16 h after their transfer, coinciding with the formation of the conidia (Li and Schmidhauser 1995). This induction is also observed under continuous

illumination, when *al-1* and *al-2* transcript levels are attenuated by photoadaptation, and it is absent in regulatory mutants defective in conidiation, such as *fluffy* or *fluffyoid*. However, the *wc* mutants produce pigmented conidia irrespective of illumination. The separation of the stimulatory effects of light by the WC complex and of conidiation by FLUFFY or FLUFFYOID is clearly shown when the mycelium is simultaneously exposed to light and air. In this case there are two different inductions, a rapid and transient one produced by light and a late one after 16 h, which coincides with the formation of the major constrictions during the conidiation process. In the case of gene *al-3*, the separate regulations by light and conidiation are also proved by the synthesis of specific transcripts for each inducing condition from different transcription start sites: with a size of 1.6 kb in the first case that requires a functional WC complex and 2.2 kb in the second, dependent of a functional FLUFFY protein (Arpaia et al. 1995). The complexity of the regulation of this gene is also shown by the finding of more than one translation start site (Vittorioso et al. 1994).

Different observations indicate that the cAMP signaling pathway, involved in other biological processes, participates in the regulation of neurosporaxanthin biosynthesis in *N. crassa* and *F. fujikuroi*. During the lag phase preceding carotenoid photoinduction, there is a transient increase of the cAMP levels in *N. crassa*, and the *cr-1* (*crisp-1*) mutants, defective in adenylyl-cyclase activity, contain more carotenoids in the dark (Kritsky et al. 1982). A similar phenotype is exhibited by the mutants of the adenylate cyclase gene *acyA* of *F. fujikuroi* in the dark (García-Martínez et al. 2012). Moreover, carotenoid photoinduction in *N. crassa* is reduced by exogenous cAMP addition (Harding 1973), and artificial upregulation of the gene *gna-1*, coding for a Gα component of a heterotrimeric G complex, exhibited developmental alterations and contained less carotenoids than the control strain (Yang and Borkovich 1999). As a later observation, the lack of histidine kinase DCC-1 increases conidia formation and carotenoid production in *N. crassa* (Barba-Ostria et al. 2011), but this effect is reversed by cAMP addition. Taken together, these observations indicate an inverse correlation between cAMP levels and carotenoid production in these fungi.

Neurosporaxanthin biosynthesis in *N. crassa* and *F. fujikuroi* is stimulated by nitrogen starvation. In *N. crassa*, the mRNA levels for the genes *al-1* and *al-2* in the dark are higher when the wild type is grown in a medium with 2 mM NH_4Cl (nitrogen limitation) than with 50 mM NH_4Cl (nitrogen excess) (Sokolovsky et al. 1992). The same pattern was found in *wc* mutants, indicating that the effect of nitrogen is independent of the regulation by light. Likewise, nitrogen starvation stimulates carotenoid biosynthesis in *F. fujikuroi* in the dark, as showed experiments with either immobilized mycelia (Garbayo et al. 2003) or shake cultures (Rodríguez-Ortiz et al. 2009). In both cases, the transfer from high nitrogen to low nitrogen conditions resulted in an increased carotenoid production, corroborated in the case of the shake cultures with an increase in *carRA* and *carB* mRNA levels. As in *N. crassa*, the induction by nitrogen starvation in *F. fujikuroi* is

independent of the induction by light, as shown by their additive effects when both stimulating conditions are simultaneously present (Rodríguez-Ortiz et al. 2009).

Neurosporaxanthin-overproducing mutants, called *carS*, have been described in *F. fujikuroi* (Avalos and Cerdá-Olmedo 1987; Rodríguez-Ortiz et al. 2013) and *F. oxysporum* (Rodríguez-Ortiz et al. 2012). Their higher carotenoid content is consistent with the finding of an increased carotenogenic activity of their cell extracts in vitro (Avalos et al. 1988) and higher mRNA levels for the structural genes *carRA*, *carB* (Prado et al. 2004; Thewes et al. 2005), *carT* (Prado-Cabrero et al. 2007), and *carD* (Díaz-Sánchez et al. 2011b). Except for the case of *carD*, the mRNA levels for these genes in the dark are as high as those in the wild type after 1 h illumination; however, the *carS* mutants are still able to respond to light either in *F. fujikuroi* (Prado-Cabrero et al. 2007) or in *F. oxysporum* (Rodríguez-Ortiz et al. 2012). The gene *carS* encodes a protein with RF and LON domains, with clear similarities with the domain features of CrgA of *M. circinelloides* despite a considerable sequence divergence (Rodríguez-Ortiz et al. 2012, 2013), and the *carS* mutant phenotype coincides with that of the *crgA* mutants. The *carS* mutation not only affects the synthesis of carotenoids but also the production of other secondary metabolites, such as gibberellins or bikaverin (Candau et al. 1991a; Rodríguez-Ortiz et al. 2009). The mechanism of action of the CarS protein in *Fusarium* is currently object of detailed investigation.

In contrast to *Fusarium*, no *carS*-like mutants are known in *N. crassa*. Two mutants of this fungus, *ccb-1* and *ccb-2*, have an increased carotenoid content in the dark (Linden et al. 1997), but their carotenoid levels are quite low compared to those of the *carS* strains of *Fusarium* (Avalos and Cerdá-Olmedo 1987; Rodríguez-Ortiz et al. 2012). The *ccb-1* and *cbb-2* mutants are also affected in hyphal morphology, more severely in the case of *ccb-1*, which is the only one exhibiting an increase in *al-1* and *al-2* mRNA levels. Other carotenoid-overproducing mutants described in this fungus, as *vvd* (Shrode et al. 2001) and *ovc* (Harding et al. 1984), exhibit this phenotype only under light. In the latter case, the *ovc* phenotype is accompanied by osmotic sensitivity (Youssar et al. 2005), and it is due to a large deletion covering 21 genes (Youssar and Avalos 2007), none of them with a predictable relation with the regulation of carotenogenesis. Carotenoid production may be also augmented in *N. crassa* by genetic engineering, as reveals the visible increase resulting from the expression of the catalytic domain of 3-hydroxy-3-methylglutaryl coenzyme A reductase from *S. cerevisiae* under control of a strong promoter (Wang and Keasling 2002).

8.3 Carotenoid Biosynthesis in Yeasts

The color of many pigmented yeasts, frequently in the phylum of basidiomycetes, is due to the production of carotenoids. β-carotene biosynthesis has been described in some species, as *Rhodosporidium* sp. (de Miguel et al. 1997), *Sporidiobolus pararoseus* (Han et al. 2012), *Ustilago maydis* (Estrada et al. 2010), and other *Ustilago* species (Will et al. 1984, 1985). However, yeasts usually produce

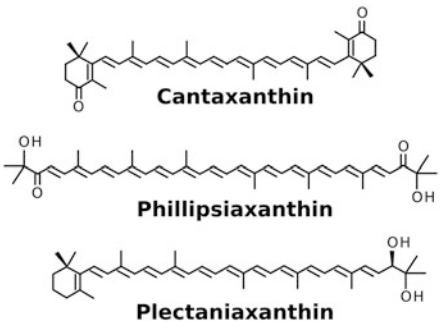

Fig. 8.7 Chemical structures of some yeast carotenoids

xanthophylls. To this group belong canthaxanthin, found in *Cantharellus cinnabarinus* (Haxo 1950), plectaniaxanthin in *Dioszegia* sp. (Madhour et al. 2005), and phillipsiaxanthin, a lycopene derivative with hydroxyl and keto groups, in the ascomycete *Phillipsia carminea* (Arpin and Liaaen-Jensen 1967). The structures of these xanthophylls are depicted in Fig. 8.7. However, the most investigated cases correspond to the productions of astaxanthin by *Xanthophyllomyces dendrorhous*, and torularhodin by *Rhodotorula* sp., summarized in the next sections. The biotechnological production of carotenoids by yeast has been object of a recent review (Mata-Gómez et al. 2014).

8.3.1 Astaxanthin Biosynthesis in *Xanthophyllomyces*

Astaxanthin is a red xanthophyll of economic importance because of its benefiting effects on human health (Ambati et al. 2014). The high antioxidant activity and health-promoting properties of this xanthophyll have led to an increasing use in the nutraceutical market, where it is sold as an encapsulated product. Astaxanthin is also widely used as animal feed additive, especially as a source of pigmentation for some fishes and crustaceans in aquaculture industries, as those of trouts, salmons, or shrimps (Higuera-Ciapara et al. 2006). Astaxanthin is produced by different microorganisms, including algae, bacteria, and fungi (Alcaino et al. 2014), and it is industrially obtained from the alga *Haemematococcus pluvialis* (Ambati et al. 2014) and the yeast *X. dendrorhous* (Rodríguez-Sáiz et al. 2010), also known as *Phaffia rhodozyma* (the anamorph form) (Johnson 2003).

Astaxanthin is synthesized from β-carotene through the introduction of keto and hydroxyl groups in the β-rings of the molecule (Fig. 8.8). Therefore, the steps up to β-carotene coincide with those described in former sections for β-carotene-producing fungi (Fig. 8.2). All the genes specifically involved in astaxanthin biosynthesis are known in *X. dendrorhous*, as well as some involved in early steps of the terpenoid pathway, as the one coding for the enzyme that isomerizes IPP to DMAPP (Kajiwara et al. 1997), the FPP synthase gene *fpp* (Alcaíno et al. 2014), and the GGPP synthase gene *crtE* (Niklitschek et al. 2008). The phytoene synthase/lycopene cyclase is encoded by the gene *crtYB*, the first one in fungi where the

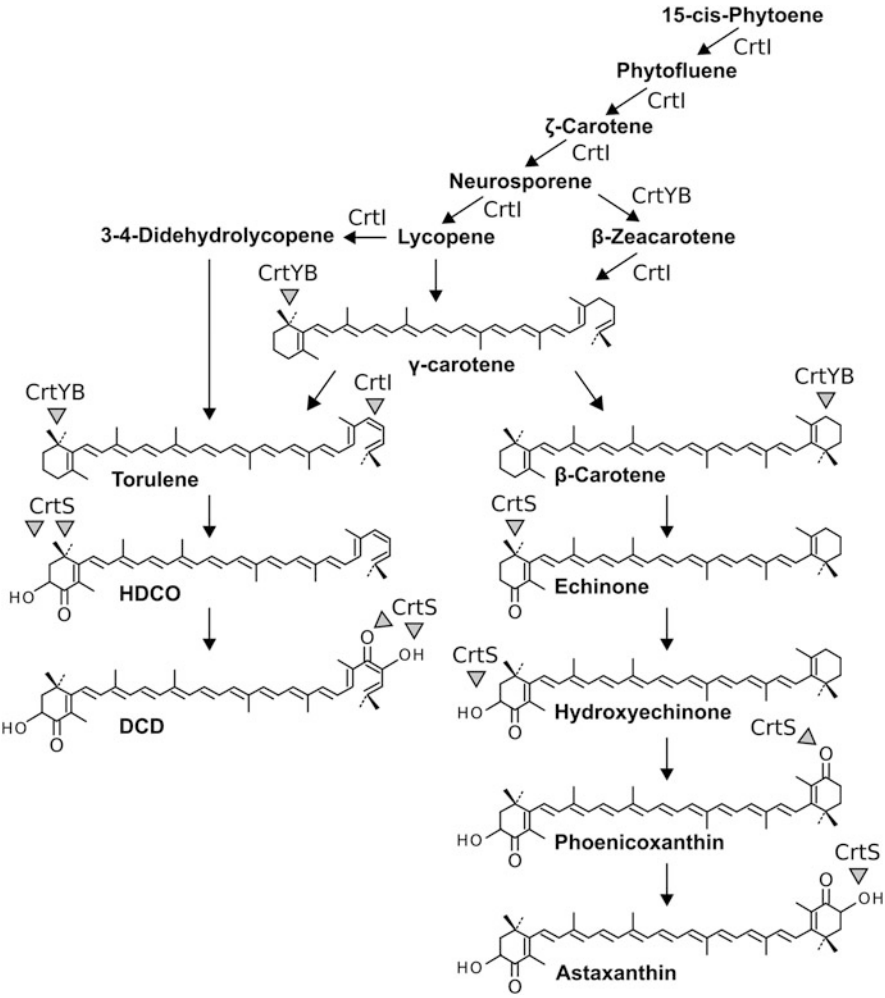

Fig. 8.8 Astaxanthin biosynthesis in *X. dendrorhous*. In the reactions achieved by CrtS, the presumed participation of CrtR is omitted. Reactions and chemical structures up to lycopene and 3-4-didehydrolycopene are as shown in Figs. 8.2 and 8.5. Enzymes responsible for each reaction are indicated close to the chemical modification produced in each enzymatic step (*gray arrowhead*). The uncycled end of torulene and derivatives is represented in a folded form, as drawn in original works (Visser et al. 2003; Martín et al. 2008). *HDCO* 3-hydroxy-3′4′-didehydro-β,-ψ-carotene-4-one, *DCD* 3,3′-dihydroxy-β,ψ-carotene-4,4′dione

double function of the enzyme was discovered (Verdoes et al. 1999a). Between the synthase and cyclase reactions carried out by CrtYB, the four desaturations are achieved by the desaturase encoded by the gene *crtI* (Verdoes et al. 1999b). Albino mutants accumulating phytoene or no carotenes, affected in *crtI* and presumably in *crtYB*, are readily obtained by mutagenesis (Girard et al. 1994), but no mutants

affected in the cyclase activity have been described. However, such activity is blocked in the presence of nicotine, resulting in the accumulation of lycopene (Ducrey Sanpietro and Kula 1998). On the other hand, diphenylamine blocks the desaturase activity and, at a lower concentration, the activity of CrtS-CrtR on β-carotene, described below.

The conversion of β-carotene to astaxanthin might result in a diversity of intermediates depending in the order of introduction of the keto and hydroxyl groups. However, early chemical analysis of the carotenoids in this yeast revealed the presence of significant amounts of echinenone, 3-hydroxy-echinenone and phoenicoxanthin (Andrewes et al. 1976), suggesting the order of reactions displayed in Fig. 8.8. The 3-hydroxylations and 4-ketolations are achieved by a single enzyme of the cytochrome P450 monooxygenase family. The responsible gene was simultaneously cloned by two different research groups, that named it *crtS* (Álvarez et al. 2006) and *asy* (Ojima et al. 2006), referred to *crtS* hereafter. The achievement of both types of oxidations by a single enzyme differs from what was found in astaxanthin-producing algae and bacteria, where independent ketolases and hydroxylases are involved in the conversion of β-carotene to astaxanthin (Fraser et al. 1997). However, the expression of *crtS* in a heterologous system, as *M. circinelloides*, results only in the production of hydroxylated derivatives, indicating the participation in *X. dendrorhous* of at least an additional protein (Martín et al. 2008). This turned out to be a cytochrome P450 reductase, encoded by the gene *crtR*, that provides the electrons for the oxygenation reactions achieved by CrtS, as indicates the lack of astaxanthin in *crtR* deletion mutants (Alcaíno et al. 2008). When CrtS is expressed in a metabolically engineered *S. cerevisiae* strain (see next section), it is only active when co-expressed with *crtR*; despite this yeast having its own cytochrome P450 reductase (Ukibe et al. 2009). This indicates a specific interaction between CrtS and CrtR for proper activity, a conclusion that has been supported by protein modeling and molecular dynamics simulations (Alcaíno et al. 2012).

The chemical analyses of the carotenoids accumulated in *X. dendrorhous* identified a novel xanthophyll, 3-hydroxy-3′4′-didehydro-β,ψ-carotene-4-one (HDCO, Fig. 8.8), that implies the occurrence of a side biosynthetic branch of monocycled carotenoids in this yeast. More detailed chemical analyses found also torulene and postulated the origin of HDCO through an additional desaturation on γ-carotene and the introduction of keto and hydroxyl groups at the only β-ionone ring of the molecule (An et al. 1999). Thus, as already described for *N. crassa* and *Fusarium* sp., this side pathway in *X. dendrorhous* shows the capacity of the CrtI desaturase to carry out a fifth desaturation that impedes the introduction of the second cyclization in the molecule. Moreover, the detection of minor amounts of the HDCO derivative 3,3′-dihydroxy-β,ψ-carotene-4,4′dione (DCD) indicates that the CrtS-CrtR enzymatic machinery is also able to recognize as a substrate the uncycled end of HDCO (Fig. 8.8).

8.3.2 Biotechnological Astaxanthin Production

The yeast *X. dendrorhous* (*P. rhodozyma*) is the only fungus used for industrial astaxanthin production. The yields may vary considerably depending on the strain and the growth conditions (Schmidt et al. 2011), and the biotechnological use of this yeast implies the improvement of the production through the isolation of astaxanthin-overproducing strains and the development of more efficient culture conditions (see, e.g., the study described by Meyer et al. 1993). Despite this fungus is diploid (Kucsera et al. 1998), deep-pigmented mutants producing more carotenoids are easily detected after adequate mutagenesis treatments (Visser et al. 2003), allowing increases in astaxanthin content ranging from 2–3-fold (Fang and Cheng 1992; Stachowiak 2013; Barbachano-Torres et al. 2014) to 10–15-fold (Miao et al. 2010). In addition, different enrichment methods have been successfully used to obtain mutants with higher astaxanthin production, as those based on antymicin resistance (An et al. 1989), β-ionone inhibition (Lewis et al. 1990), flow cytometry sorting (An et al. 1991; Brehm-Stecher and Johnson 2012), photosensitization (An 1997), DPA resistance (Chumpolkulwong et al. 1997), or low-dose gamma irradiation (Sun et al. 2004). Incubation under continuous illumination has no relevant effect on the accumulation of carotenoids in this yeast (Johnson and Lewis 1979), or it even has a detrimental effect (An and Johnson 1990), but it results in increase of the carotenoid content in antimycin-treated cells (An and Johnson 1990), in some astaxanthin-overproducing mutants (Meyer and Du Preez 1994), and in an industrial strain (de la Fuente et al. 2010).

X. dendrorhous is amenable to genetic transformation (Martínez et al. 1998), allowing to obtain strains with enhanced astaxanthin production through engineered metabolic alterations (Visser et al. 2003). In this way, different yield improvements were obtained through the increased expression of early genes of the terpenoid pathway (Hara et al. 2014), the GGPP synthase *crtE* gene (Breitenbach et al. 2011), the bifunctional *crtYB* gene (Ledetzky et al. 2014), and the *crtS* gene (Chi et al. 2015) or through the deletion of genes related to ergosterol biosynthesis (Loto et al. 2012; Yamamoto et al. 2016), thus releasing the repression of HMG-CoA reductase. A similar stimulation was obtained by the targeted deletion of *mig1*, encoding the orthologous global regulator of catabolite repression, indicating that glucose availability plays a regulatory role in carotenoid biosynthesis in *X. dendrorhous* (Alcaíno et al. 2016). Actually, glucose exerts a negative effect on astaxanthin biosynthesis (Yamane et al. 1997). Recently, the introduction of several vectors to an overproducing mutant to obtain the combined increased expression of *crtYB* and *crtS* (Gassel et al. 2013), and later with the addition of a truncated version of the HMG-CoA reductase gene *hmg1* from *S. cerevisiae* and the endogenous *crtE* gene (Gassel et al. 2014), allowed to reach carotenoid levels comparable to the extremely high production obtained with the alga *H. pluvialis*. However, the modified expression of carotenogenic genes may eventually result in undesired effects for astaxanthin production. Thus, overexpression of *crtYB* or *crtI* may lead to increased proportions of β-carotene and echinone or monocyclic carotenoids, respectively (Verdoes et al. 2003).

As described for *B. trispora*, oxidative stress affects the synthesis of astaxanthin in *X. dendrorhous*. A positive correlation has been found between oxygen levels in the medium and astaxanthin biosynthesis (Yamane et al. 1997). Photochemical generation of singlet oxygen with rose bengal or alpha-terthienyl results in a higher astaxanthin accumulation, but H_2O_2 or the peroxyl radical-generating agent *t*-butylhydroperoxide decreases the content of astaxanthin, although it is compensated by a higher β-carotene accumulation (Schroeder and Johnson 1995a). Other authors, however, found the opposite effect for H_2O_2, its addition increasing the astaxanthin proportion at expenses of that of β-carotene (Liu and Wu 2006b). Moreover, the presence of H_2O_2 under continuous culture renewal conditions that allow higher astaxanthin yields compared to standard batch cultures leads to a further improvement in the production (Liu and Wu 2007). The increased astaxanthin production in the presence of oxidative agents must be related with the antioxidant properties of this xanthophyll, as indicates the positive correlation between carotenoid content and survival of the yeast in media supplemented with H_2O_2 or under chemical conditions generating superoxide (Schroeder and Johnson 1993) or singlet oxygen (Schroeder and Johnson 1995b). The proteomes in cells at different culture stages are consistent with a relation of astaxanthin production with the defense against reactive oxygen species generated during metabolism (Martinez-Moya et al. 2011).

The composition of the medium exerts a strong influence on astaxanthin production in *X. dendrorhous* cultures (Johnson and Lewis 1979). The use of a non-fermentable carbon source, succinate, instead of the fermentable glucose results in a duplication of the carotenoid level (Wozniak et al. 2011), which has been associated to an increased acetyl-CoA availability and cellular respiration rate and presumably also to enhanced oxidative stress (Martinez-Moya et al. 2015). A significant increase in total carotenoid content is also obtained with low nitrogen or phosphate concentrations and with citrate addition, but changes in astaxanthin are not so marked (Flores-Cotera et al. 2001). An increase in carotenoid content is also produced in different genetic backgrounds by addition of ethanol, possibly also related with enhanced oxidative stress, but the proportion of astaxanthin was not determined (Gu et al. 1997). In this case, increased HMG-CoA reductase was detected in the presence of ethanol, which may be the result of a regulatory effect. In support to this observation, addition of mevalonic acid, the product of this enzyme, enhances the accumulation of astaxanthin (Calo et al. 1995). In other cases, the chemical basis of the inducing agents remains to be identified. The presence of a fungal contaminant, identified as *Epicoccum nigrum*, in Petri dishes with *X. dendrorhous* colonies, or the incubation of the yeast in media with filtered extracts from *E. nigrum* cultures, results in increased carotenoid production, suggesting that the contaminant fungus produces a stimulatory metabolite (Echavarri-Erasun and Johnson 2004). Similarly, unidentified fungal elicitors from diverse fungi (Wang et al. 2006) or certain plant extracts (Kim et al. 2007) have inducing effects.

Considering the variations in astaxanthin content depending on the culture conditions and media composition, many efforts have been addressed to improve

the culture parameters for optimal production (see, e.g., Ramírez et al. 2001; Liu et al. 2008). The changes in basic fermentation parameters, as pH, temperature, inoculum, and carbon or nitrogen concentrations, usually done on batch cultures because of experimental amenability, may lead to tenfold variations in carotene production (Ramírez et al. 2001). As already mentioned for β-carotene production with *B. trispora*, the use of cheap substrates contributes to increase the profitability of astaxanthin production as an industrial activity, and many different substrates proved useful for industrial growth of *X. dendrorhous*. The alternative nutrient sources successfully used include corn wet-milling coproducts (Hayman et al. 1995), peat hydrolysates (Acheampong and Martin 1995; Vázquez and Martin 1998), sugarcane juice (Fontana et al. 1996), wood hemicellulosic hydrolysates (Parajo et al. 1998), beet blackstrap molasses (An et al. 2001), raw coconut milk (Domínguez-Bocanegra and Torres-Muñoz 2004), mustard wastes (Tinoi et al. 2006), pineapple juice (Jirasripongpun et al. 2008), sugarcane bagasse and barley straw hydroxylates (Montanti et al. 2011), mussel-processing wastewater (Amado and Vázquez 2015), and others (Frengova and Beshkova 2009). Although behavior of this yeast may be similar in aerated fermenters or in batch cultures (Acheampong and Martin 1995), the growth conditions in industrial fermenters require to optimize other parameters. A representative example is found in the search for the optimal pH and dilution rate combination using a response surface methodology (Vázquez and Martin 1998).

Contrary to the many efforts dedicated to develop the industrial astaxanthin production by *X. dendrorhous*, the regulation of the expression of the carotenogenic genes has received little attention. The amplification of the cDNA products for the genes *crtI* and *crtYB* revealed the occurrence of alternative splicing events, leading to the formation of predictably nonfunctional proteins (Lodato et al. 2003). Interestingly, the proportion of correctly spliced mRNAs decreased with aging for the gene *crtI*, suggesting a possible regulatory mechanism of CrtI levels mediated through transcript maturation. Some expression studies have focused the attention on the relation between enhanced carotenoid biosynthetic activity and mRNA levels for the structural genes. In a comparison between a wild type and two carotenoid-overproducing mutants, no correlation was found in RT-PCR analyses between transcript levels for the genes *idi*, *crtE*, *crtYB*, and *crtI* and carotenoid production (Lodato et al. 2004). A later detailed study of the kinetics of growth, carotenoid content, and mRNA levels for the same genes, in this case extended to *crtS*, revealed only minor differences between the wild type and one mutant (Lodato et al. 2007). In both strains, the transcript levels for the genes *idi* and *crtE* were similar at either the exponential or the stationary phase, while those for *crtYB*, *crtI*, and *crtS* reached maximal levels at the end of the exponential phase and decreased afterward. On the other hand, RT-qPCR analyses of the expression of the whole set of genes involved in astaxanthin biosynthesis found increased mRNA levels for *crtE* and *crtR* in an overproducing mutant, but against expected, the pattern was the contrary for *crtYB* and *crtS* in advanced culture stages (Castelblanco-Matiz et al. 2015). Even so, in this strain *crtYB* and *crtS* contained mutations in their coding sequences, which might result in a higher enzymatic

activity. However, other overproducing mutants may have a different molecular explanation, as indicates the much higher levels for genes *crtE*, *crtYB* (called in this case *pbs*), *crtI*, and *crtS* (called here *ast*) in a particular mutant strain compared to the wild type, while genes for earlier steps in the terpenoid pathway were basically unaffected (Miao et al. 2011). The overproducing phenotype in this case was also related with a decrease in ergosterol and fatty acids production.

Other reports have described the effects on gene expression of culture conditions leading to a higher carotenoid content. The inducing effect of succinate on astaxanthin biosynthesis is accompanied by some changes in the expression patterns of the carotenogenic genes, with a delay in the maximal mRNA levels in succinate for *crtS* and for the alternative spliced versions of *crtYB* and *crtI*, although such changes seem insufficient to explain the differences in carotenoid production (Wozniak et al. 2011). However, the inducing effect of ethanol may be explained by a transient but significant increase in *crtYB*, *crtI*, and *crtS* transcript levels, while the opposite pattern was found upon addition of glucose (Marcoleta et al. 2011). The stimulating effect of oxygen on astaxanthin biosynthesis may be at least partially explained by an effect on gene expression, as indicates the positive correlation between dissolved oxygen and mRNA levels for *crtE*, *crtYB*, *crtI*, and *crtS* (Wu et al. 2011).

8.3.3 Biosynthesis of Torularhodin and Other Xanthophylls

Torularhodin is well known because of its production by the yeasts of the genus *Rhodotorula* (Moliné et al. 2012), but it has been also found in other yeasts, as those of the genera *Peniophora*, *Cystofilobasidium*, *Rhodosporidium*, *Sporobolomyces*, and *Sporidiobolus* (Buzzini et al. 2007; Iurkov et al. 2008). Torularhodin has a chemical structure similar to that of neurosporaxanthin, but with 40 carbon atoms instead of 35 (Sperstad et al. 2006). A first biosynthetic pathway for this xanthophyll was early proposed for *Rhodotorula glutinis*, based on the intermediates accumulated by their cultures under unfavorable biosynthetic conditions, as low temperature or the presence of methylheptenone and β-ionone vapors, and from former chemical studies (Simpson et al. 1964). The sequence of reactions to produce torularhodin (Fig. 8.9) coincides with that of neurosporaxanthin biosynthesis up to torulene (see Fig. 8.5). However, instead of the cleaving reaction characteristic of this pathway, in *R. glutinis*, torulene is object of an oxidation reaction to generate an aldehyde group, which is oxidized further to generate the final torularhodin carboxylic group. Chemical studies in *Cystofilobasidium* demonstrated the occurrence not only of the aldehyde intermediate, torularhodinaldehyde, but also of 16′-hydroxytorulene, indicating three successive oxidation steps from torulene to the final product (Herz et al. 2007), as depicted in Fig. 8.9. The chemical analysis of the carotenoids produced by 13 different *Rhodotorula* species showed considerable differences in their concentrations and the presence of variable proportions of β-carotene in addition to torularhodin and its precursors γ-carotene and torulene (Buzzini et al. 2007). Therefore, as found in the

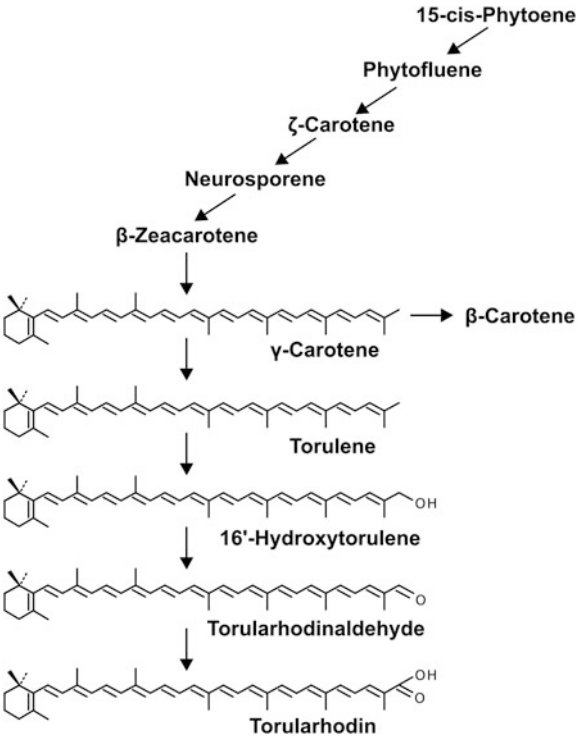

Fig. 8.9 Proposed torularhodin biosynthetic pathway. Reactions and chemical structures up to β-zeacarotene are as shown in Figs. 8.2 and 8.5. Reactions between torulene and torularhodin are as proposed by Herz et al. (2007)

neurosporaxanthin pathway, the γ-carotene intermediate in torularhodin biosynthesis may be subject of a second cyclization instead of the fifth desaturation (Fig. 8.9).

In contrast to the synthesis of astaxanthin or other fungal carotenoids described in former sections, no reports are available on the identification or characterization of the genes involved in torularhodin biosynthesis in the producing yeasts. However, many reports have been dedicated to the improvement of the production of this xanthophyll in *Rhodotorula* species. Mutagenesis methods, based in the exposure to either chemical mutagens (Frengova et al. 2004b) or UV-B radiation (Moliné et al. 2012), have been successfully employed to obtain torularhodin-overproducing mutants. Illumination has a retarding effect on growth of *R. glutinis*, but blue light stimulates significantly the accumulation of torularhodin biosynthesis (Sakaki et al. 2000, 2001), an effect that is more pronounced in an overproducing mutant (Sakaki et al. 2000, 2001). Actually, torularhodin exerts a photoprotective role, as indicates the positive correlation between the presence of this xanthophyll and the resistance to UV-B in strains of *Rhodotorula mucilaginosa* with different carotenoid content (Moliné et al. 2010). In another report, illumination of the yeast in a fermentor resulted in a duplication of the carotenoid content, but in this case β-carotene was predominant (68%) while torularhodin content only reached 21.5% (Zhang et al. 2014). In contrast, oxidative stress artificially induced

by addition of chemicals generating reactive oxygen species, as methyl viologen or methylene blue, increased both the carotenoid content and the torularhodin proportion in *R. glutinis* (Sakaki et al. 2002b). The protective role of torularhodin against oxidative stress was formerly observed in *R. mucilaginosa*, in which the absence of colored carotenoids produced by addition of diphenylamine resulted in cells more sensitive to duroquinone or to hyperoxia than those with a normal carotenoid content (Moore et al. 1989).

Because of its characteristics, torularhodin is a xanthophyll with potential applications in food and cosmetic industries (Hernández-Almanza et al. 2014a; Zoz et al. 2015). Recent reports describe different efforts to develop industrial culture conditions allowing improved carotenoid production with *Rhodotorula* species or with other yeasts, usually containing torularhodin mixed with other carotenoids, as torulene and β-carotene. In a classical optimization work, the effect of initial pH, temperature, aeration rate, carbon (glucose, molasses, sucrose, and whey lactose), nitrogen concentration, as well as cotton seed oil and Tween 80 as potentially activating agents, was investigated in batch cultures of strains of *R. mucilaginosa* (Aksu and Eren 2005) and *R. glutinis* (Aksu and Eren 2007). As already described for β-carotene and astaxanthin productions, research efforts with *Rhodotorula* species have been frequently addressed to check the potential use of low-cost substrates for carotenoid production, although the torularhodin proportion in the carotenoid mixtures was frequently disregarded. The cheap substrates tested include grape must, glucose syrup, beet molasses, soybean or maize flour extracts (Buzzini and Martini 1999), hydrolyzed mung bean waste flour (Tinoi et al. 2005), crude glycerol from biodiesel plants (Saenge et al. 2011), and brewery effluents (Schneider et al. 2013). In a different approach, some *Rhodotorula* species were cocultivated with yogurt starter bacteria in media with whey ultrafiltrates (Frengova et al. 1994, 2004a). In some cases β-carotene is particularly abundant, leading to different reports on the potential use of *Rhodotorula* strains for its biotechnological production, e.g., in stirred or airlift tanks (Yen and Chang 2015), in solid-state fermentation (Hernández-Almanza et al. 2014b), or to obtain β-carotene-enriched biomass with different processed waste substrates (Marova et al. 2012).

In addition to *Rhodotorula*, other torularhodin-producing yeasts have been considered as alternative biotechnological carotenoid sources. Glycerol is particularly efficient for torularhodin biosynthesis by *Sporobolomyces ruberrimus* (Razani et al. 2007), even using raw glycerol from biodiesel production (Cardoso et al. 2016). Basic fermentation parameters and their effects on carotenoid production were also investigated in *Sporidiobolus pararoseus* (Valduga et al. 2009), which was more recently optimized in a cheap glycerol-based medium (Valduga et al. 2014), but the proportion of torularhodin in the total carotenoid mixtures was not determined in these studies. Carob pulp syrup and sugarcane molasses proved efficient for the production of carotenoids by *Rhodosporidium toruloides* (Freitas et al. 2014). A particularly innovative strategy to enhance the carotenoid production by this species was the expression of the Pdr10 multidrug transporter from *S. cerevisiae*, adapted for the codon usage of *R. toruloides* and the culture of this yeast in two-phase media containing oil (Lee et al. 2016). Unexpectedly, a higher

proportion of torulene was exported when *pdr10* was expressed, indicating that torularhodin export was less efficient. Torularhodin may be also obtained from *Sporobolomyces salmonicolor* biomass, as a potential side product in the use of this yeast for exopolysaccharides production (Dimitrova et al. 2013).

8.3.4 Use of Yeasts for Heterologous Carotenoid Production

Heterologous expression in non-carotenogenic microorganisms, as *E. coli* and some yeasts, has been a powerful tool in functional studies of carotenoid biosynthetic genes and in the development of new carotenoid producing systems (Schmidt-Dannert 2000; Sandmann 2002). In the case of yeasts, the experimental amenability derived from its extensive use in basic research and in brewing and fermentative industries has made *S. cerevisiae* a favorite system for heterologous expression. The first report on the use of *S. cerevisiae* for heterologous carotenoid production consisted in the introduction of a plasmid with the *Erwinia uredovora* genes *crtE*, *crtB*, and *crtI* (coding for GGPP synthase, phytoene synthase, and phytoene desaturase, respectively) under control of yeast promoters. The expression of these foreign genes led to lycopene production, which was mostly replaced by β-carotene if the lycopene cyclase *crtY* was also included (Yamano et al. 1994). Later, the overexpression of *crtI* and *crtYB* from *X. dendrorhous* in *S. cerevisiae* allowed the accumulation of variable amounts of β-carotene and their precursors. The production was enhanced by increasing substrate supply through the expression of either the homologous GGPP synthase gene or its ortholog from *X. dendrorhous* *crtE* and a truncated version of the *S. cerevisiae* HMG-CoA reductase gene *hmg1* (Verwaal et al. 2007), mentioned in a former section. Improved production was also attained by expressing in parallel to *ctrI* and *crtYB* from *X. dendrorhous* the mevalonate kinase gene *mvaK1* from *Staphylococcus aureus* (Lange and Steinbüchel 2011), by codon optimization for *S. cerevisiae* of the *crtI* and *crtYB* genes (Li et al. 2013) or simply incubating at 20 °C instead of its usual growth temperature of 30 °C (Shi et al. 2014).

The biosynthesis of β-carotene in *S. cerevisiae* has been subsequently altered in different ways. Production of lycopene was also attained with *X. dendrorhous* genes through the elimination of the cyclase activity of the *crtYB* gene of *X. dendrorhous*, co-expressed with *crtE* and *crtI* (Xie et al. 2015). In this case, the carotene levels were raised further improving the catalytic activity of CrtE by directed evolution and varying the copy number of the *crt* genes. In other study, lycopene production was particularly efficient by combining the *crtE* (GGPP synthase) and *crtB* (phytoene synthase) genes from the bacterium *Pantoea agglomerans* with the *carB* gene from *B. trispora* (called BtCrtI in this study), accompanied by other host alterations (Xie et al. 2015). As an innovative approach, the *crtI*, *crtE*, and *crtYB* coding sequences were also expressed in *S. cerevisiae* as a single polycistronic transcript separated by the T2A sequence of the *Thosea asigna* virus, resulting in the subsequent separation in independent polypeptides during mRNA translation (Beekwilder et al. 2014); the additional expression of the β-carotene cleavage dioxygenase *RiCCD1* from raspberry in these *S. cerevisiae* cells allowed the production of β-ionone. This

apocarotenoid was also produced by separate expression in *S. cerevisiae* of the *crtI*, *crtE*, and *crtYB* genes in parallel with the *Petunia hybrida PhCCD1* gene (López et al. 2015), while the expression of algal β-carotene ketolase genes in similar *S. cerevisiae* β-carotene producing strains allowed the accumulation of significant amounts of canthaxanthin (Chang et al. 2015).

The production of carotenoids in *S. cerevisiae*, presumably at expenses of the deviation of substrates normally used for sterols biosynthesis, results in stressed cells. This conclusion is supported by the specific induction in the carotenoid-producing cells of genes of the pleiotropic drug resistance response (PDR), involved in secretion of toxic compounds (Verwaal et al. 2010). Actually, the addition of an appropriate solvent leads to secretion of carotenoids, indicating that the cells are unable to secrete it in the usual hydrophilic environment of the laboratory cultures. Accordingly, the synthesis of different xanthophyll mixtures, obtained through the expression of *crtI* and *crtYB* in combination with *crtS* and *crtR* from *X. dendrorhous* or with the bacterial ketolase and hydroxylase genes *crtW* and *crtZ*, results in a reduced growth compared to the wild type, although these xanthophyll-producing strains exhibit a better growth capacity in the presence of 1.7 mM H_2O_2 (Ukibe et al. 2009). In contrast, the canthaxanthin-producing strains obtained by expression of algal β-carotene ketolase genes exhibit a slightly faster growth than the control strain in the absence of artificially induced oxidative stress (Chang et al. 2015).

Besides *S. cerevisiae*, other biotechnological yeasts lacking carotenoid biosynthesis have been used for heterologous carotenoid production. The sequences of the *crtE*, *crtB*, and *crtI* genes from *E. uredovora* were modified for optimal codon usage and expressed in *Candida utilis* under control of constitutive promoters from this yeast, leading to lycopene production (Miura et al. 1998), accumulated in higher levels if the cells were engineered for increased HMGCoA reductase activity and reduced squalene synthesis (Shimada et al. 1998). Lycopene was efficiently converted to β-carotene in the same yeast by the additional expression of the cyclase gene *crtY* from *E. uredovora* and to astaxanthin if the β-carotene ketolase (*crtW*) and hydroxylase (*crtZ*) genes from *Agrobacterium aurantiacum* were also expressed (Miura et al. 1998). Another yeast successfully used for carotenoid synthesis is *Pichia pastoris*, known as industrial producer of heterologous proteins. Two plasmid combinations, one with the already mentioned *crtE*, *crtB*, and *crtI* genes from *E. uredovora* and another carrying these genes together with the cyclase *crtL* gene from the plant *Ficus carica*, in all cases under control of yeast promoters, led, respectively, to notable lycopene and β-carotene productions (Araya-Garay et al. 2012b), while the supplementary expression of *crtW* and *crtZ* genes from *A. aurantiacum* added astaxanthin to the produced carotenoids (Araya-Garay et al. 2012a).

8.4 Conclusions

Because of their easy growth and manipulation, the fungi have been a major choice by the researchers as a tool to investigate the molecular basis of carotenoid biosynthesis and its regulation in microorganisms and by biotechnologists as a

source for carotenoids demanded by the market. Usually, depending on their specific features, different species have been used for one purpose or the other. *P. blakesleeanus,* and later also *M. circinelloides,* have been extensively used to learn about the biochemistry and the genetics of β-carotene production, while *N. crassa* and *F. fujikuroi* have been the major sources of information on neurosporaxanthin biosynthesis. In the case of β-carotene, most of the efforts on its biotechnological production have been dedicated to *B. trispora*, which was benefited from the scientific background generated with *P. blakesleeanus* and *M. circinelloides*. In contrast, the possible biotechnological interest of neurosporaxanthin has not received attention. Regarding the yeasts, *X. dendrorhous* (*P. rhodozyma*) has been the major object of attention for astaxanthin production, both from the scientific and biotechnological points of view, while genetics and biochemistry of the carotenoids pathways for the production of other xanthophylls have received very limited attention. Torularhodin production is a notable exception, but the efforts on this xanthophyll were mostly focused on its biotechnological production. The concentration of the research on very few species has allowed a deep knowledge on the molecular basis of carotenoid biosynthesis of the cases investigated, and the high conservation between the ortholog genes of the carotenoid pathways facilitates the prediction for the capacity to produce carotenoids by organisms for which the genome sequences become available. Nevertheless, the low number of fungi investigated in detail allows anticipating that novel fungal carotenoids or more suitable carotenoid producers could be available in the future with more extensive screenings, widening the biotechnological potential of the fungi.

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References

Aasen AJ, Jensen SL (1965) Fungal carotenoids II. The structure of the carotenoid acid neurosporaxanthin. Acta Chem Scand 19:1843–1853

Acheampong EA, Martin AM (1995) Kinetic studies on the yeast *Phaffia rhodozyma*. J Basic Microbiol 35:147–155

Ádám AL, García-Martínez J, Szücs EP et al (2011) The MAT1-2-1 mating-type gene upregulates photo-inducible carotenoid biosynthesis in *Fusarium verticillioides*. FEMS Microbiol Lett 318:76–83

Ahrazem O, Gómez-Gómez L, Rodrigo M et al (2016) Carotenoid cleavage oxygenases from microbes and photosynthetic organisms: features and functions. Int J Mol Sci 17:1781

Aksu Z, Eren AT (2005) Carotenoids production by the yeast *Rhodotorula mucilaginosa*: use of agricultural wastes as a carbon source. Process Biochem 40:2985–2991

Aksu Z, Eren AT (2007) Production of carotenoids by the isolated yeast of *Rhodotorula glutinis*. Biochem Eng J 35:107–113

Alcaíno J, Barahona S, Carmona M et al (2008) Cloning of the cytochrome p450 reductase (*crtR*) gene and its involvement in the astaxanthin biosynthesis of *Xanthophyllomyces dendrorhous*. BMC Microbiol 8:169

Alcaíno J, Fuentealba M, Cabrera R et al (2012) Modeling the interfacial interactions between CrtS and CrtR from *Xanthophyllomyces dendrorhous*, a P450 system involved in astaxanthin production. J Agric Food Chem 60:8640–8647

Alcaino J, Baeza M, Cifuentes V (2014) Astaxanthin and related xanthophylls. In: Martín J-F, García-Estrada C, Zeilinger S (eds) Biosynthesis and molecular genetics of fungal secondary metabolites. Springer, New York, NY, pp 187–208

Alcaíno J, Romero I, Niklitschek M et al (2014) Functional characterization of the *Xanthophyllomyces dendrorhous* farnesyl pyrophosphate synthase and geranylgeranyl pyrophosphate synthase encoding genes that are involved in the synthesis of isoprenoid precursors. PLoS One 9:e96626

Alcaíno J, Bravo N, Córdova P et al (2016) The involvement of Mig1 from *Xanthophyllomyces dendrorhous* in catabolic repression: an active mechanism contributing to the regulation of carotenoid production. PLoS One 11:e0162838

Almeida ER, Cerdá-Olmedo E (2008) Gene expression in the regulation of carotene biosynthesis in *Phycomyces*. Curr Genet 53:129–137

Álvarez V, Rodríguez-Sáiz M, de la Fuente JL et al (2006) The *crtS* gene of *Xanthophyllomyces dendrorhous* encodes a novel cytochrome-P450 hydroxylase involved in the conversion of β-carotene into astaxanthin and other xanthophylls. Fungal Genet Biol 43:261–272

Amado IR, Vázquez JA (2015) Mussel processing wastewater: a low-cost substrate for the production of astaxanthin by *Xanthophyllomyces dendrorhous*. Microb Cell Factories 14:177

Ambati RR, Phang SM, Ravi S, Aswathanarayana RG (2014) Astaxanthin: sources, extraction, stability, biological activities and its commercial applications—a review. Mar Drugs 12:128–152

An GH (1997) Photosensitization of the yeast *Phaffia rhodozyma* at a low temperature for screening carotenoid hyperproducing mutants. Appl Biochem Biotechnol 66:263–268

An GH, Johnson EA (1990) Influence of light on growth and pigmentation of the yeast *Phaffia rhodozyma*. Antonie Van Leeuwenhoek 57:191–203

An GH, Schuman DB, Johnson EA (1989) Isolation of *Phaffia rhodozyma* mutants with increased astaxanthin content. Appl Environ Microbiol 55:116–124

An GH, Bielich J, Auerbach R, Johnson EA (1991) Isolation and characterization of carotenoid hyperproducing mutants of yeast by flow cytometry and cell sorting. Biotechnology (N Y) 9:70–73

An GH, Cho MH, Johnson EA (1999) Monocyclic carotenoid biosynthetic pathway in the yeast *Phaffia rhodozyma* (*Xanthophyllomyces dendrorhous*). J Biosci Bioeng 88:189–193

An GH, Jang BG, Cho MH (2001) Cultivation of the carotenoid-hyperproducing mutant 2A2N of the red yeast *Xanthophyllomyces dendrorhous* (*Phaffia rhodozyma*) with molasses. J Biosci Bioeng 92:121–125

Andrewes AG, Phaff HJ, Starr MP (1976) Carotenoids of *Phaffia rhodozyma*, a red-pigmented fermenting yeast. Phytochemistry 15:1003–1007

Aragón CM, Murillo FJ, de la Guardia MD, Cerdá-Olmedo E (1976) An enzyme complex for the dehydrogenation of phytoene in *Phycomyces*. Eur J Biochem FEBS 63:71–75

Araya-Garay JM, Ageitos JM, Vallejo JA et al (2012a) Construction of a novel *Pichia pastoris* strain for production of xanthophylls. AMB Express 2:24

Araya-Garay JM, Feijoo-Siota L, Rosa-dos-Santos F et al (2012b) Construction of new *Pichia pastoris* X-33 strains for production of lycopene and β-carotene. Appl Microbiol Biotechnol 93:2483–2492

Arpaia G, Carattoli A, Macino G (1995) Light and development regulate the expression of the *albino-3* gene in *Neurospora crassa*. Dev Biol 170:626–635

Arpin N, Liaaen-Jensen S (1967) Chemotaxonomic research on fungi. Fungal carotenoids. IV. Carotenoids of *Phillipsia carminea* (Pat.) Le Gal; isolation and identification of a new natural xanthophyll. Bull Soc Chim Biol (Paris) 49:527–536

Arrach N, Fernández-Martín R, Cerdá-Olmedo E, Avalos J (2001) A single gene for lycopene cyclase, phytoene synthase, and regulation of carotene biosynthesis in *Phycomyces*. Proc Natl Acad Sci U S A 98:1687–1692

Arrach N, Schmidhauser TJ, Avalos J (2002) Mutants of the carotene cyclase domain of *al-2* from *Neurospora crassa*. Mol Gen Genomics 266:914–921

Austin DG, Bu'Lock JD, Winstanley DJ (1969) Trisporic acid biosynthesis and carotenogenesis in *Blakesleea trispora*. Biochem J 113:34P

Austin DJ, Bu'Lock JD, Drake D (1970) The biosynthesis of trisporic acids from β-carotene via retinal and trisporol. Experientia 26:348–349

Avalos J, Cerdá-Olmedo E (1987) Carotenoid mutants of *Gibberella fujikuroi*. Curr Genet 25:1837–1841

Avalos J, Cerdá-Olmedo E (2004) Fungal carotenoid production. In: Arora DK (ed) Handbook of fungal biotechnology, 2nd edn. Marcel Dekker, New York, pp 367–378

Avalos J, Corrochano LM (2013) Carotenoid biosynthesis in *Neurospora*. In: Kasbekar DP, McCluskey K (eds) *Neurospora*: genomics and molecular biology. Caister Academic Press, Norfolk, pp 227–241

Avalos J, Estrada AF (2010) Regulation by light in *Fusarium*. Fungal Genet Biol 47:930–938

Avalos J, Schrott EL (1990) Photoinduction of carotenoid biosynthesis in *Gibberella fujikuroi*. FEMS Microbiol Lett 66:295–298

Avalos J, Mackenzie A, Nelki DS, Bramley PM (1988) Terpenoid biosynthesis in cell-extracts of wild type and mutant strains of *Gibberella fujikuroi*. Biochim Biophys Acta 966:257–265

Avalos J, Díaz-Sánchez V, García-Martínez J et al (2014a) Carotenoids. In: Martín JF, García-Estrada C, Zeilinger S (eds) Biosynthesis and molecular genetics of fungal secondary metabolites. Springer, New York, pp 149–185

Baima S, Macino G, Morelli G (1991) Photoregulation of the *albino-3* gene in *Neurospora crassa*. J Photochem Photobiol B 11:107–115

Barbachano-Torres A, Castelblanco-Matiz LM, Ramos-Valdivia AC et al (2014) Analysis of proteomic changes in colored mutants of *Xanthophyllomyces dendrorhous* (*Phaffia rhodozyma*). Arch Microbiol 196:411–421

Barba-Ostria C, Lledias F, Georgellis D (2011) The *Neurospora crassa* DCC-1 protein, a putative histidine kinase, is required for normal sexual and asexual development and carotenogenesis. Eukaryot Cell 10:1733–1739

Barbato C, Calissano M, Pickford A et al (1996) Mild RIP-an alternative method for *in vivo* mutagenesis of the *albino-3* gene in *Neurospora crassa*. Mol Gen Genet 252:353–361

Barredo J-L (ed) (2012) Microbial carotenoids from fungi: methods and protocols. Humana Press, New York

Barrero AF, Herrador MM, Arteaga P et al (2011) New apocarotenoids and β-carotene cleavage in *Blakeslea trispora*. Org Biomol Chem 9:7190–7195

Bartley GE, Schmidhauser TJ, Yanofsky C, Scolnik PA (1990) Carotenoid desaturases from *Rhodobacter capsulatus* and *Neurospora crassa* are structurally and functionally conserved and contain domains homologous to flavoprotein disulfide oxidoreductases. J Biol Chem 265:16020–16024

Beekwilder J, van Rossum HM, Koopman F et al (2014) Polycistronic expression of a β-carotene biosynthetic pathway in *Saccharomyces cerevisiae* coupled to β-ionone production. J Biotechnol 192(Pt B):383–392

Bejarano ER, Cerdá-Olmedo E (1989) Inhibition of phytoene dehydrogenation and activation of carotenogenesis in *Phycomyces*. Phytochemistry 28:1623–1626

Bejarano ER, Avalos J, Lipson ED, Cerdá-Olmedo E (1991) Photoinduced accumulation of carotene in *Phycomyces*. Planta 183:1–9

Bejarano ER, Cerdá-Olmedo E (1992) Independence of the carotene and sterol pathways of *Phycomyces*. FEBS Lett 306:209–212

Bejarano ER, Govind NS, Cerdá-Olmedo E (1987) ξ-carotene and other carotenes in a *Phycomyces* mutant. Phytochemistry 26:2251–2254

Bejarano ER, Parra F, Murillo FJ, Cerdá-Olmedo E (1988) End-product regulation of carotenogenesis in *Phycomyces*. Arch Microbiol 150:209–214

Bergman K, Eslava AP, Cerdá-Olmedo E (1973) Mutants of *Phycomyces* with abnormal phototropism. Mol Gen Genet 123:1–16

Bieszke JA, Braun EL, Bean LE et al (1999a) The *nop-1* gene of *Neurospora crassa* encodes a seven transmembrane helix retinal-binding protein homologous to archaeal rhodopsins. Proc Natl Acad Sci U S A 96:8034–8039

Bieszke JA, Spudich EN, Scott KL et al (1999b) A eukaryotic protein, NOP-1, binds retinal to form an archaeal rhodopsin-like photochemically reactive pigment. Biochemistry (Mosc) 38:14138–14145

Bindl E, Lang W, Rau W (1970) Untersuchungen über die lichtabhängige Carotinoidsynthese. VI Zeitlicher Verlauf der Synthese der einzelnen Carotinoide bei *Fusarium aquaeductuum* unter verschiedenen Induktionsbedingungen. Planta 94:156–174

Blasco JL, Roeßink D, Iturriaga EA et al (2001) Photocarotenogenesis in phycomyces: expression of the carB gene encoding phytoene dehydrogenase. J Plant Res 114:25–31

Blomhoff R, Blomhoff HK (2006) Overview of retinoid metabolism and function. J Neurobiol 66:606–630

Böhme K, Richter C, Pätz R (2006) New insights into mechanisms of growth and β-carotene production in *Blakeslea trispora*. Biotechnol J 1:1080–1084

Bramley PM (1985) The *in vitro* biosynthesis of carotenoids. Adv Lipid Res 21:243–279

Brehm-Stecher BF, Johnson EA (2012) Isolation of carotenoid hyperproducing mutants of *Xanthophyllomyces dendrorhous* (*Phaffia rhodozyma*) by flow cytometry and cell sorting. Methods Mol Biol 898:207–217

Breitenbach J, Fraser PD, Sandmann G (2012) Carotenoid synthesis and phytoene synthase activity during mating of *Blakeslea trispora*. Phytochemistry 76:40–45

Breitenbach J, Visser H, Verdoes JC et al (2011) Engineering of geranylgeranyl pyrophosphate synthase levels and physiological conditions for enhanced carotenoid and astaxanthin synthesis in *Xanthophyllomyces dendrorhous*. Biotechnol Lett 33:755–761

Britton G, Liaaen-Jensen S, Pfander H (1998) Carotenoids. Birkhäuser Verlag, Basel

Britton G, Liaaen-Jensen S, Pfander H (2004) Carotenoids: handbook. Birkhauser, Boston

Burgeff H (1924) Untersuchungen über Sexualität und Parasitismus bei Mucorineen. Gustav Fischer, Jena

Burmester A, Richter M, Schultze K et al (2007) Cleavage of β-carotene as the first step in sexual hormone synthesis in zygomycetes is mediated by a trisporic acid regulated β-carotene oxygenase. Fungal Genet Biol 44:1096–1108

Buzzini P, Innocenti M, Turchetti B et al (2007) Carotenoid profiles of yeasts belonging to the genera Rhodotorula, *Rhodosporidium*, *Sporobolomyces*, and *Sporidiobolus*. Can J Microbiol 53:1024–1031

Buzzini P, Martini A (1999) Production of carotenoids by strains of *Rhodotorula glutinis* cultured in raw materials of agro-industrial origin. Bioresour Technol 71:41–44

Calo P, de Miguel T, Velázquez JB, Villa TG (1995) Mevalonic acid increases trans-astaxanthin and carotenoid biosynthesis in *Phaffia rhodozyma*. Biotechnol Lett 17:575–578

Candau R, Avalos J, Cerdá-Olmedo E (1991a) Gibberellins and carotenoids in the wild type and mutants of *Gibberella fujikuroi*. Appl Environ Microbiol 57:3378–3382

Candau R, Bejarano ER, Cerdá-Olmedo E (1991b) *In vivo* channeling of substrates in an enzyme aggregate for β-carotene biosynthesis. Proc Natl Acad Sci U S A 88:4936–4940

Carattoli A, Cogoni C, Morelli G, Macino G (1994) Molecular characterization of upstream regulatory sequences controlling the photoinduced expression of the *albino-3* gene of *Neurospora crassa*. Mol Microbiol 13:787–795

Carattoli A, Romano N, Ballario P et al (1991) The *Neurospora crassa* carotenoid biosynthetic gene (*albino 3*) reveals highly conserved regions among prenyltransferases. J Biol Chem 266:5854–5859

Cardoso LA, Jäckel S, Karp SG et al (2016) Improvement of *Sporobolomyces ruberrimus* carotenoids production by the use of raw glycerol. Bioresour Technol 200:374–379
Castelblanco-Matiz LM, Barbachano-Torres A, Ponce-Noyola T et al (2015) Carotenoid production and gene expression in an astaxanthin-overproducing *Xanthophyllomyces dendrorhous* mutant strain. Arch Microbiol 197:1129–1139
Castrillo M, Avalos J (2015) The flavoproteins CryD and VvdA cooperate with the white collar protein WcoA in the control of photocarotenogenesis in *Fusarium fujikuroi*. PLoS One 10: e0119785
Castrillo M, Bernhardt A, Avalos J et al (2015) Biochemical characterization of the DASH-type cryptochrome CryD from *Fusarium fujikuroi*. Photochem Photobiol 91:1356–1367
Castrillo M, García-Martínez J, Avalos J (2013) Light-dependent functions of the *Fusarium fujikuroi* CryD DASH cryptochrome in development and secondary metabolism. Appl Environ Microbiol 79:2777–2788
Cederberg E, Neujahr HY (1969) Activation of β-carotene synthesis in *Blakeslea trispora* by certain terpenes. Acta Chem Scand 23:957–961
Cerdá-Olmedo E (1987) Carotene. In: Cerdá-Olmedo E, Lipson ED (eds) *Phycomyces*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, pp 199–222
Cerdá-Olmedo E (1985) Carotene mutants of *Phycomyces*. Methods Enzymol 110:220–243
Cerdá-Olmedo E (1989) Production of carotenoids with fungi. In: Vandamme E (ed) Biotechnology of vitamin, growth factor and pigment production. Elsevier Applied Science, London, pp 27–42
Cerdá-Olmedo E, Hüttermann A (1986) Förderung und Hemmung der Carotinsynthese bei *Phycomyces* durch Aromaten. Angew Bot 60:59–70
Cerdá-Olmedo E, Mehta BJ (2012) Isolation of mutants and construction of intersexual heterokaryons of *Blakeslea trispora*. Methods Mol Biol 898:75–84
Chang J-J, Thia C, Lin H-Y et al (2015) Integrating an algal β-carotene hydroxylase gene into a designed carotenoid-biosynthesis pathway increases carotenoid production in yeast. Bioresour Technol 184:2–8
Chen CH, Dunlap JC, Loros JJ (2010) *Neurospora* illuminates fungal photoreception. Fungal Genet Biol 47:922–929
Chi S, He Y, Ren J et al (2015) Overexpression of a bifunctional enzyme, CrtS, enhances astaxanthin synthesis through two pathways in *Phaffia rhodozyma*. Microb Cell Factories 14:90
Choudhari S, Singhal R (2008) Media optimization for the production of β-carotene by *Blakeslea trispora*: a statistical approach. Bioresour Technol 99:722–730
Choudhari SM, Ananthanarayan L, Singhal RS (2008) Use of metabolic stimulators and inhibitors for enhanced production of β-carotene and lycopene by *Blakeslea trispora* NRRL 2895 and 2896. Bioresour Technol 99:3166–3173
Chumpolkulwong N, Kakizono T, Nagai S, Nishio N (1997) Increased astaxanthin production by *Phaffia rhodozyma* mutants isolated as resistant to diphenylamine. J Ferment Bioeng 83:429–434
Corrochano LM, Cerdá-Olmedo E (1992) Sex, light and carotenes: the development of *Phycomyces*. Trends Genet TIG 8:268–274
Corrochano LM, Cerdá-Olmedo E (1990) Photomorphogenesis in behavioural and colour mutants of *Phycomyces*. J Photochem Photobiol B 6:325–335
Corrochano LM, Garre V (2010) Photobiology in the Zygomycota: multiple photoreceptor genes for complex responses to light. Fungal Genet Biol 47:893–899
Csernetics A, Nagy G, Iturriaga EA et al (2011) Expression of three isoprenoid biosynthesis genes and their effects on the carotenoid production of the zygomycete *Mucor circinelloides*. Fungal Genet Biol 48:696–703
Csernetics Á, Tóth E, Farkas A et al (2015) Expression of *Xanthophyllomyces dendrorhous* cytochrome-P450 hydroxylase and reductase in *Mucor circinelloides*. World J Microbiol Biotechnol 31:321–336

Daub ME, Payne GA (1989) The role of carotenoids in resistance of fungi to cercosporin. Phytopathology 79:180–185

de Fabo EC, Harding RW, Shropshire W Jr (1976) Action spectrum between 260 and 800 nanometers for the photoinduction of carotenoid biosynthesis in *Neurospora crassa*. Plant Physiol 57:440–445

de la Fuente JL, Rodríguez-Sáiz M, Schleissner C et al (2010) High-titer production of astaxanthin by the semi-industrial fermentation of *Xanthophyllomyces dendrorhous*. J Biotechnol 148:144–146

De la Guardia MD, Aragón CM, Murillo FJ, Cerdá-Olmedo E (1971) A carotenogenic enzyme aggregate in *Phycomyces*: evidence from quantitive complementation. Proc Natl Acad Sci U S A 68:2012–2015

de Miguel T, Calo P, Díaz A, Villa TG (1997) The genus *Rhodosporidium*: a potential source of β-carotene. Microbiologia 13:67–70

DeBose-Boyd RA (2008) Feedback regulation of cholesterol synthesis: sterol-accelerated ubiquitination and degradation of HMG CoA reductase. Cell Res 18:609–621

Degli-Innocenti F, Russo VE (1984) Isolation of new white collar mutants of *Neurospora crassa* and studies on their behavior in the blue light-induced formation of protoperithecia. J Bacteriol 159:757–761

Díaz-Sánchez V, Estrada AF, Limón MC et al (2013) The oxygenase CAO-1 of *Neurospora crassa* is a resveratrol cleavage enzyme. Eukaryot Cell 12:1305–1314

Díaz-Sánchez V, Estrada AF, Trautmann D et al (2011a) Analysis of *al-2* mutations in *Neurospora*. PLoS One 6:e21948

Díaz-Sánchez V, Estrada AF, Trautmann D et al (2011b) The gene *carD* encodes the aldehyde dehydrogenase responsible for neurosporaxanthin biosynthesis in *Fusarium fujikuroi*. FEBS J 278:3164–3176

Dimitrova S, Pavlova K, Lukanov L et al (2013) Production of metabolites with antioxidant and emulsifying properties by antarctic strain *Sporobolomyces salmonicolor* AL_1. Appl Biochem Biotechnol 169:301–311

Domenech CE, Giordano W, Avalos J, Cerdá-Olmedo E (1996) Separate compartments for the production of sterols, carotenoids and gibberellins in *Gibberella fujikuroi*. Eur J Biochem 239:720–725

Domínguez-Bocanegra AR, Torres-Muñoz JA (2004) Astaxanthin hyperproduction by *Phaffia rhodozyma* (now *Xanthophyllomyces dendrorhous*) with raw coconut milk as sole source of energy. Appl Microbiol Biotechnol 66:249–252

Domonkos I, Kis M, Gombos Z, Ughy B (2013) Carotenoids, versatile components of oxygenic photosynthesis. Prog Lipid Res 52:539–561

Ducrey Sanpietro LM, Kula MR (1998) Studies of astaxanthin biosynthesis in *Xanthophyllomyces dendrorhous* (*Phaffia rhodozyma*). Effect of inhibitors and low temperature. Yeast Chichester Engl 14:1007–1016

Echavarri-Erasun C, Johnson EA (2004) Stimulation of astaxanthin formation in the yeast *Xanthophyllomyces dendrorhous* by the fungus *Epicoccum nigrum*. FEMS Yeast Res 4:511–519

Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 32:1792–1797

Ehrenshaft M, Daub ME (1994) Isolation, sequence, and characterization of the *Cercospora nicotianae* phytoene dehydrogenase gene. Appl Environ Microbiol 60:2766–2771

Elahi M, Chichester CO, Simpson KL (1973a) Biosynthesis of carotenoids by *Phycomyces blakesleeanus* mutants in the presence of nitrogenous heterocyclic compounds. Phytochemistry 12:1627–1632

Elahi M, Lee TH, Simpson KL, Chichester CO (1973b) Effect of CPTA and cycocel on the biosynthesis of carotenoids by *Phycomyces blakesleeanus* mutants. Phytochemistry 12:1633–1639

El-Jack M, Mackenzie A, Bramley PM (1988) The photoregulation of carotenoid biosynthesis in *Aspergillus giganteus* mut. *alba*. Planta 174:59–66

Eslava AP, Alvarez MI, Cerdá-Olmedo E (1974) Regulation of carotene biosynthesis in *Phycomyces* by vitamin A and β-ionone. Eur J Biochem 48:617–623

Estrada AF, Avalos J (2009) Regulation and targeted mutation of *opsA*, coding for the NOP-1 opsin orthologue in *Fusarium fujikuroi*. J Mol Biol 387:59–73

Estrada AF, Avalos J (2008) The White Collar protein WcoA of *Fusarium fujikuroi* is not essential for photocarotenogenesis, but is involved in the regulation of secondary metabolism and conidiation. Fungal Genet Biol 45:705–718

Estrada AF, Brefort T, Mengel C et al (2010) *Ustilago maydis* accumulates β-carotene at levels determined by a retinal-forming carotenoid oxygenase. Fungal Genet Biol 46:803–813

Estrada AF, Maier D, Scherzinger D et al (2008a) Novel apocarotenoid intermediates in *Neurospora crassa* mutants imply a new biosynthetic reaction sequence leading to neurosporaxanthin formation. Fungal Genet Biol 45:1497–1505

Estrada AF, Youssar L, Scherzinger D et al (2008b) The *ylo-1* gene encodes an aldehyde dehydrogenase responsible for the last reaction in the *Neurospora* carotenoid pathway. Mol Microbiol 69:1207–1220

Fang TJ, Cheng YS (1992) Isolation of astaxanthin over-producing mutants of *Phaffia rhodozyma* and their fermentation kinetics. Chin J Microbiol Inmunol 25:209–222

Fernández-Martín R, Cerdá-Olmedo E, Avalos J (2000) Homologous recombination and allele replacement in transformants of *Fusarium fujikuroi*. Mol Gen Genet 263:838–845

Flores R, Cerdá-Olmedo E, Corrochano LM (1998) Separate sensory pathways for photomorphogenesis in *Phycomyces*. Photochem Photobiol 67:467–472

Flores-Cotera LB, Martín R, Sánchez S (2001) Citrate, a possible precursor of astaxanthin in *Phaffia rhodozyma*: influence of varying levels of ammonium, phosphate and citrate in a chemically defined medium. Appl Microbiol Biotechnol 55:341–347

Fontana JD, Guimarães MF, Martins NT et al (1996) Culture of the astaxanthinogenic yeast *Phaffia rhodozyma* in low-cost media. Appl Biochem Biotechnol 57–58:413–422

Fraser PD, Bramley PM (1994) The purification of phytoene dehydrogenase from *Phycomyces blakesleeanus*. Biochim Biophys Acta 1212:59–66

Fraser PD, Miura Y, Misawa N (1997) *In vitro* characterization of astaxanthin biosynthetic enzymes. J Biol Chem 272:6128–6135

Fraser PD, Ruiz-Hidalgo MJ, López-Matas MA et al (1996) Carotenoid biosynthesis in wild type and mutant strains of *Mucor circinelloides*. Biochim Biophys Acta 1289:203–208

Freitas C, Parreira TM, Roseiro J et al (2014) Selecting low-cost carbon sources for carotenoid and lipid production by the pink yeast *Rhodosporidium toruloides*> NCYC 921 using flow cytometry. Bioresour Technol 158:355–359

Frengova G, Simova E, Beshkova D (2004a) Use of whey ultrafiltrate as a substrate for production of carotenoids by the yeast *Rhodotorula rubra*. Appl Biochem Biotechnol 112:133–141

Frengova G, Simova E, Pavlova K et al (1994) Formation of carotenoids by *Rhodotorula glutinis* in whey ultrafiltrate. Biotechnol Bioeng 44:888–894

Frengova GI, Beshkova DM (2009) Carotenoids from *Rhodotorula* and *Phaffia*: yeasts of biotechnological importance. J Ind Microbiol Biotechnol 36:163–180

Frengova GI, Simova ED, Beshkova DM (2004b) Improvement of carotenoid-synthesizing yeast *Rhodotorula rubra* by chemical mutagenesis. Z Naturforschung C J Biosci 59:99–103

Garbayo I, Vílchez C, Nava-Saucedo JE, Barbotin JN (2003) Nitrogen, carbon and light-mediated regulation studies of carotenoid biosynthesis in immobilized mycelia of *Gibberella fujikuroi*. Enzym Microb Technol 33:629–634

García-Martínez J, Ádám AL, Avalos J (2012) Adenylyl cyclase plays a regulatory role in development, stress resistance and secondary metabolism in *Fusarium fujikuroi*. PLoS One 7:e28849

García-Martínez J, Brunk M, Avalos J, Terpitz U (2015) The CarO rhodopsin of the fungus *Fusarium fujikuroi* is a light-driven proton pump that retards spore germination. Sci Rep 5:7798

Gassel S, Breitenbach J, Sandmann G (2014) Genetic engineering of the complete carotenoid pathway towards enhanced astaxanthin formation in *Xanthophyllomyces dendrorhous* starting from a high-yield mutant. Appl Microbiol Biotechnol 98:345–350

Gassel S, Schewe H, Schmidt I et al (2013) Multiple improvement of astaxanthin biosynthesis in *Xanthophyllomyces dendrorhous* by a combination of conventional mutagenesis and metabolic pathway engineering. Biotechnol Lett 35:565–569

Georgiou CD, Tairis N, Polycratis A (2001a) Production of β-carotene by *Sclerotinia sclerotiorum* and its role in sclerotium differentiation. Mycol Res 105:1110–1115

Georgiou CD, Zervoudakis G, Tairis N, Kornaros M (2001b) β-Carotene production and its role in sclerotial differentiation of *Sclerotium rolfsii*. Fungal Genet Biol 34:11–20

Gessler NN, Sokolov AV, Bykhovsky VY, Belozerskaya TA (2002) Superoxide dismutase and catalase activities in carotenoid-synthesizing fungi *Blakeslea trispora* and *Neurospora crassa* in oxidative stress. Appl Biochem Microbiol 38:205–209

Girard P, Falconnier B, Bricout J, Vladescu B (1994) β-Carotene producing mutants of *Phaffia rhodozyma*. Appl Microbiol Biotechnol 41:183–191

Goksungur Y, Mantzouridou F, Roukas T, Kotzekidou P (2004) Production of β-carotene from beet molasses by *Blakeslea trispora* in stirred-tank and bubble column reactors: development of a mathematical modeling. Appl Biochem Biotechnol 112:37–54

Goldie AH, Subden RE (1973) The neutral carotenoids of wild-type and mutant strains of *Neurospora crassa*. Biochem Genet 10:275–284

Goodwin TW (1980) The biochemistry of the carotenoids, 2nd edn. Chapman & Hall, London

Govind NS, Cerdá-Olmedo E (1986) Sexual activation of carotenogenesis in *Phycomyces blakesleeanus*. J Gen Microbiol 132:2775–2780

Gu WL, An GH, Johnson EA (1997) Ethanol increases carotenoid production in *Phaffia rhodozyma*. J Ind Microbiol Biotechnol 19:114–117

Han JR, Zhao WJ, Gao YY, Yuan JM (2005) Effect of oxidative stress and exogenous β-carotene on sclerotial differentiation and carotenoid yield of *Penicillium* sp. PT95. Lett Appl Microbiol 40:412–417

Han M, He Q, Zhang WG (2012) Carotenoids production in different culture conditions by *Sporidiobolus pararoseus*. Prep Biochem Biotechnol 42:293–303

Hara KY, Morita T, Mochizuki M et al (2014) Development of a multi-gene expression system in *Xanthophyllomyces dendrorhous*. Microb Cell Factories 13:175

Harding RW (1974) The effect of temperature on photo-induced carotenoid biosynthesis in *Neurospora crassa*. Plant Physiol 54:142–147

Harding RW (1973) Inhibition of conidiation and photoinduced carotenoid biosynthesis by cyclic AMP. Neurospora Newsl 20:20–21

Harding RW, Huang PC, Mitchell HK (1969) Photochemical studies of the carotenoid biosynthetic pathway in *Neurospora crassa*. Arch Biochem Biophys 129:696–707

Harding RW, Philip DQ, Drozdowicz BZ, Williams NP (1984) A *Neurospora crassa* mutant which overaccumulates carotenoid pigments. Neurospora Newsl 31:23–25

Harding RW, Turner RV (1981) Photoregulation of the carotenoid biosynthetic pathway in albino and white collar mutants of *Neurospora crassa*. Plant Physiol 68:745–749

Hausmann A, Sandmann G (2000) A single five-step desaturase is involved in the carotenoid biosynthesis pathway to β-carotene and torulene in *Neurospora crassa*. Fungal Genet Biol 30:147–153

Haxo F (1950) Carotenoids of the mushroom *Cantharellus cinnabarinus*. Bot Gaz 112:228–232

Hayman GT, Mannarelli BM, Leathers TD (1995) Production of carotenoids by *Phaffia rhodozyma* grown on media composed of corn wet-milling co-products. J Ind Microbiol 14:389–395

He Q, Liu Y (2005) Molecular mechanism of light responses in *Neurospora*: from light-induced transcription to photoadaptation. Genes Dev 19:2888–2899

Hernández-Almanza A, Cesar Montanez J, Aguilar-González MA et al (2014a) *Rhodotorula glutinis* as source of pigments and metabolites for food industry. Food Biosci 5:64–72

Hernández-Almanza A, Montañez-Sáenz J, Martínez-Ávila C et al (2014b) Carotenoid production by *Rhodotorula glutinis* YB-252 in solid-state fermentation. Food Biosci 7:31–36

Herz S, Weber RW, Anke H et al (2007) Intermediates in the oxidative pathway from torulene to torularhodin in the red yeasts *Cystofilobasidium infirmominiatum* and *C. capitatum* (Heterobasidiomycetes, Fungi). Phytochemistry 68:2503–2511

Higuera-Ciapara I, Félix-Valenzuela L, Goycoolea FM (2006) Astaxanthin: a review of its chemistry and applications. Crit Rev Food Sci Nutr 46:185–196

Hsu W-J, Poling SM, Yokoyama H (1974) Effects of amines on the carotenogenesis in *Blakeslea trispora*. Phytochemistry 13:415–419

Hsu WJ, Yokoyama H, Coggins CW (1972) Carotenoid biosynthesis in *Blakeslea trispora*. Phytochemistry 11:2985–2990

Hu X, Li H, Tang P et al (2013a) GC-MS-based metabolomics study of the responses to arachidonic acid in *Blakeslea trispora*. Fungal Genet Biol 57:33–41

Hu X, Ma X, Tang P, Yuan Q (2013b) Improved β-carotene production by oxidative stress in *Blakeslea trispora* induced by liquid paraffin. Biotechnol Lett 35:559–563

Hu X, Sun J, Yuan Q (2012) Improved β-carotene biosynthesis and gene transcription in *Blakeslea trispora* with arachidonic acid. Biotechnol Lett 34:2107–2111

Huang PC (1964) Recombination and complementation of albino mutants in *Neurospora*. Genetics 49:453–469

Idnurm A, Rodríguez-Romero J, Corrochano LM et al (2006) The *Phycomyces madA* gene encodes a blue-light photoreceptor for phototropism and other light responses. Proc Natl Acad Sci U S A 103:4546–4551

Iurkov AM, Vustin MM, Tiaglov BV et al (2008) Pigmented basidiomycete yeasts are a promising source of carotenoids and ubiquinone Q10. Mikrobiologiia 77:5–10

Jayaram M, Leutwiler L, Delbrück M (1980) Light-induced carotene synthesis in mutants of *Phycomyces* with abnormal phototropism. Photochem Photobiol 32:241–245

Jeong JC, Lee IY, Kim SW, Park YH (1999) Stimulation of β-carotene synthesis by hydrogen peroxide in *Blakeslea trispora*. Biotechnol Lett 21:683–686

Jin JM, Lee J, Lee YW (2010) Characterization of carotenoid biosynthetic genes in the ascomycete *Gibberella zeae*. FEMS Microbiol Lett 302:197–202

Jirasripongpun K, Pewlong W, Kitraksa P, Krudngern C (2008) Carotenoid production by *Xanthophyllomyces dendrorhous*: use of pineapple juice as a production medium. Lett Appl Microbiol 47:112–116

Johnson EA (2003) *Phaffia rhodozyma*: colorful odyssey. Int Microbiol 6:169–174

Johnson EA, Lewis MJ (1979) Astaxanthin formation by the yeast *Phaffia rhodozyma*. J Gen Microbiol 115:173–183

Kajiwara S, Fraser PD, Kondo K, Misawa N (1997) Expression of an exogenous isopentenyl diphosphate isomerase gene enhances isoprenoid biosynthesis in *Escherichia coli*. Biochem J 324:421–426

Kim S-K, Lee J-H, Lee C-H, Yoon Y-C (2007) Increased carotenoid production in *Xanthophyllomyces dendrorhous* G276 using plant extracts. J Microbiol Seoul Korea 45:128–132

Kirti K, Amita S, Priti S et al (2014) Colorful world of microbes: carotenoids and their applications. Adv Biol 2014:1–13

Krinsky NI, Johnson EJ (2005) Carotenoid actions and their relation to health and disease. Mol Asp Med 26:459–516

Kritsky MS, Sokolovsky VY, Belozerskaya TA, Chernysheva EK (1982) Relationship between cyclic AMP level and accumulation of carotenoid pigments in *Neurospora crassa*. Arch Microbiol 133:206–208

Kucsera J, Pfeiffer I, Ferenczy L (1998) Homothallic life cycle in the diploid red yeast *Xanthophyllomyces dendrorhous* (*Phaffia rhodozyma*). Antonie Van Leeuwenhoek 73:163–168

Kuzina V, Cerdá-Olmedo E (2006) Modification of sexual development and carotene production by acetate and other small carboxylic acids in *Blakeslea trispora* and *Phycomyces blakesleeanus*. Appl Environ Microbiol 72:4917–4922

Kuzina V, Domenech C, Cerdá-Olmedo E (2006) Relationships among the biosyntheses of ubiquinone, carotene, sterols, and triacylglycerols in Zygomycetes. Arch Microbiol 186:485–493

Kuzina V, Ramírez-Medina H, Visser H et al (2008) Genes involved in carotene synthesis and mating in *Blakeslea trispora*. Curr Genet 54:143–152

Lampila LE, Wallen SE, Bullerman LB (1985) A review of factors affecting biosynthesis of carotenoids by the order *Mucorales*. Mycopathologia 90:65–80

Lange N, Steinbüchel A (2011) β-carotene production by *Saccharomyces cerevisiae* with regard to plasmid stability and culture media. Appl Microbiol Biotechnol 91:1611–1622

Ledetzky N, Osawa A, Iki K et al (2014) Multiple transformation with the *crtYB* gene of the limiting enzyme increased carotenoid synthesis and generated novel derivatives in *Xanthophyllomyces dendrorhous*. Arch Biochem Biophys 545:141–147

Lee JJL, Chen L, Cao B, Chen WN (2016) Engineering *Rhodosporidium toruloides* with a membrane transporter facilitates production and separation of carotenoids and lipids in a bi-phasic culture. Appl Microbiol Biotechnol 100:869–877

Lewis MJ, Ragot N, Berlant MC, Miranda M (1990) Selection of astaxanthin-overproducing mutants of *Phaffia rhodozyma* with β-Ionone. Appl Environ Microbiol 56:2944–2945

Li C, Schmidhauser TJ (1995) Developmental and photoregulation of *al-1* and *al-2*, structural genes for two enzymes essential for carotenoid biosynthesis in *Neurospora*. Dev Biol 169:90–95

Li Q, Sun Z, Li J, Zhang Y (2013) Enhancing beta-carotene production in *Saccharomyces cerevisiae* by metabolic engineering. FEMS Microbiol Lett 345:94–101

Linden H, Rodríguez-Franco M, Macino G (1997) Mutants of *Neurospora crassa* defective in regulation of blue light perception. Mol Gen Genet 254:111–118

Linnemannstöns P, Prado MM, Fernández-Martín R et al (2002) A carotenoid biosynthesis gene cluster in *Fusarium fujikuroi*: the genes *carB* and *carRA*. Mol Gen Genomics 267:593–602

Liu X-J, Liu R-S, Li H-M, Tang Y-J (2012) Lycopene production from synthetic medium by *Blakeslea trispora* NRRL 2895 (+) and 2896 (-) in a stirred-tank fermenter. Bioprocess Biosyst Eng 35:739–749

Liu YS, Wu JY (2006a) Use of n-hexadecane as an oxygen vector to improve *Phaffia rhodozyma* growth and carotenoid production in shake-flask cultures. J Appl Microbiol 101:1033–1038

Liu YS, Wu JY (2006b) Hydrogen peroxide-induced astaxanthin biosynthesis and catalase activity in *Xanthophyllomyces dendrorhous*. Appl Microbiol Biotechnol 73:663–668

Liu YS, Wu JY (2007) Perfusion culture process plus H_2O_2 stimulation for efficient astaxanthin production by Xanthophyllomyces dendrorhous. Biotechnol Bioeng 97:568–573

Liu Z-Q, Zhang J-F, Zheng Y-G, Shen Y-C (2008) Improvement of astaxanthin production by a newly isolated *Phaffia rhodozyma* mutant with low-energy ion beam implantation. J Appl Microbiol 104:861–872

Lodato P, Alcaino J, Barahona S et al (2003) Alternative splicing of transcripts from *crtI* and *crtYB* genes of *Xanthophyllomyces dendrorhous*. Appl Environ Microbiol 69:4676–4682

Lodato P, Alcaíno J, Barahona S et al (2007) Expression of the carotenoid biosynthesis genes in *Xanthophyllomyces dendrorhous*. Biol Res 40:73–84

Lodato P, Alcaíno J, Barahona S et al (2004) Study of the expression of carotenoid biosynthesis genes in wild-type and deregulated strains of *Xanthophyllomyces dendrorhous* (Ex.: *Phaffia rhodozyma*). Biol Res 37:83–93

López J, Essus K, Kim I et al (2015) Production of β-ionone by combined expression of carotenogenic and plant CCD1 genes in *Saccharomyces cerevisiae*. Microb Cell Factories 14:84

López-Díaz I, Cerdá-Olmedo E (1980) Relationship of photocarotenogenesis to other behavioural and regulatory responses in *Phycomyces*. Planta 150:134–139
López-Nieto MJ, Costa J, Peiro E et al (2004) Biotechnological lycopene production by mated fermentation of *Blakeslea trispora*. Appl Microbiol Biotechnol 66:153–159
Lorca-Pascual JM, Murcia-Flores L, Garre V et al (2004) The RING-finger domain of the fungal repressor *crgA* is essential for accurate light regulation of carotenogenesis. Mol Microbiol 52:1463–1474
Loto I, Gutiérrez MS, Barahona S et al (2012) Enhancement of carotenoid production by disrupting the C22-sterol desaturase gene (CYP61) in *Xanthophyllomyces dendrorhous*. BMC Microbiol 12:235
Madhour A, Anke H, Mucci A et al (2005) Biosynthesis of the xanthophyll plectaniaxanthin as a stress response in the red yeast *Dioszegia* (Tremellales, Heterobasidiomycetes, Fungi). Phytochemistry 66:2617–2626
Mantzouridou F, Naziri E, Tsimidou MZ (2008) Industrial glycerol as a supplementary carbon source in the production of β-carotene by *Blakeslea trispora*. J Agric Food Chem 56:2668–2675
Mantzouridou F, Roukasa T, Kotzekidoua P, Liakopoulou M (2002) Optimization of β-carotene production from synthetic medium by *Blakeslea trispora*: a mathematical modeling. Appl Biochem Biotechnol 101:153–175
Mantzouridou F, Tsimidou MZ (2008) Lycopene formation in *Blakeslea trispora*. Chemical aspects of a bioprocess. Trends Food Sci Technol 19:363–371
Mantzouridou F, Tsimidou MZ, Roukas T (2006) Performance of crude olive pomace oil and soybean oil during carotenoid production by *Blakeslea trispora* in submerged fermentation. J Agric Food Chem 54:2575–2581
Marcoleta A, Niklitschek M, Wozniak A et al (2011) Glucose and ethanol-dependent transcriptional regulation of the astaxanthin biosynthesis pathway in *Xanthophyllomyces dendrorhous*. BMC Microbiol 11:190
Marova I, Carnecka M, Halienova A et al (2012) Use of several waste substrates for carotenoid-rich yeast biomass production. J Environ Manag 95:S338–S342
Martín JF, Gudiña E, Barredo JL (2008) Conversion of β-carotene into astaxanthin: two separate enzymes or a bifunctional hydroxylase-ketolase protein? Microb Cell Factories 7:3
Martínez C, Hermosilla G, León R et al (1998) Genetic transformation of astaxanthin mutants of *Phaffia rhodozyma*. Antonie Van Leeuwenhoek 73:147–153
Martinez-Moya P, Niehaus K, Alcaíno J et al (2015) Proteomic and metabolomic analysis of the carotenogenic yeast *Xanthophyllomyces dendrorhous* using different carbon sources. BMC Genomics 16:289
Martinez-Moya P, Watt SA, Niehaus K et al (2011) Proteomic analysis of the carotenogenic yeast *Xanthophyllomyces dendrorhous*. BMC Microbiol 11:131
Mata-Gómez LC, Montañez JC, Méndez-Zavala A, Aguilar CN (2014) Biotechnological production of carotenoids by yeasts: an overview. Microb Cell Factories 13:12
Medina HR, Cerdá-Olmedo E, Al-Babili S (2011) Cleavage oxygenases for the biosynthesis of trisporoids and other apocarotenoids in *Phycomyces*. Mol Microbiol 82:199–208
Mehta BJ, Cerdá-Olmedo E (1995) Mutants of carotene production in *Blakeslea trispora*. Appl Microbiol Biotechnol 42:836–838
Mehta BJ, Cerdá-Olmedo E (1999) Lycopene cyclization in *Blakeslea trispora*. Mycoscience 40:307–310
Mehta BJ, Cerdá-Olmedo E (2001) Intersexual partial diploids of *Phycomyces*. Genetics 158:635–641
Mehta BJ, Obraztsova IN, Cerdá-Olmedo E (2003) Mutants and intersexual heterokaryons of *Blakeslea trispora* for production of β-carotene and lycopene. Appl Environ Microbiol 69:4043–4048
Mehta BJ, Salgado LM, Bejarano ER, Cerdá-Olmedo E (1997) New mutants of *Phycomyces blakesleeanus* for β-carotene production. Appl Environ Microbiol 63:3657–3661

Mende K, Homann V, Tudzynski B (1997) The geranylgeranyl diphosphate synthase gene of *Gibberella fujikuroi*: isolation and expression. Mol Gen Genet 255:96–105
Meyer PS, Du Preez JC (1994) Photo-regulated astaxanthin production by *Phaffia rhodozyma* mutants. Syst Appl Microbiol 17:24–31
Meyer PS, du Preez JC, Kilian SG (1993) Selection and evaluation of astaxanthin-overproducing mutants of *Phaffia rhodozyma*. World J Microbiol Biotechnol 9:514–520
Miao L, Chi S, Tang Y et al (2011) Astaxanthin biosynthesis is enhanced by high carotenogenic gene expression and decrease of fatty acids and ergosterol in a *Phaffia rhodozyma* mutant strain. FEMS Yeast Res 11:192–201
Miao L, Wang Y, Chi S et al (2010) Reduction of fatty acid flux results in enhancement of astaxanthin synthesis in a mutant strain of *Phaffia rhodozyma*. J Ind Microbiol Biotechnol 37:595–602
Mitzka U, Rau W (1977) Composition and photoinduced biosynthesis of the carotenoids of a protoplast-like *Neurospora crassa* "slime" mutant. Arch Microbiol 111:261–263
Mitzka-Schnabel U (1985) Carotenogenic enzymes from *Neurospora*. Pure Appl Chem 57:667–669
Mitzka-Schnabel U, Rau W (1980) The subcellular distribution of carotenoids in *Neurospora crassa*. Phytochemistry 19:1409–1413
Miura Y, Kondo K, Saito T et al (1998) Production of the carotenoids lycopene, β-carotene, and astaxanthin in the food yeast *Candida utilis*. Appl Environ Microbiol 64:1226–1229
Moliné M, Flores MR, Libkind D et al (2010) Photoprotection by carotenoid pigments in the yeast *Rhodotorula mucilaginosa*: the role of torularhodin. Photochem Photobiol Sci 9:1145–1151
Moliné M, Libkind D, van Broock M (2012) Production of torularhodin, torulene, and β-carotene by *Rhodotorula yeasts*. Methods Mol Biol 898:275–283
Montanti J, Nghiem NP, Johnston DB (2011) Production of astaxanthin from cellulosic biomass sugars by mutants of the yeast *Phaffia rhodozyma*. Appl Biochem Biotechnol 164:655–665
Moore MM, Breedveld MW, Autor AP (1989) The role of carotenoids in preventing oxidative damage in the pigmented yeast, *Rhodotorula mucilaginosa*. Arch Biochem Biophys 270:419–431
Moran NA, Jarvik T (2010) Lateral transfer of genes from fungi underlies carotenoid production in aphids. Science 328:624–627
Murcia-Flores L, Lorca-Pascual JM, Garre V et al (2007) Non-AUG translation initiation of a fungal RING finger repressor involved in photocarotenogenesis. J Biol Chem 282:15394–15403
Murillo FJ, Calderón IL, López-Díaz I, Cerdá-Olmedo E (1978) Carotene-superproducing strains of *Phycomyces*. Appl Environ Microbiol 36:639–642
Murillo FJ, Cerdá-Olmedo E (1976) Regulation of carotene synthesis in *Phycomyces*. Mol Gen Genet 148:19–24
Murillo FJ, Torres-Martínez S, Aragón CM, Cerdá-Olmedo E (1981) Substrate transfer in carotene biosynthesis in *Phycomyces*. Eur J Biochem 119:511–516
Nanou K, Roukas T (2011) Stimulation of the biosynthesis of carotenes by oxidative stress in *Blakeslea trispora* induced by elevated dissolved oxygen levels in the culture medium. Bioresour Technol 102:8159–8164
Nanou K, Roukas T (2010) Oxidative stress response and morphological changes of *Blakeslea trispora* induced by butylated hydroxytoluene during carotene production. Appl Biochem Biotechnol 160:2415–2423
Nanou K, Roukas T (2013) Oxidative stress response of *Blakeslea trispora* induced by iron ions during carotene production in shake flask culture. Appl Biochem Biotechnol 169:2281–2289
Nanou K, Roukas T (2016) Waste cooking oil: a new substrate for carotene production by *Blakeslea trispora* in submerged fermentation. Bioresour Technol 203:198–203
Navarro E, Lorca-Pascual JM, Quiles-Rosillo MD et al (2001) A negative regulator of light-inducible carotenogenesis in *Mucor circinelloides*. Mol Gen Genomics 266:463–470

Navarro E, Ruiz-Pérez VL, Torres-Martínez S (2000) Overexpression of the *crgA* gene abolishes light requirement for carotenoid biosynthesis in *Mucor circinelloides*. Eur J Biochem 267:800–807

Navarro E, Sandmann G, Torres-Martínez S (1995) Mutants of the carotenoid biosynthetic pathway of *Mucor circinelloides*. Exp Mycol 19:186–190

Navarro-Sampedro L, Yanofsky C, Corrochano LM (2008) A genetic selection for *Neurospora crassa* mutants altered in their light regulation of transcription. Genetics 178:171–183

Nelson MA, Morelli G, Carattoli A et al (1989) Molecular cloning of a *Neurospora crassa* carotenoid biosynthetic gene (albino-3) regulated by blue light and the products of the white collar genes. Mol Cell Biol 9:1271–1276

Neupert W, Ludwig GD (1971) Sites of biosynthesis of outer and inner membrane proteins of *Neurospora crassa* mitochondria. Eur J Biochem 19:523–532

Niklitschek M, Alcaíno J, Barahona S et al (2008) Genomic organization of the structural genes controlling the astaxanthin biosynthesis pathway of *Xanthophyllomyces dendrorhous*. Biol Res 41:93–108

Ninet L, Renaut J, Tissier R (1969) Activation of the biosynthesis of carotenoids by *Blakeslea trispora*. Biotechnol Bioeng 11:1195–1210

Ojima K, Breitenbach J, Visser H et al (2006) Cloning of the astaxanthin synthase gene from *Xanthophyllomyces dendrorhous* (*Phaffia rhodozyma*) and its assignment as a β-carotene 3-hydroxylase/4-ketolase. Mol Gen Genomics 275:148–158

Ootaki T, Crafts-Lighty A, Delbrück M, Hsu WJ (1973) Complementation between mutants of *Phycomyces* deficient with respect to carotenogenesis. Mol Gen Genet 121:57–70

Paietta J, Sargent ML (1981) Photoreception in *Neurospora crassa*: correlation of reduced light sensitivity with flavin deficiency. Proc Natl Acad Sci U S A 78:5573–5577

Paietta J, Sargent ML (1983) Modification of blue light photoresponses by riboflavin analogs in *Neurospora crassa*. Plant Physiol 72:764–766

Papaioannou EH, Liakopoulou-Kyriakides M (2012) Agro-food wastes utilization by *Blakeslea trispora* for carotenoids production. Acta Biochim Pol 59:151–153

Papp T, Csernetics A, Nagy G et al (2013) Canthaxanthin production with modified *Mucor circinelloides* strains. Appl Microbiol Biotechnol 97:4937–4950

Papp T, Velayos A, Bartok T et al (2006) Heterologous expression of astaxanthin biosynthesis genes in *Mucor circinelloides*. Appl Microbiol Biotechnol 69:526–531

Parajo JC, Santos VV, Vazquez M (1998) Production of carotenoids by *Phaffia rhodozyma* growing on media made from hemicellulosic hydrolysates of *Eucalyptus globulus* wood. Biotechnol Bioeng 59:501–506

Pegklidou K, Mantzouridou F, Tsimidou MZ (2008) Lycopene production using *Blakeslea trispora* in the presence of 2-methyl imidazole: yield, selectivity, and safety aspects. J Agric Food Chem 56:4482–4490

Polaino S, Herrador MM, Cerdá-Olmedo E, Barrero AF (2010) Splitting of β-carotene in the sexual interaction of *Phycomyces*. Org Biomol Chem 8:4229–4231

Prado MM, Prado-Cabrero A, Fernández-Martín R, Avalos J (2004) A gene of the opsin family in the carotenoid gene cluster of *Fusarium fujikuroi*. Curr Genet 46:47–58

Prado-Cabrero A, Estrada AF, Al-Babili S, Avalos J (2007) Identification and biochemical characterization of a novel carotenoid oxygenase: elucidation of the cleavage step in the *Fusarium* carotenoid pathway. Mol Microbiol 64:448–460

Prado-Cabrero A, Schaub P, Díaz-Sánchez V et al (2009) Deviation of the neurosporaxanthin pathway towards β-carotene biosynthesis in *Fusarium fujikuroi* by a point mutation in the phytoene desaturase gene. FEBS J 276:4582–4597

Qiang W, Ling-ran F, Luo W et al (2014) Mutation breeding of lycopene-producing strain *Blakeslea trispora* by a novel atmospheric and room temperature plasma (ARTP). Appl Biochem Biotechnol 174:452–460

Quiles-Rosillo MD, Ruiz-Vázquez RM, Torres-Martínez S, Garre V (2005) Light induction of the carotenoid biosynthesis pathway in *Blakeslea trispora*. Fungal Genet Biol 42:141–153

Quiles-Rosillo MD, Torres-Martínez S, Garre V (2003) *cigA*, a light-inducible gene involved in vegetative growth in *Mucor circinelloides* is regulated by the carotenogenic repressor *crgA*. Fungal Genet Biol 38:122–132

Ramírez J, Gutierrez H, Gschaedler A (2001) Optimization of astaxanthin production by *Phaffia rhodozyma* through factorial design and response surface methodology. J Biotechnol 88:259–268

Rao AV, Rao LG (2007) Carotenoids and human health. Pharmacol Res 55:207–216

Rau W (1962) Über den Einfluss der Temperatur auf die lichtabhängige Carotinoidbildung von *Fusarium aquaeductuum*. Planta 59:123–137

Rau W (1967) Untersuchungen über die lichtabhängige Carotinoidsynthese. I Das Wirkungsspektrum von *Fusarium aquaeductuum*. Planta 72:14–28

Rau W, Lindemann I, Rau-Hund A (1968) Untersuchungen über die lichtabhängige Carotinoidsynthese. III Die Farbstoffbildung von *Neurospora crassa* in Submerskultur. Planta 80:309–316

Razani SH, Mousavi SM, Yeganeh HM, Marc I (2007) Fatty acid and carotenoid production by *Sporobolomyces ruberrimus* when using technical glycerol and ammonium sulfate. J Microbiol Biotechnol 17:1591–1597

Revuelta JL, Eslava AP (1983) A new gene (*carC*) involved in the regulation of carotenogenesis in *Phycomyces*. Mol Gen Genet 192:225–229

Ribeiro BD, Barreto DW, Coelho MAZ (2011) Technological aspects of β-carotene production. Food Bioprocess Technol 4:693–701

Rodríguez-Ortiz R, Limón MC, Avalos J (2009) Regulation of carotenogenesis and secondary metabolism by nitrogen in wild-type *Fusarium fujikuroi* and carotenoid-overproducing mutants. Appl Environ Microbiol 75:405–413

Rodríguez-Ortiz R, Limón MC, Avalos J (2013) Functional analysis of the *carS* gene of *Fusarium fujikuroi*. Mol Gen Genomics 288:157–173

Rodríguez-Ortiz R, Michielse C, Rep M et al (2012) Genetic basis of carotenoid overproduction in *Fusarium oxysporum*. Fungal Genet Biol 49:684–696

Rodríguez-Sáiz M, de la Fuente JL, Barredo JL (2010) *Xanthophyllomyces dendrorhous* for the industrial production of astaxanthin. Appl Microbiol Biotechnol 88:645–658

Rodríguez-Saiz M, Paz B, De La Fuente JL et al (2004) *Blakeslea trispora* genes for carotene biosynthesis. Appl Environ Microbiol 70:5589–5594

Rohmer M, Knani M, Simonin P et al (1993) Isoprenoid biosynthesis in bacteria: a novel pathway for the early steps leading to isopentenyl diphosphate. Biochem J 15:517–524

Roukas T (2016) The role of oxidative stress on carotene production by *Blakeslea trispora* in submerged fermentation. Crit Rev Biotechnol 36:424–433

Roukas T, Varzakakou M, Kotzekidou P (2015) From cheese whey to carotenes by *Blakeslea trispora* in a bubble column reactor. Appl Biochem Biotechnol 175:182–193

Ruiz-Hidalgo MJ, Benito EP, Sandmann G, Eslava AP (1997a) The phytoene dehydrogenase gene of *Phycomyces*: regulation of its expression by blue light and vitamin A. Mol Gen Genet 253:734–744

Ruiz-Roldán MC, Garre V, Guarro J et al (2008) Role of the white collar 1 photoreceptor in carotenogenesis, UV resistance, hydrophobicity, and virulence of *Fusarium oxysporum*. Eukaryot Cell 7:1227–1230

Saelices L, Youssar L, Holdermann I et al (2007) Identification of the gene responsible for torulene cleavage in the *Neurospora* carotenoid pathway. Mol Gen Genomics 278:527–537

Saenge C, Cheirsilp B, Suksaroge TT, Bourtoom T (2011) Potential use of oleaginous red yeast *Rhodotorula glutinis* for the bioconversion of crude glycerol from biodiesel plant to lipids and carotenoids. Process Biochem 46:210–218

Sahadevan Y, Richter-Fecken M, Kaerger K et al (2013) Early and late trisporoids differentially regulate β-carotene production and gene transcript levels in the mucoralean fungi *Blakeslea trispora* and *Mucor mucedo*. Appl Environ Microbiol 79:7466–7475

Sakaki H, Kaneno H, Sumiya Y et al (2002a) A new carotenoid glycosyl ester isolated from a marine microorganism, *Fusarium* strain T-1. J Nat Prod 65:1683–1684

Sakaki H, Nakanishi T, Satonaka K et al (2000) Properties of a high-torularhodin-producing mutant of *Rhodotorula glutinis* cultivated under oxidative stress. J Biosci Bioeng 89:203–205

Sakaki H, Nakanishi T, Tada A et al (2001) Activation of torularhodin production by *Rhodotorula glutinis* using weak white light irradiation. J Biosci Bioeng 92:294–297

Sakaki H, Nochide H, Komemushi S, Miki W (2002b) Effect of active oxygen species on the productivity of torularhodin by *Rhodotorula glutinis* No. 21. J Biosci Bioeng 93:338–340

Salgado LM, Avalos J, Bejarano ER, Cerdá-Olmedo E (1991) Correlation between *in vivo* and *in vitro* carotenogenesis in *Phycomyces*. Phytochemistry 30:2587–2591

Salgado LM, Bejarano ER, Cerdá-Olmedo E (1989) Carotene-superproducing mutants of *Phycomyces blakesleeanus*. Exp Mycol 13:332–336

Salgado LM, Cerdá-Olmedo E (1992) Genetic interactions in the regulation of carotenogenesis in *Phycomyces*. Curr Genet 21:67–71

Sandmann G (2002) Combinatorial biosynthesis of carotenoids in a heterologous host: a powerful approach for the biosynthesis of novel structures. Chembiochem Eur J Chem Biol 3:629–635

Sandmann G, Misawa N (2002) Fungal carotenoids. In: Osiewacz HD (ed) The mycota X. Industrial applications. Springer, Berlin, pp 247–262

Sandmann G, Misawa N, Wiedemann M et al (1993) Functional identification of *al-3* from *Neurospora crassa* as the gene for geranylgeranyl pyrophosphate synthase by complementation with *crt* genes, *in vitro* characterization of the gene product and mutant analysis. J Photochem Photobiol B 18:245–251

Sandmann G, Takaichi S, Fraser PD (2008) C(35)-apocarotenoids in the yellow mutant *Neurospora crassa* YLO. Phytochemistry 69:2886–2890

Sandmann G, Zhu C, Krubasik P, Fraser PD (2006) The biotechnological potential of the *al-2* gene from *Neurospora crassa* for the production of monocyclic keto hydroxy carotenoids. Biochim Biophys Acta 1761:1085–1092

Sanz C, Alvarez MI, Orejas M et al (2002) Interallelic complementation provides genetic evidence for the multimeric organization of the *Phycomyces blakesleeanus* phytoene dehydrogenase. Eur J Biochem 269:902–908

Sanz C, Benito EP, Orejas M et al (2010) Protein-DNA interactions in the promoter region of the *Phycomyces carB* and *carRA* genes correlate with the kinetics of their mRNA accumulation in response to light. Fungal Genet Biol 47:773–781

Sanz C, Rodríguez-Romero J, Idnurm A et al (2009) *Phycomyces* MADB interacts with MADA to form the primary photoreceptor complex for fungal phototropism. Proc Natl Acad Sci U S A 106:7095–7100

Sanz C, Velayos A, Alvarez MI et al (2011) Functional analysis of the *Phycomyces carRA* gene encoding the enzymes phytoene synthase and lycopene cyclase. PLoS One 6:e23102

Schachtschabel D, David A, Menzel K-D et al (2008) Cooperative biosynthesis of trisporoids by the (+) and (-) mating types of the zygomycete *Blakeslea trispora*. Chembiochem Eur J Chem Biol 9:3004–3012

Schmidhauser TJ, Lauter FR, Russo VE, Yanofsky C (1990) Cloning, sequence, and photoregulation of *al-1*, a carotenoid biosynthetic gene of *Neurospora crassa*. Mol Cell Biol 10:5064–5070

Schmidhauser TJ, Lauter FR, Schumacher M et al (1994) Characterization of *al-2*, the phytoene synthase gene of *Neurospora crassa*. Cloning, sequence analysis, and photoregulation. J Biol Chem 269:12060–12066

Schmidt AD, Heinekamp T, Matuschek M et al (2005) Analysis of mating-dependent transcription of *Blakeslea trispora* carotenoid biosynthesis genes *carB* and *carRA* by quantitative real-time PCR. Appl Microbiol Biotechnol 67:549–555

Schmidt I, Schewe H, Gassel S et al (2011) Biotechnological production of astaxanthin with *Phaffia rhodozyma*/*Xanthophyllomyces dendrorhous*. Appl Microbiol Biotechnol 89:555–571

Schmidt-Dannert C (2000) Engineering novel carotenoids in microorganisms. Curr Opin Biotechnol 11:255–261

Schneider T, Graeff-Hönninger S, French WT et al (2013) Lipid and carotenoid production by oleaginous red yeast *Rhodotorula glutinis* cultivated on brewery effluents. Energy 61:34–43

Schroeder WA, Johnson EA (1995a) Singlet oxygen and peroxyl radicals regulate carotenoid biosynthesis in *Phaffia rhodozyma*. J Biol Chem 270:18374–18379
Schroeder WA, Johnson EA (1993) Antioxidant role of carotenoids in *Phaffia rhodozyma*. J Gen Microbiol 139:907–912
Schroeder WA, Johnson EA (1995b) Carotenoids protect *Phaffia rhodozyma* against singlet oxygen damage. J Ind Microbiol 14:502–507
Schrott EL (1980) Fluence response relationship of carotenogenesis in *Neurospora crassa*. Planta 150:174–179
Schrott EL (1981) The biphasic fluence response of carotenogenesis in *Neurospora crassa*: temporary insensitivity of the photoreceptor system. Planta 151:371–374
Schwerdtfeger C, Linden H (2003) VIVID is a flavoprotein and serves as a fungal blue light photoreceptor for photoadaptation. EMBO J 22:4846–4855
Shi F, Zhan W, Li Y, Wang X (2014) Temperature influences β-carotene production in recombinant *Saccharomyces cerevisiae* expressing carotenogenic genes from *Phaffia rhodozyma*. World J Microbiol Biotechnol 30:125–133
Shi Y, Xin X, Yuan Q (2012) Improved lycopene production by *Blakeslea trispora* with isopentenyl compounds and metabolic precursors. Biotechnol Lett 34:849–852
Shimada H, Kondo K, Fraser PD et al (1998) Increased carotenoid production by the food yeast *Candida utilis* through metabolic engineering of the isoprenoid pathway. Appl Environ Microbiol 64:2676–2680
Shrode LB, Lewis ZA, White LD et al (2001) *vvd* is required for light adaptation of conidiation-specific genes of *Neurospora crassa*, but not circadian conidiation. Fungal Genet Biol 32:169–181
Sieiro C, Poza M, de Miguel T, Villa TG (2003) Genetic basis of microbial carotenogenesis. Int Microbiol 6:11–16
Silva F, Navarro E, Peñaranda A et al (2008) A RING-finger protein regulates carotenogenesis via proteolysis-independent ubiquitylation of a White Collar-1-like activator. Mol Microbiol 70:1026–1036
Silva F, Torres-Martínez S, Garre V (2006) Distinct *white collar-1* genes control specific light responses in *Mucor circinelloides*. Mol Microbiol 61:1023–1037
Simpson KL, Nakayama TO, Chichester CO (1964) Biosynthesis of yeast carotenoids. J Bacteriol 88:1688–1694
Sokolovsky VY, Lauter FR, Müller-Röber B et al (1992) Nitrogen regulation of blue light-inducible genes in *Neurospora crassa*. J Gen Microbiol 138:2045–2049
Sperstad S, Lutnaes BF, Stormo SK et al (2006) Torularhodin and torulene are the major contributors to the carotenoid pool of marine *Rhodosporidium babjevae* (Golubev). J Ind Microbiol Biotechnol 33:269–273
Spurgeon SL, Turner RV, Harding RW (1979) Biosynthesis of phytoene from isopentenyl pyrophosphate by a *Neurospora enzyme* system. Arch Biochem Biophys 195:23–29
Stachowiak B (2013) Efficiency of selected mutagens in generating *Xanthophyllomyces dendrorhous* strains hyperproducing astaxanthin. Pol J Microbiol 62:67–72
Stahl W, Sies H (2005) Bioactivity and protective effects of natural carotenoids. Biochim Biophys Acta 1740:101–107
Stahl W, Sies H (2003) Antioxidant activity of carotenoids. Mol Asp Med 24:345–351
Strobel I, Breitenbach J, Scheckhuber CQ et al (2009) Carotenoids and carotenogenic genes in *Podospora anserina*: engineering of the carotenoid composition extends the life span of the mycelium. Curr Genet 55:175–184
Sun J, Li H, Yuan Q (2012) Metabolic regulation of trisporic acid on *Blakeslea trispora* revealed by a GC-MS-based metabolomic approach. PLoS One 7:e46110
Sun N, Lee S, Song KB (2004) Characterization of a carotenoid-hyperproducing yeast mutant isolated by low-dose gamma irradiation. Int J Food Microbiol 94:263–267
Sutter RP (1970) Effect of light on β-carotene accumulation in *Blakeslea trispora*. J Gen Microbiol 64:215–221

Sutter RP (1987) Sexual development. In: Cerdá-Olmedo E, Lipson ED (eds) *Phycomyces*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, pp 317–336

Tagua VG, Medina HR, Martín-Domínguez R et al (2012) A gene for carotene cleavage required for pheromone biosynthesis and carotene regulation in the fungus *Phycomyces blakesleeanus*. Fungal Genet Biol 49:398–404

Tang Q, Li Y, Yuan QP (2008) Effects of an ergosterol synthesis inhibitor on gene transcription of terpenoid biosynthesis in *Blakeslea trispora*. Curr Microbiol 57:527–531

Tapiero H, Townsend DM, Tew KD (2004) The role of carotenoids in the prevention of human pathologies. Biomed Pharmacother Biomedecine Pharmacother 58:100–110

Thewes S, Prado-Cabrero A, Prado MM et al (2005) Characterization of a gene in the car cluster of *Fusarium fujikuroi* that codes for a protein of the carotenoid oxygenase family. Mol Gen Genomics 274:217–228

Tinoi J, Rakariyatham N, Deming RL (2006) Utilization of mustard waste isolates for improved production of astaxanthin by *Xanthophyllomyces dendrorhous*. J Ind Microbiol Biotechnol 33:309–314

Tinoi J, Rakariyatham N, Deming RL (2005) Simplex optimization of carotenoid production by *Rhodotorula glutinis* using hydrolyzed mung bean waste flour as substrate. Process Biochem 40:2551–2557

Torres-Martínez S, Murillo FJ, Cerdá-Olmedo E (1980) Genetics of lycopene cyclization and substrate transfer in β-carotene biosynthesis in *Phycomyces*. Genet Res 36:299–309

Torres-Martínez S, Ruiz-Vázquez RM, Garre V et al (2012) Molecular tools for carotenogenesis analysis in the zygomycete *Mucor circinelloides*. Methods Mol Biol 898:85–107

Tsolakis G, Parashi E, Galland P, Kotzabasis K (1999) Blue light signaling chains in *Phycomyces*: phototransduction of carotenogenesis and morphogenesis involves distinct protein kinase/phosphatase elements. Fungal Genet Biol 28:201–213

Tudzynski B (2005) Gibberellin biosynthesis in fungi: genes, enzymes, evolution, and impact on biotechnology. Appl Microbiol Biotechnol 66:597–611

Ukibe K, Hashida K, Yoshida N, Takagi H (2009) Metabolic engineering of *Saccharomyces cerevisiae* for astaxanthin production and oxidative stress tolerance. Appl Environ Microbiol 75:7205–7211

Valadon LRG, Cooke RC (1963) Carotenoid pigments of the nematode-trapping hyphomycete *Arthrobotrys oligospora* (fres.) Phytochemistry 2:103–105

Valadon LRG, Mummery RS (1969) Biosynthesis of neurosporaxanthin. Microbios 1A:3–8

Valadon LRG, Mummery RS (1977) Natural β-apo-4′-carotenoic acid methyl ester in the fungus *Verticillium agaricinum*. Phytochemistry 16:613–614

Valduga E, Rausch Ribeiro AH, Cence K et al (2014) Carotenoids production from a newly isolated Sporidiobolus pararoseus strain using agroindustrial substrates. Biocatal Agric Biotechnol 3:207–213

Valduga E, Valerio A, Treichel H et al (2009) Kinetic and stoichiometric parameters in the production of carotenoids by *Sporidiobolus salmonicolor* (CBS 2636) in synthetic and agroindustrial media. Appl Biochem Biotechnol 157:61–69

van Eijk GW, Mummery RS, Roeymans HJ, Valadon LR (1979) A comparative study of carotenoids of *Aschersonia aleyroides* and *Aspergillus giganteus*. Antonie Van Leeuwenhoek 45:417–422

Varzakakou M, Roukas T (2010) Identification of carotenoids produced from cheese whey by *Blakeslea trispora* in submerged fermentation. Prep Biochem Biotechnol 40:76–82

Varzakakou M, Roukas T, Papaioannou E et al (2011) Autolysis of *Blakeslea trispora* during carotene production from cheese whey in an airlift reactor. Prep Biochem Biotechnol 41:7–21

Vázquez M, Martin AM (1998) Optimization of *Phaffia rhodozyma* continuous culture through response surface methodology. Biotechnol Bioeng 57:314–320

Velayos A, Blasco JL, Álvarez MI et al (2000a) Blue-light regulation of phytoene dehydrogenase (*carB*) gene expression in *Mucor circinelloides*. Planta 210:938–946

Velayos A, Eslava AP, Iturriaga EA (2000b) A bifunctional enzyme with lycopene cyclase and phytoene synthase activities is encoded by the *carRP* gene of *Mucor circinelloides*. Eur J Biochem 267:5509–5519

Velayos A, López-Matas MA, Ruiz-Hidalgo MJ, Eslava AP (1997) Complementation analysis of carotenogenic mutants of *Mucor circinelloides*. Fungal Genet Biol 22:19–27

Velayos A, Papp T, Aguilar-Elena R et al (2003) Expression of the *carG* gene, encoding geranylgeranyl pyrophosphate synthase, is up-regulated by blue light in *Mucor circinelloides*. Curr Genet 43:112–120

Verdoes JC, Krubasik P, Sandmann G, van Ooyen AJJ (1999a) Isolation and functional characterisation of a novel type of carotenoid biosynthetic gene from *Xanthophyllomyces dendrorhous*. Mol Gen Genet 262:453–461

Verdoes JC, Misawa N, van Ooyen AJJ (1999b) Cloning and characterization of the astaxanthin biosynthetic gene encoding phytoene desaturase of *Xanthophyllomyces dendrorhous*. Biotechnol Bioeng 63:750–755

Verdoes JC, Sandmann G, Visser H et al (2003) Metabolic engineering of the carotenoid biosynthetic pathway in the yeast *Xanthophyllomyces dendrorhous* (*Phaffia rhodozyma*). Appl Environ Microbiol 69:3728–3738

Verwaal R, Jiang Y, Wang J et al (2010) Heterologous carotenoid production in *Saccharomyces cerevisiae* induces the pleiotropic drug resistance stress response. Yeast Chichester Engl 27:983–998

Verwaal R, Wang J, Meijnen J-P et al (2007) High-level production of beta-carotene in *Saccharomyces cerevisiae* by successive transformation with carotenogenic genes from *Xanthophyllomyces dendrorhous*. Appl Environ Microbiol 73:4342–4350

Visser H, van Ooyen AJJ, Verdoes JC (2003) Metabolic engineering of the astaxanthin-biosynthetic pathway of *Xanthophyllomyces dendrorhous*. FEMS Yeast Res 4:221–231

Vittorioso P, Carattoli A, Londei P, Macino G (1994) Internal translational initiation in the mRNA from the *Neurospora crassa albino-3* gene. J Biol Chem 269:26650–26654

Wang GY, Keasling JD (2002) Amplification of HMG-CoA reductase production enhances carotenoid accumulation in *Neurospora crassa*. Metab Eng 4:193–201

Wang H-B, Luo J, Huang X-Y et al (2014) Oxidative stress response of *Blakeslea trispora* induced by H_2O_2 during β-carotene biosynthesis. J Ind Microbiol Biotechnol 41:555–561

Wang Q, Luo W, Gu Q-Y et al (2013) Enhanced lycopene content in *Blakeslea trispora* by effective mutation-screening method. Appl Biochem Biotechnol 171:1692–1700

Wang W, Yu L, Zhou P (2006) Effects of different fungal elicitors on growth, total carotenoids and astaxanthin formation by *Xanthophyllomyces dendrorhous*. Bioresour Technol 97:26–31

Wang Y, Chen X, Hong X et al (2016) Cyclase inhibitor tripropylamine significantly enhanced lycopene accumulation in *Blakeslea trispora*. J Biosci Bioeng 122(5):570–576

Will OH, Ruddat M, Garber ED, Kezdy FJ (1984) Characterization of carotene accumulation in *Ustilago violacea* using high-performance liquid chromatography. Curr Microbiol 10:57–63

Will OH, Ruddat M, Newland NA (1985) Characterization of carotene accumulation in species of the fungal genus *Ustilago* using high-performance liquid chromatography. Bot Gaz 146:204–207

Woodside JV, McGrath AJ, Lyner N, McKinley MC (2015) Carotenoids and health in older people. Maturitas 80:63–68

Wozniak A, Lozano C, Barahona S et al (2011) Differential carotenoid production and gene expression in *Xanthophyllomyces dendrorhous* grown in a nonfermentable carbon source. FEMS Yeast Res 11:252–262

Wu W, Lu M, Yu L (2011) Expression of carotenogenic genes and astaxanthin production in *Xanthophyllomyces dendrorhous* as a function of oxygen tension. Z Naturforschung C J Biosci 66:283–286

Xie W, Lv X, Ye L et al (2015) Construction of lycopene-overproducing *Saccharomyces cerevisiae* by combining directed evolution and metabolic engineering. Metab Eng 30:69–78

Xu F, Yuan Q-P, Zhu Y (2007) Improved production of lycopene and β-carotene by *Blakeslea trispora* with oxygen-vectors. Process Biochem 42:289–293

Yamamoto K, Hara KY, Morita T et al (2016) Enhancement of astaxanthin production in *Xanthophyllomyces dendrorhous* by efficient method for the complete deletion of genes. Microb Cell Factories 15:155

Yamane Y, Higashida K, Nakashimada Y et al (1997) Influence of oxygen and glucose on primary metabolism and astaxanthin production by *Phaffia rhodozyma* in batch and fed-batch cultures: kinetic and stoichiometric analysis. Appl Environ Microbiol 63:4471–4478

Yamano S, Ishii T, Nakagawa M et al (1994) Metabolic engineering for production of β-carotene and lycopene in *Saccharomyces cerevisiae*. Biosci Biotechnol Biochem 58:1112–1114

Yang Q, Borkovich KA (1999) Mutational activation of a Gαi causes uncontrolled proliferation of aerial hyphae and increased sensitivity to heat and oxidative stress in *Neurospora crassa*. Genetics 151:107–117

Yen H-W, Chang J-T (2015) Growth of oleaginous *Rhodotorula glutinis* in an internal-loop airlift bioreactor by using lignocellulosic biomass hydrolysate as the carbon source. J Biosci Bioeng 119:580–584

Youssar L, Avalos J (2007) Genetic basis of the *ovc* phenotype of *Neurospora*: identification and analysis of a 77 kb deletion. Curr Genet 51:19–30

Youssar L, Schmidhauser TJ, Avalos J (2005) The *Neurospora crassa* gene responsible for the *cut* and *ovc* phenotypes encodes a protein of the haloacid dehalogenase family. Mol Microbiol 55:828–838

Zahra Bathaie S, Ashrafi M, Azizian M, Tamanoi F (2016) Mevalonate pathway and human cancers. Curr Mol Pharmacol 10:77–85

Zalokar M (1954) Studies on biosynthesis of carotenoids in *Neurospora crassa*. Arch Biochem Biophys 50:71–80

Zalokar M (1957) Isolation of an acidic pigment in *Neurospora*. Arch Biochem Biophys 70:568–571

Zalokar M (1955) Biosynthesis of carotenoids in *Neurospora*. Action spectrum of photoactivation. Arch Biochem Biophys 56:318–325

Zhang Z, Zhang X, Tan T (2014) Lipid and carotenoid production by *Rhodotorula glutinis* under irradiation/high-temperature and dark/low-temperature cultivation. Bioresour Technol 157:149–153

Zoz L, Carvalho JC, Soccol VT et al (2015) Torularhodin and torulene: bioproduction, properties and prospective applications in food and cosmetics—a review. Braz Arch Biol Technol 58:278–288

Molecular Studies of the Flavinogenic Fungus *Ashbya gossypii* and the Flavinogenic Yeast *Candida famata*

9

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Abstract

Riboflavin (vitamin B_2) is an important nutrient used as precursor for synthesis of flavin nucleotides, flavin mononucleotide (FMN), and flavin adenine dinucleotide (FAD), which are used as coenzymes in many enzymatic, mostly oxidoreductive, reactions. Riboflavin is widely used in food industry, agriculture, and medicine. Annual market for this vitamin is around 250 million US dollars. Yeasts and filamentous fungi are prototrophs for riboflavin and mostly produce small amounts of this vitamin, which correspond to their needs. However, there is a group of yeasts and filamentous fungi, known as flavinogenic organisms, which are able to produce huge amounts of riboflavin. Flavinogenic yeasts overproduce riboflavin under iron starvation and flavinogenic fungi did it during sporulation under stationary phase of growth. Some mutants of the flavinogenic fungus *Ashbya gossypii* and the yeast *Candida famata* belong to the most effective riboflavin overproducers known. This review describes development of molecular tools for studying *A. gossypii* and *C. famata* and examples of successful utilization of these approaches for construction of the even more effective riboflavin producers.

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Contents

9.1 Introduction

In most cases, primary metabolites are synthesized in optimal amounts; it is thought that otherwise organism loses its competitiveness and could be eliminated by natural selection. There are however several exceptions of this general picture, an overproduction of glutamic acid by *Corynebacterium glutamicum* being apparently the best known example (Kimura 2003). Other examples deal with the ability to overproduce riboflavin (vitamin B_2) by several species of filamentous fungi and yeasts. Current review describes two such natural riboflavin-overproducing organisms, *Ashbya gossypii* and *Candida famata*. *Ashbya gossypii* is a filamentous fungus isolated as a plant pathogen and characterized as a natural riboflavin (vitamin B_2) producer (Wickerham et al. 1946). *A. gossypii* belongs to the group of the most flavinogenic organisms known (Demain 1972). Riboflavin accumulates in stationary phase (Stahmann et al. 2000), giving mycelium a bright yellow color (Förster et al. 1999). *Candida famata* is an asporogenic yeast and representative of so named flavinogenic yeasts—group of organisms capable of riboflavin oversynthesis under iron limitation (Tanner et al. 1945; Abbas and Sibirny 2011). This group also includes alkane-utilizing yeast *Meyerozyma (Pichia* or *Candida) guilliermondii*, starch-utilizing yeast *Schwanniomyces occidentalis*, pathogenic yeast *Candida albicans*, and some others (Knight et al. 2002; Abbas and Sibirny 2011; Papon et al. 2015). They all belong to the so-named CTG clade of yeasts, the organisms which decode leucine CTG codon to serine. *C. famata* apparently is the most flavinogenic yeast as under iron limitation conditions, it accumulates around 500 μg/ml of riboflavin in the medium, whereas under optimal concentration of iron ions for growth, accumulation of riboflavin does not exceed 1–1.5 μg of riboflavin/ml, i.e., similarly to that in non-flavinogenic yeasts.

The first microbial riboflavin production processes developed in the 1940s used *Eremothecium ashbyi* and *Ashbya gossypii*, two natural riboflavin-overproducing filamentous fungal species, as production strains (Revuelta et al. 2016a, b). Commercial riboflavin fermentation production plants were built in the 1960s using these fungi, but closed them after several years of operation because they could not compete with the chemical production plants.

A riboflavin production process based on *Candida famata* was developed and used by ADM Company (USA) for commercial riboflavin production using hydrolyzed cornstarch as feedstock. However, due to genetic instability of the overproducing strain dep8, resulting in non-overproducing revertants, the *C. famata*-based production process was stopped several years ago, and it is not currently used at the industrial scale. In the early 1970s, an *A. gossypii*-based riboflavin process was developed, which was later implemented at industrial scale and used it in parallel with the traditional chemical process for several years (Revuelta et al. 2016a, b). Since then, the *A. gossypii* process was continuously improved with the consequence that the chemical production was shut down in 1996, and the microbial fermentation plant was enlarged to provide sufficient production capacity.

The one-step *A. gossypii* fermentation process using highly efficient production strains is economically advantageous to the chemical production technologies because of lower technical complexity and reduced consumption of raw materials, mainly renewable carbon sources like vegetable oils or carbohydrates. It should be stressed that the microbial riboflavin production also is favorable relative to the chemical synthesis from the ecologic point of view. The riboflavin-producing biotechnological potential of the *C. famata* is also very high as some mutants of this species are the most flavinogenic organisms known (Dmytruk and Sibirny 2012). The success of the *A. gossypii* and *C. famata* in biotechnological processes is based on the development of efficient molecular tools that enable the application of metabolic engineering approaches to improve the productivity of the industrial strains. In addition, *C. famata* can also be used as a model organism in studying the iron metabolism as changes in iron acquisition could be easily detected due to changes in production of the color substance, riboflavin. This chapter describes the molecular tools developed for *A. gossypii* and *C. famata* and their application to the development of riboflavin-overproducing strains. In addition, some features of the regulation of riboflavin synthesis in the flavinogenic yeasts *C. famata, M. guilliermondii*, and *C. albicans* are discussed.

9.2 *A. gossypii*

9.2.1 Transformation

There are two procedures that achieve efficient transformation of *A. gossypii*. The first method reported is based in the enzymatic treatment of growing cells to remove their cell walls and the resulting spheroplasts exposed to DNA in the presence of

calcium ions and polyethylene glycol, before being embedded in hypertonic selective agar to facilitate cell wall regeneration and subsequent colonies formation. Transformation frequencies of up to 63 transformants per μg of plasmid DNA were obtained (Wright and Philippsen 1991).

Intact germling cells can be transformed by electroporation, which involves using a brief voltage pulse to facilitate entry of DNA, plasmid, or PCR products into the cells (Förster et al. 1999). Transformation by electroporation is simple and at least as efficient as the spheroplast transformation (50–100 transformants/μg DNA). Since *A. gossypii* grows as multinucleate mycelium, the transformant colonies are heterokarytic and contain a mixture of transformed and untransformed nuclei. Therefore, to obtain homokaryotic transformant strains, the primary transformant colonies are subjected to clonal selection. This is achieved by plating the uninucleate spores obtained from the primary transformant colonies onto selectable medium.

9.2.2 Selectable Markers

Selectable markers are one of the important molecular tools because they allow distinguishing transformed cells from untransformed cells. They generally can be classified into three categories: nutritional markers, resistance markers, and bidirectional or counterselectable markers. Besides, a few reporters (see below) also serve as convenient visual markers to select transformants.

Resistance markers allow the growth of transformants in the presence of inhibitors (antimetabolite or antibiotic). They are routinely used to transform wild-type strains because they circumvent the need to create an auxotrophic host background. The only requirement is that the recipient organism is sensitive to the selection pressure applied. The most commonly used resistance markers used in *A. gossypii* gene manipulations (gene knockout, gene over- or underexpression, and gene tagging) are *kan*MX4, *nat*R, and *hyg*R, which confer resistance to Geneticin (G418®), nourseothricin (Clonat®), and hygromycin B, respectively (Wright and Philippsen 1991; Revuelta et al. 2016a, b; Jiménez et al. 2008; Santos et al. 2005). Whereas antibiotic or nutritional markers are in general dominant and provide only a tool for positive selection (the gene imparting a survival phenotype to the recipient on the respective selection medium), counterselectable markers enabling both positive and negative selection are powerful molecular tools in genetic engineering because they allow selection for loss of a genetic marker rather than its presence. Interestingly, *hsv-1 tk* (coding for herpes simplex virus type1 thymidine kinase) provides for a negative selection—its expression is lethal to the host organism when grown in the presence of ganciclovir. Lethality is due to the conversion of ganciclovir into a cytotoxic nucleoside analogue. In combination with *kan*MX4, *hsv-1 tk* was used to provide both positive and negative selection in *A. gossypii* (Revuelta JL, personal communication).

The list of markers at our disposal is not extensive and therefore recycling/reusing a marker is of essence. The issue is particularly problematic when the same

recipient strain has to be transformed repeatedly. A very efficient method for marker recycling has been developed in a wide variety of fungi based in the Cre-*loxP* recombination system adapted from bacteriophage P1. This approach comprises the excision of a DNA fragment that is flanked by loxP sites by the catalytic activity of a Cre recombinase, which needs to be expressed in the fungus. Marker rescue based on the Cre/*loxP* recombination system has been also developed for *A. gossypii* (Aguiar et al. 2014a, b; Ledesma-Amaro et al. 2014a, b, c).

9.2.3 Reporters and Promoters

A. gossypii has been extensively used as a model to study hyphae formation, and therefore several reporters have been developed. Some of them are *LacZ* and fluorescence proteins that can be used and fused to target genes/promoters, such as yEGFP, yEmCFP, yEVenus, yECitrine, yEMCitrine, mCherry, mStrawberry, and tdTomoto (Kaufmann 2009).

The wide use of *A. gossypii* in both basic research (hyphae formation) and applied research (bioproduction of vitamins and other industrially relevant compounds) required the identification and use of different promoters. Some of the constitutive promoters are *GPD1* and *TEF1*. Interestingly, *AgTEF1* promoter is also widely used to control gene expression in *S. cerevisiae* (Monschau et al. 1998). On the contrary, *HIS3*, *TEF1*, *TEF2*, and *PGK1* promoters from *S. cerevisiae* have been used to express genes in *A. gossypii* (Kemper et al. 2011; Ravasio et al. 2014; Ribeiro et al. 2010). *GPD1* is the strongest promoter described so far, and therefore, it has been used in most of the gene overexpressions carried out for metabolic engineering approaches. In addition to the constitutive promoters, inducible and repressible ones such as *AgICLp*, *AgMET3p*, and *ScTH13p* have been developed. The promoter from *AgICL1* is repressed by glucose, derepressed by glycerol, partially activated by acetate and ethanol, and fully activated by oil (Maeting et al. 1999). The *AgMET3* promoter can be repressed by the presence of methionine (Dünkler and Wendland 2007) while the *ScTH12* promoter is repressed by thiamine (Kaufmann 2009).

9.2.4 Metabolic Engineering to Increase Riboflavin Production

Ashbya gossypii naturally produces a yellow pigment that was identified as riboflavin. In the last decade, genetic and biochemical studies have determined the metabolic pathways and the enzymes involved in the production of this vitamin (Abbas and Sibirny 2011; Revuelta et al. 2016a, b). The pathway has two branches, each with a precursor, ribulose-5-phosphate and GTP. GTP is synthesized through the purine nucleotide pathway, which is regulated by the product of *BAS1* gene. GTP and ribulose-5-phosphate are converted into riboflavin by the action of the enzymes encoded by six *RIB* genes (Ledesma-Amaro et al. 2013a, b; Jimenez et al. 2016).

The deep knowledge of the pathway has allowed its manipulation by rational metabolic engineering in order to increase the production of the vitamin. The overexpression and deregulation of the first two enzymes in the pathway of purine nucleotide synthesis de novo (phosphoribosyl pyrophosphate synthetase and phosphoribosyl pyrophosphate amidotransferase) increased the amount of riboflavin synthesized by *A. gossypii* (Jiménez et al. 2005, 2008). In addition, the elimination of a negative regulatory domain in the Bas1 enzyme further increased vitamin production (Mateos et al. 2006). The flux toward GTP and, thus, riboflavin was also enhanced by the overexpression of the IMP dehydrogenase (*IMD3*), an intermediate gene of the pathway (Buey et al. 2015a, b).

Recently, the *RIB* genes were found to be limiting riboflavin production as well as the competing gene *ADE12*, when analyzed in silico using the genome-scale metabolic model iRL776 (Ledesma-Amaro et al. 2014a, b, c). Both, the overexpression of the *RIB* genes and deletion of the gene *ADE12* lead to an increase of vitamin production as predicted by the model (Ledesma-Amaro et al. 2015a, b, c).

The combination of such rational engineering approaches with random mutagenesis and selection has created vitamin B_2 overproducer strains, which are currently in use for the large-scale production of this product by the company BASF, Germany.

9.2.5 Other Biotechnological Products of *A. gossypii*

Besides the production of riboflavin, *A. gossypii* has been engineered for the production of other biotechnological products such as lipids, nucleosides, folic acid, or proteins. Microbial lipids or single-cell oils are considered to be an ideal source for biodiesel and chemicals (Ledesma-Amaro et al. 2015a, b, c, 2016a, b; Ledesma-Amaro and Nicaud 2016a, b). *A. gossypii* is known to have a very active lipid metabolism, and this characteristic has been exploited to maximize lipid production by metabolic engineering. In one work, the capacity of this fungus to accumulate lipids was increased by blocking beta oxidation and by expressing genes from the oleaginous yeast *Yarrowia lipolytica* (Ledesma-Amaro et al. 2014a, b, c, 2015a, b, c). In another work, the profile of fatty acids produced was tuned by the manipulation of the elongase and desaturase pathways (Ledesma-Amaro et al. 2014a, b, c) or the expression of hydroxylase (Lozano-Martínez et al. 2016a, b). Later on, this host was engineered to enhance lipid production using low-cost carbon sources such as molasses (Lozano-Martínez et al. 2016a, b) or xylose (Díaz-Fernández et al. 2017).

Nucleosides, especially inosine and guanosine, are metabolites with industrial applications as flavor enhancers or pharmaceuticals (Ledesma-Amaro et al. 2013a, b). These compounds are synthesized from the purine pathway, which is a very active pathway in *A. gossypii* used for the production of riboflavin, and thus, metabolic engineering strategies have redirected the metabolic flux toward inosine and guanosine by overexpression of purine pathway genes and the deletion of

competing pathways, e.g., minimizing riboflavin production (Ledesma-Amaro et al. 2015a, b, c, 2016a, b). These works represent the highest nucleoside titer ever produced in a eukaryote organism, which suggests its potential use as industrial producer.

A. gossypii also present the capacity to produce and secrete high amount of proteins. In this regard, *A. gossypii* has been proposed as a potential producer of recombinant proteins. As pioneers, our laboratory patented the use of this fungus to produce proteins specifically heterologous cellulases (Althöefer et al. 2001). Afterward the expression of cellulases from *Trichoderma reesei* (Ribeiro et al. 2010) and a beta-galactosidase from *Aspergillus niger* (Magalhes et al. 2014) have been reported. Additionally, the natural capability of *A. gossypii* to produce proteins has been improved by both random and targeted mutagenesis (Ribeiro et al. 2013). Besides, the characteristics of protein N-glycosylation in this fungus has been studied in detail (Aguiar et al. 2014a, b).

A recent work has generated strains of *A. gossypii* able to produce substantial amounts of folic acid. The folic acid or vitamin B_9, is an essential compound for human diet, which, as riboflavin, is synthesized from GTP. These overproducing strains accumulate up to 300 times more folic acid than the wild type, and their production titers are the highest described so far (Serrano-Amatriain et al. 2016).

9.3 *C. famata*

9.3.1 Transformation

An efficient transformation method is crucial for genetic engineering of yeasts as a way to introduce foreign DNA into their cells that is necessary for construction of new strains. In the case of *C. famata*, there were adapted a few protocols which were previously used for other species transformation (Voronovsky et al. 2002).

C. famata can be modified with both spheroplast transformation and electroporation. The first one requires number of various buffers, lyticase as cell wall lysing agent, and depends on a quality of all components; thereby electroporation is the main method of *C. famata* transformation. It requires cells in their exponential phase of growth to be shortly incubated with DTT and then washed with water and with 1M sucrose before electroporation. The frequency of transformation is up to 6.3×10^4 and 1×10^5 transformants per μg DNA, respectively.

9.3.2 Selectable Markers

Development of selectable markers is an important step of genetic engineering, because it is usually the main way to select transformants. However, as *C. famata* is still a poorly studied yeast, there are only few selectable markers available at the moment that significantly limits research potential of this species.

The first selectable marker adapted for *C. famata* was gene *LEU2*. Using UV mutagenesis with subsequent growth analysis, the 16 strains unable to grow in leucine-depleted conditions were isolated among more than 40,000 colonies. The further analysis using spheroplast transformation with plasmids harboring *LEU2* from *Saccharomyces cerevisiae* allowed to identify among leucine auxotrophs *C. famata leu2* mutants and proved the gene *S. cerevisiae LEU2* as a suitable marker (Voronovsky et al. 2002).

Another auxotrophy-based selectable marker, *ADE2* gene, was recently developed for *C. famata* using CRISPR-cas9 mutagenesis. Obtained strain has pink/red colony color and unable to grow without adenine in the medium. Gene *ADE2* was isolated from *C. famata* using BLAST for identification of its sequence based on homology with the corresponding genes of *C. albicans* and *D. hansenii*. Additional benefit of using this marker is the change to white/light-pink color of transformants that makes selection easier (Lyzak et al. 2016).

Gene *ble* conferring resistance to bleomycin family of antibiotics was a first dominant marker used to select the *C. famata* transformants. It was found that growth of *C. famata* is inhibited by phleomycin (2.0 mg/l) in rich YPD medium. The *Staphylococcus aureus* version of this gene was used due to the absence of CTG codons in its sequence, and it was expressed under control of *C. famata TEF1* promoter. A frequency of transformation obtained was approximately 100 transformants per μg of DNA (Dmytruk et al. 2006).

Gene *IMH3* which encodes IMP dehydrogenase can also serve as a selective marker (Köhler et al. 1997). *D. hansenii IMH3* gene homologue and the minimal toxic concentration of mycophenolic acid (15 mg/l) were used for *C. famata* transformation. The transformation frequency was approximately 30 transformants per μg of DNA. In addition, as *IMH3* takes part in purine biosynthesis, it also provides an additional increase in riboflavin synthesis (Dmytruk et al. 2011).

Modified *D. hansenii ARO4* gene encoding 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase not sensitive to retroinhibition with tyrosine, which provides first step of aromatic amino acid biosynthesis, also can be used as dominant selectable marker. It confers resistance to fluorophenylalanine (Cebollero and Gonzalez 2004). The transformants can be selected on mineral medium containing 2.5 g/l fluorophenylalanine and supplemented with 0.8 g/l tyrosine. Transformation frequency for the *ARO4m* marker was around 100 transformants per μg of DNA (Dmytruk et al. 2011). In addition, *ARO4m* could confer a multicopy plasmid integration when toxic agent concentration is increased (Dmytruk et al. 2014).

Gene *SAT1* allows yeasts to grow on medium with nourseothricin as a selective agent. It encodes nourseothricin acetyltransferase and its sequence was recently adapted for use in CTG clade of yeasts (Reuss et al. 2004; Millerioux et al. 2011). It is extremely useful as to allow selection of *C. famata* transformants at low concentration of antibiotic, 2–4 mg/l.

9.3.3 Insertional Mutagenesis

Insertional mutagenesis is a method for identification genes and their functions and is suitable even for yeasts in which genomes are not sequenced yet. The main idea of this method is the generation of mutant phenotype library with integration-insertion cassette, a small nucleotide sequence, into a genome of a host cell that can lead to changes or full arrest of disrupted gene expression. In addition, the mutated gene is marked with the insertion cassette. Identification of altered gene is conducted by isolating the insertion cassette with flanking DNA regions. To achieve good results with insertional mutagenesis, the insertion cassette has to provide high-frequency transformation and integrate into genome randomly and in single-copy manner. Thus, the most simple insertion cassette is linearized plasmid, harboring selectable marker gene, bacterial origin of replication (ori), and bacterial selectable marker and, preferably, be lacking restriction endonuclease sites. Transformants with desired phenotype selected after transformation undergo the next step—verification of insertion cassette copy number and localization of disrupted locus. For this, genomic DNA is cleaved with endonuclease, in which recognition site is absent in cassette, and then is self-ligated and used for *Escherichia coli* transformation. Flanking regions can be identified with sequencing (Kahmann and Basse 1999). The last step of this process is complementation of obtained mutation with wild-type gene or re-obtaining of mutant phenotype with homologous integration of insertional cassette with flanking regions (Dmytruk and Sibirny 2007).

In *C. famata*, insertional mutagenesis was used to identify genes involved in the regulation of riboflavin synthesis using pL2 plasmid as the insertion cassette (Dmytruk et al. 2006). Only three mutants unable to overproduce riboflavin were obtained after transformation and were picked for further analysis. Although, in one transformant, the structural gene of riboflavin biosynthesis (*RIB1*) was disrupted, the other two had mutations in genes *MET2* and *SEF1*.

9.3.4 Reporter System and Promoters

Gene promoters are the main regulators of gene expression. Constitutive promoters are usually used for expression of genes, in which products are desirable to be in the cell all the time, whereas inducible ones can be regulated using specific chemical compounds. Thus, it is extremely important to have operating promoters from host cell as they usually are the most efficient. For evaluation of promoter activity, the report system could be a useful tool.

For *C. famata*, it was proposed to use gene encoding β-galactosidase as a reporter gene because activity of this enzyme can be easily detected and measured (Uhl and Johnson 2001; Fu and Xiao 2006). The first step of this work was isolation of *C. famata* mutants unable to grow on medium with lactose as a sole carbon source with UV mutagenesis. One mutant, βg6, was used as parental strain for report system (Ishchuk et al. 2008). As was already mentioned, *C. famata* belongs

to the yeast clade with alternative genetic code (it reassigns leucine CTG codon as serine). This fact could be one of the obstacles in search for active variant of gene encoding β-galactosidase. *LAC4* from *Kluyveromyces lactis* has only two CTG codons. Hence, it was a suitable candidate for being the report gene. Several promoters from *C. famata* and closely relative flavinogenic yeast *Debaryomyces hansenii* have been isolated, which are homologues to that previously used in other yeasts. Only one promoter from *D. hansenii* demonstrated reasonable level of expression—*DhPGK1*—the others, *DhPGI*, *DhPHO5*, and *DhTPI1*, were quite weak. Promoters from *C. famata*, *CfTEF1* and *CfPGI*, were active; however, the activity of *CfTEF1* was threefold higher relative to that of *CfPGI*. Thus, *TEF1* promoter was chosen as a strongest promoter for *C. famata*.

9.3.5 CRISPR-cas9 System

CRISPR-cas9 system is a recently developed method of genome editing and modification. It is based on one of bacterial defensive mechanisms against foreign DNA and consists of two components: RNA-guided DNA endonuclease cas9 and small guide RNA (gRNA). Complex cas9-gRNA is able to recognize DNA sequence complementary to 20 nucleotides on gRNA 5′-end followed by NGG. After recognition of target site, cas9 generates double-strand break (DSB) in this region, approximately 3–4 nucleotides before NGG. This DSB can be repaired with host cell via two possible mechanisms: nonhomologous end joining (NHEJ) or homology-directed repair (HDR). In the case of NHEJ, it is possible to generate only small mutations like mono- or oligonucleotide insertions or deletions which usually result in premature translation stop or frameshift, so the general outcome is gene knockout. Although HDR needs additional component—repair template, it has much more wide capabilities for precise gene editing, tagging, or integration. CRISPR-cas9 system demonstrates high efficiency and flexibility and might be adapted for various hosts (Gilbert et al. 2013; Xie and Yang 2013; Sander and Joung 2014).

CRISPR-cas9 system becomes available for application in *C. famata* after its adaptation from another flavinogenic yeast of CTG clade, the pathogenic yeast *C. albicans* (Vyas et al. 2015). It has been demonstrated that CRISPR-cas9 system works in *C. famata* by knocking out the model gene *ADE2* (Fig. 9.1) (Lyzak et al. 2016). Although currently the frequency of the mutation events is still quite low, this parameter could be significantly improved via promoter modification.

9.3.6 Regulation of Riboflavin Biosynthesis

As was mentioned earlier, some species of *Pichia*, *Debaryomyces*, *Schwanniomyces*, *Torulopsis*, and *Candida* genera are able to overproduce riboflavin under cultivation in iron-depleted medium. The purpose of regulation of riboflavin biosynthesis with iron is unknown. It could be hypothesized that

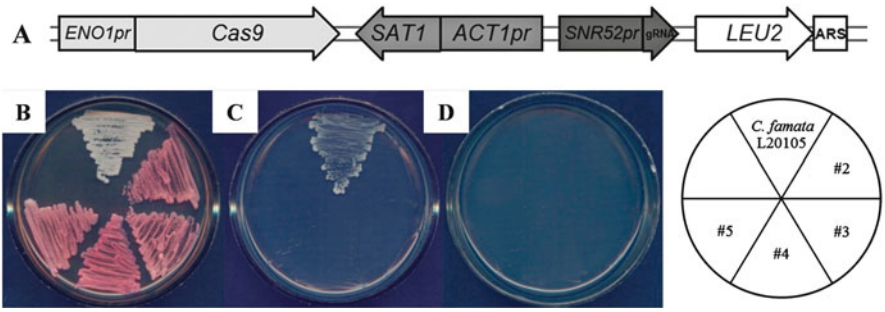

Fig. 9.1 Linear scheme of CRISPR-Cas9 plasmid harboring Cas9 protein under control of *CaENO1* promoter, guide targeting region of *CfADE2* gene controlling with *SNR52* promoter from *C. albicans*, *SAT1* and *ScLEU2* as selective markers and *Cf*ARS-element (**a**) Growth test of parental (*C. famata* L20105, *leu2*) and selected *ade2* strains (#2-5) on YPD (**b**), on YNB with leucine (**c**), and on YNB without leucine (**d**)

riboflavin secreted by flavinogenic yeasts under iron deficiency could reduce practically insoluble Fe^{3+} ions to more soluble Fe^{2+} ions providing iron in such a way to the cells.

During riboflavin overproduction, derepression of riboflavin biosynthetic enzymes (GTP-cyclohydrolase, desaminase, riboflavin synthase, etc.) occurs. In *M. guilliermondii*, nine groups of mutants, *rib80*, *rib81*, *hit1*, and *red1–red6*, which overproduce riboflavin under high iron content in the medium, have been described (Papon et al. 2015). Mutation *rib80* is recessive and has pleiotropic effect. It stimulates not only expression of riboflavin biosynthesis genes but also transport and accumulation of iron. Mutations *rib81* and *red6* lead to constitutive expression of *RIB1* even in iron-supplemented condition (Boretsky et al. 2005). In addition, it was shown that mutations *rib80*, *rib81*, and *hit1*, iron deficiency, and cell exposure to Co^{2+} lead to oxidative stress. Deletion of *YFH1* gene encoding frataxin homologue involved in iron storage and transferring also leads to riboflavin overproduction (Pynyaha et al. 2009). Although, *YFH* deletion had the same effect on the cell, it was nonallelic to the mutations mentioned above. Probably, mutations in different genes involved in iron metabolism can cause riboflavin oversynthesis. Using insertional mutagenesis in *M. guilliermondii*, the role of *VMA1* gene in regulation of riboflavin biosynthesis has been proved (Boretsky et al. 2011). On the other hand, the mutations in *RIB83* gene, which lead to loss of riboflavin overproduction under iron deficiency, have been selected. The *rib83* mutation is epistatic to mutations, which cause riboflavin overproduction, like *rib80* or *rib81*. Later on it was shown that *M. guilliermondii RIB83* is homologous to *C. famata SEF1* coding for transcription activator (Boretsky and Sibirny, unpublished).

Only one regulatory gene, *SEF1*, involved in regulation of riboflavin synthesis has been identified for *C. famata* so far. Apparently *SEF1* is involved in the positive regulation of riboflavin biosynthesis. Moreover, its overexpression leads both to increasing the riboflavin production and increasing stability of the industrial overproducing strain dep8 (Dmytruk et al. 2006). Product of *SEF1* gene,

transcriptional regulator Sef1, belongs to Gal4 family of transcriptional activators and possesses zinc finger domain at its N-terminus. To understand interaction between Sef1 and its putative DNA targets, one-hybrid system on the base of non-flavinogenic yeast *S. cerevisiae* was developed by us as a useful tool. One-hybrid system is a derivative of the yeast two-hybrid system. This specific variation is generated to investigate protein-DNA interactions and uses the following scheme: DNA sequence of interest is fused with gene, which encodes reporter protein and gene of the studied protein under control of the regulated promoter. Promoters of structural genes of riboflavin biosynthesis pathway or of *SEF1* promoter were fused with gene *LAC4* encoding β-galactosidase and were co-transformed with plasmid harboring *C. famata SEF1* under control of *S. cerevisiae GAL1* promoter. The β-galactosidase activity of the resulted strains cultivated on glucose- or galactose-containing media represented the strength of binding of the transcriptional factor with promoter. The strongest level of activation was observed for promoters of *RIB1*, *RIB6* and *RIB7* genes. These genes encode enzymes of the first and last steps of riboflavin biosynthetic pathway, so they have to be tightly regulated. Furthermore, truncation of *RIB1* promoter allowed localizing the specific region apparently involved in Sef1 binding. In addition, the evidence on *SEF1* autoregulation has been obtained (Lyzak et al. 2015).

To find out the mechanisms of *SEF1* upregulation, the expression level of this gene was assayed using qRT-PCR in three yeast species, *C. famata*, *S. cerevisiae*, and *Scheffersomyces stipitis*, cultivated in the media with high or low content of iron. In *S. cerevisiae*, the level of *SEF1* expression in the media with low and high iron content was unchanged. In *C. famata*, as expected, an inverse correlation between iron supply and *SEF1* expression was observed, i.e., iron deficiency led to increasing *SEF1* expression level. It is interesting to note that in the non-flavinogenic yeast of CUG clade *S. stipitis* also, an inverse correlation between iron supply and *SEF1* expression was found (though expression level was quite low relative to *C. famata* even in the cells cultivated under iron limitation) (Lyzak et al. unpublished).

Sef1 is involved in the regulatory system of iron homeostasis in *C. albicans*, pathogenic flavinogenic yeast with weak flavinogenic potential. This system involves two additional transcriptional factors: Sfu1 and Hap43. Sfu1 suppresses expression of *SEF1* and iron uptake; however, Hap43, in turn, inhibits *SFU1*, and its expression is activated with Sef1. At least *RIB1* gene is under positive control of *SEF1* in *C. albicans* as it takes place in *C. famata* (Chen et al. 2011). The role of orthologs of *C. albicans SFU1* and *HAP43* in *C. famata* is currently unknown. However, it could be suggested that both *C. famata* and *C. albicans* have similar systems for regulation of iron metabolism and connected to it system of riboflavin oversynthesis.

9.4 Conclusions

The *A. gossypii* and *C. famata* are intensively studied microorganisms. Advanced progress on the development of efficient molecular tools promises more complete realization of biotechnological potential of both species. *A. gossypii* is an important

industrial riboflavin producer. Application of recently developed tools of molecular research opens possibility for its further improvements. Besides riboflavin production, *A. gossypii* has been genetically modified for production of lipids, inosine and guanosine, folic acid (vitamin B_9), and industrially important proteins. Until now, flavinogenic potential of *C. famata* has not been explored extensively. Industrial riboflavin production based on *C. famata* has been shut down due to instability of the producer used (Abbas and Sibirny 2011). Much effort still is needed for elucidation of the molecular mechanisms responsible for iron-dependent regulation of riboflavin synthesis in *C. famata*. Genetically stable riboflavin-overproducing strains of *C. famata* are known. Recent molecular studies of *C. famata* permit to envisage fast future progress in construction of the robust competitive biotechnological producers of riboflavin and its derivatives (flavin nucleotides, antibiotic roseoflavin, etc.).

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References

Abbas CA, Sibirny AA (2011) Genetic control of biosynthesis and transport of riboflavin and flavin nucleotides and construction of robust biotechnological producers. Microbiol Mol Biol Rev 75(2):321–360

Aguiar TQ, Dinis C, Domingues L (2014a) Cre-loxP-based system for removal and reuse of selection markers in *Ashbya gossypii* targeted engineering. Fungal Genet Biol 68:1–8

Aguiar TQ, Dinis C, Magalhães F et al (2014b) Molecular and functional characterization of an invertase secreted by *Ashbya gossypii*. Mol Biotechnol 56:524–534

Althöefer H, Pompeus M, Revuelta JL, Santos M, Jiminez A, Benito R, Santamaria R, Fernandez J (2001) Protein production using Ashbya gossypii. Publication date: 05-04-2001. International filling date: 05-04-2001. Patent WO/2001/023576

Boretsky YR, Kapustyak KY, Fayura LR, Stasyk OV, Stenchuk MM, Bobak YP, Drobot LB, Sibirny AA (2005) Positive selection of mutants defective in transcriptional repression of riboflavin synthesis by iron in the flavinogenic yeast *Pichia guilliermondii*. FEMS Yeast Res 5 (9):829–837

Boretsky YR, Pynyaha YV, Boretsky VY, Fedorovych DV, Fayura LR, Protchenko O, Philpott CC, Sibirny AA (2011) Identification of the genes affecting the regulation of riboflavin synthesis in the flavinogenic yeast *Pichia guilliermondii* using insertion mutagenesis. FEMS Yeast Res 11(3):307–314

Buey RM, Ledesma-Amaro R, Balsera M, de Pereda JM, Revuelta JL (2015a) Increased riboflavin production by manipulation of inosine 5′-monophosphate dehydrogenase in *Ashbya gossypii*. Appl Microbiol Biotechnol 99(22):9577–9589

Buey RM, Ledesma-Amaro R, Velázquez-Campoy A, Balsera M, Chagoyen M, De Pereda JM, Revuelta JL (2015b) Guanine nucleotide binding to the Bateman domain mediates the allosteric inhibition of eukaryotic IMP dehydrogenases. Nat Commun 6:8923

Cebollero E, Gonzalez R (2004) Comparison of two alternative dominant selectable markers for wine yeast transformation. Appl Environ Microbiol 70(12):7018–7023

Chen C, Pande K, French SD, Tuch BB, Noble SM (2011) An iron homeostasis regulatory circuit with reciprocal roles in *Candida albicans* commensalism and pathogenesis. Cell Host Microbe 10(2):118–135

Demain AL (1972) Riboflavin oversynthesis. Annu Rev Microbiol 26:369–388

Díaz-Fernández D, Lozano-Martínez P, Buey RM, Revuelta JL, Jiménez A (2017) Utilization of xylose by engineered strains of *Ashbya gossypii* for the production of microbial oils. Biotechnol Biofuels 10(1):3

Dmytruk KV, Sibirny AA (2007) Molecular mechanisms of insertional mutagenesis in yeasts and mycelium fungi. Russ J Genet 43(8):835–845

Dmytruk KV, Sibirny AA (2012) *Candida famata* (*Candida flareri*). Yeast 29(11):453–458

Dmytruk KV, Voronovsky AY, Sibirny AA (2006) Insertion mutagenesis of the yeast *Candida famata* (*Debaryomyces hansenii*) by random integration of linear DNA fragments. Curr Genet 50(3):183–191

Dmytruk KV, Yatsyshyn VY, Sybirna NO, Fedorovych DV, Sibirny AA (2011) Metabolic engineering and classic selection of the yeast *Candida famata* (*Candida flar*eri) for construction of strains with enhanced riboflavin production. Metab Eng 13(1):82–88

Dmytruk K, Lyzak O, Yatsyshyn V, Kluz M, Sibirny V, Puchalski C, Sibirny A (2014) Construction and fed-batch cultivation of *Candida famata* with enhanced riboflavin production. J Biotechnol 172:11–17

Dünkler A, Wendland J (2007) Use of *MET3* promoters for regulated gene expression in *Ashbya gossypii*. Curr Genet 52(1):1–10

Förster C, Santos MA, Ruffert S, Krämer R, Revuelta JL (1999) Physiological consequence of disruption of the *VMA1* gene in the riboflavin overproducer *Ashbya gossypii*. J Biol Chem 274 (14):9442–9448

Fu Y, Xiao W (2006) Study of transcriptional regulation using a reporter gene assay. Methods Mol Biol 313:257–264

Gilbert LA, Larson MH, Morsut L, Liu Z, Brar GA, Torres SE, Stern-Ginossar N, Brandman O, Whitehead EH, Doudna JA, Lim WA, Weissman JS, Qi LS (2013) CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. Cell 154(2):442–451

Ishchuk OP, Dmytruk KV, Rohulya OV, Voronovsky AY, Abbas CA, Sibirny AA (2008) Development of a promoter assay system for the flavinogenic yeast *Candida famata* based on the *Kluyveromyces lactis* β-galactosidase *LAC4* reporter gene. Enzym Microb Technol 42 (3):208–215

Jiménez A, Santos MA, Pompejus M, Revuelta JL (2005) Metabolic engineering of the purine pathway for riboflavin production in *Ashbya gossypii*. Appl Environ Microbiol 71 (10):5743–5751

Jiménez A, Santos MA, Revuelta JL (2008) Phosphoribosyl pyrophosphate synthetase activity affects growth and riboflavin production in *Ashbya gossypii*. BMC Biotechnol 8(1):67

Jimenez A, Ledesma-Amaro R, Revuelta J (2016) Industrial production of vitamin B_2 by microbial fermentation. In: Vandamme EJ, Revuelta JL (eds) Industrial biotechnology of vitamins, biopigments and antioxidants. Wiley-VCH, Weinheim

Kahmann R, Basse C (1999) REMI (restriction enzyme mediated integration) and its impact on the isolation of pathogenicity genes in fungi attacking plants. Eur J Plant Pathol 105(3):221–229

Kaufmann A (2009) A plasmid collection for PCR-based gene targeting in the filamentous ascomycete *Ashbya gossypii*. Fungal Genet Biol 46(8):595–603

Kemper M, Mohlzahn L, Lickfeld M, Lang C, Wählisch S, Schmitz HP (2011) A Bnr-like formin links actin to the spindle pole body during sporulation in the filamentous fungus *Ashbya gossypii*. Mol Microbiol 80(5):1276–1295

Kimura E (2003) Metabolic engineering of glutamate production. In: Microbial production of l-amino acids. Springer, Berlin, pp 37–57

Knight SA, Lesuisse E, Stearman R, Klausner RD, Dancis A (2002) Reductive iron uptake by *Candida albicans*: role of copper, iron and the *TUP1* regulator. Microbiology 148:29–40

Köhler GA, White TC, Agabian N (1997) Overexpression of a cloned IMP dehydrogenase gene of *Candida albicans* confers resistance to the specific inhibitor mycophenolic acid. J Bacteriol 179(7):2331–2338

Ledesma-Amaro R, Nicaud JM (2016a) Metabolic engineering for expanding the substrate range of *Yarrowia lipolytica*. Trends Biotechnol 34(10):798–809

Ledesma-Amaro R, Nicaud JM (2016b) *Yarrowia lipolytica* as a biotechnological chassis to produce usual and unusual fatty acids. Prog Lipid Res 61:40–50

Ledesma-Amaro R, Jimenez A, Santos M, Revuelta J (2013a) Microbial production of vitamins. In: Microbial production of food ingredients, enzymes and nutraceuticals. Woodhead Publishing Series in Food Science, Technology and Nutrition, Sawston, p 246

Ledesma-Amaro R, Jiménez A, Santos MA, Revuelta JL (2013b) Biotechnological production of feed nucleotides by microbial strain improvement. Process Biochem 48(9):1263–1270

Ledesma-Amaro R, Kerkhoven EJ, Revuelta JL, Nielsen J (2014a) Genome scale metabolic modeling of the riboflavin overproducer *Ashbya gossypii*. Biotechnol Bioeng 111 (6):1191–1199

Ledesma-Amaro R, Santos MA, Jiménez A, Revuelta JL (2014b) Strain design of *Ashbya gossypii* for single-cell oil production. Appl Environ Microbiol 80(4):1237–1244

Ledesma-Amaro R, Santos MA, Jiménez A, Revuelta JL (2014c) Tuning single-cell oil production in *Ashbya gossypii* by engineering the elongation and desaturation systems. Biotechnol Bioeng 111(9):1782–1791

Ledesma-Amaro R, Buey RM, Revuelta JL (2015a) Increased production of inosine and guanosine by means of metabolic engineering of the purine pathway in *Ashbya gossypii*. Microb Cell Fact 14(1):58

Ledesma-Amaro R, Lozano-Martínez P, Jiménez A, Revuelta JL (2015b) Engineering *Ashbya gossypii* for efficient biolipid production. Bioengineered 6(2):119–123

Ledesma-Amaro R, Serrano-Amatriain C, Jiménez A, Revuelta JL (2015c) Metabolic engineering of riboflavin production in *Ashbya gossypii* through pathway optimization. Microb Cell Fact 14 (1):163

Ledesma-Amaro R, Buey RM, Revuelta JL (2016a) The filamentous fungus *Ashbya gossypii* as a competitive industrial inosine producer. Biotechnol Bioeng 113:2060–2063

Ledesma-Amaro R, Lazar Z, Rakicka M, Guo Z, Fouchard F, Crutz-Le Coq AM, Nicaud JM (2016b) Metabolic engineering of *Yarrowia lipolytica* to produce chemicals and fuels from xylose. Metab Eng 38:115–124

Lozano-Martínez P, Buey RM, Ledesma-Amaro R, Jiménez A, Revuelta JL (2016a) Engineering *Ashbya gossypii* strains for *de novo* lipid production using industrial by-products. Microb Biotechnol 10:425–433

Lozano-Martínez P, Ledesma-Amaro R, Revuelta JL (2016b) Engineering *Ashbya gossypii* for ricinoleic and linoleic acid production. Chem Eng Trans 49:253–258

Lyzak O, Semkiv M, Dmytruk K, Sibirny A (2015) Targets of transcription factor Sef1 in the flavinogenic yeasts *Candida famata*. In: Sibirny A, Fedorovich D, Gonchar M, Grabek-Lejko D (eds) Living organisms and bioanalytical approaches for detoxification and monitoring of toxic compounds. University of Rzeszow, Rzeszow

Lyzak O, Dmytruk K, Sibirny A (2016) Generation of mutations in *ADE2* gene using a CRISPR-Cas9 system in the flavinogenic yeast *Candida famata*. Visnyk of L'viv Univ. Biol Ser 73:154–159

Maeting I, Schmidt G, Sahm H, Revuelta JL, Stierhof YD, Stahmann KP (1999) Isocitrate lyase of Ashbya gossypii–transcriptional regulation and peroxisomal localization. FEBS Lett 444 (1):15–21

Magalhes F, Aguiar TQ, Oliveira C, Domingues L (2014) High-level expression of *Aspergillus niger* β-galactosidase in *Ashbya gossypii*. Biotechnol Prog 30:261

Mateos L, Jiménez A, Revuelta JL, Santos MA (2006) Purine biosynthesis, riboflavin production, and trophic-phase span are controlled by a Myb-related transcription factor in the fungus Ashbya gossypii. Appl Environ Microbiol 72(7):5052–5060

Millerioux Y, Clastre M, Simkin AJ, Courdavault V, Marais E, Sibirny AA, Noel T, Creche J, Giglioli-Guivarc'h N, Papon N (2011) Drug-resistant cassettes for the efficient transformation of *Candida guilliermondii* wild-type strains. FEMS Yeast Res 11(6):457–463

Monschau N, Sahm H, Stahmann K (1998) Threonine aldolase overexpression plus threonine supplementation enhanced riboflavin production in *Ashbya gossypii*. Appl Environ Microbiol 64:4283

Papon N, Boretsky YR, Courdavault V, Clastre M, Sibirny AA (2015) Genetic manipulation of *Meyerozyma guilliermondii*. In: Van den Berg MA, Maruthachalam K (eds) Genetic transformation systems in fungi, vol 2. Springer, pp 245–262

Pynyaha YV, Boretsky YR, Fedorovych DV, Fayura LR, Levkiv AI, Ubiyvovk VM, Protchenko OV, Philpott CC, Sibirny AA (2009) Deficiency in frataxin homologue *YFH1* in the yeast *Pichia guilliermondii* leads to missregulation of iron acquisition and riboflavin biosynthesis and affects sulfate assimilation. Biometals 22(6):1051–1061

Ravasio D, Wendland J, Walther A (2014) Major contribution of the Ehrlich pathway for 2-phenylethanol/rose flavor production in Ashbya gossypii. FEMS Yeast Res 14(6):833–844

Reuss O, Vik A, Kolter R, Morschhauser J (2004) The *SAT1* flipper, an optimized tool for gene disruption in *Candida albicans*. Gene 341:119–127

Revuelta JL, Buey RM, Ledesma-Amaro R, Vandamme EJ (2016a) Microbial biotechnology for the synthesis of (pro) vitamins, biopigments and antioxidants: challenges and opportunities. Microb Biotechnol 9(5):564–567

Revuelta JL, Ledesma-Amaro R, Lozano-Martinez P, Díaz-Fernández D, Buey RM, Jiménez A (2016b) Bioproduction of riboflavin: a bright yellow history. J Ind Microbiol Biotechnol:1–7. doi:10.1007/s10295-016-1842-7

Ribeiro O, Wiebe M, Ilmén M, Domingues L, Penttilä M (2010) Expression of *Trichoderma reesei* cellulases CBHI and EGI in *Ashbya gossypii*. Appl Microbiol Biotechnol 87(4):1437–1446

Ribeiro O, Magalhães F, Aguiar TQ, Wiebe MG, Penttilä M, Domingues L (2013) Random and direct mutagenesis to enhance protein secretion in *Ashbya gossypii*. Bioengineered 4 (5):322–331

Sander JD, Joung JK (2014) CRISPR-Cas systems for editing, regulating and targeting genomes. Nat Biotechnol 32(4):347–355

Santos MA, Mateos L, Stahmann KP, Revuelta JL (2005) Insertional mutagenesis in the vitamin B_2 producer fungus *Ashbya gossypii*. Microb Process Prod 18:283–300

Serrano-Amatriain C, Ledesma-Amaro R, López-Nicolás R, Ros G, Jiménez A, Revuelta JL (2016) Folic acid production by engineered *Ashbya gossypii*. Metab Eng 38:473–482

Stahmann KP, Revuelta JL, Seulberger H (2000) Three biotechnical processes using *Ashbya gossypii*, *Candida famata*, or *Bacillus subtilis* compete with chemical riboflavin production. Appl Microbiol Biotechnol 53(5):509–516

Tanner F Jr, Vojnovich C, Van Lanen JM (1945) Riboflavin production by *Candida* species. Science (Washington) 101:180–161

Uhl MA, Johnson AD (2001) Development of *Streptococcus thermophilus* lacZ as a reporter gene for *Candida albicans*. Microbiology 147(Pt 5):1189–1195

Voronovsky A, Abbas C, Fayura L, Kshanovska B, Dmytruk K, Sybirna K, Sibirny A (2002) Development of a transformation system for the flavinogenic yeast. FEMS Yeast Res 2 (3):381–388

Vyas VK, Barrasa MI, Fink GR (2015) A *Candida albicans* CRISPR system permits genetic engineering of essential genes and gene families. Sci Adv 1(3):e1500248

Wickerham LJ, Flickinger MH, Johnston RM (1946) The production of riboflavin by Ashbya gossypii. Arch Biochem 9:95–98

Wright MC, Philippsen P (1991) Replicative transformation of the filamentous fungus *Ashbya gossypii* with plasmids containing *Saccharomyces cerevisiae* ARS elements. Gene 109 (1):99–105

Xie K, Yang Y (2013) RNA-guided genome editing in plants using a CRISPR-Cas system. Mol Plant 6(6):1975–1983

Cold-Active Enzymes from Cold-Adapted Yeasts

10

Marcelo Baeza, Jennifer Alcaíno, Víctor Cifuentes, Benedetta Turchetti, and Pietro Buzzini

Abstract

Cold-adapted yeasts include psychrophiles or psychrotolerant nonconventional species able to survive and grow at low temperatures. They represent an important source of biological diversity that has developed a set of structural and functional adaptation strategies to overcome the adverse effects of cold (sometimes associated with other limiting conditions). Among them, the production of cold-active enzymes is probably one of the most efficient adaptations of the eukaryotic physiology at low temperatures. Current literature reports that cold-active enzymes exhibit several advantages than their mesophilic and thermophilic homologues and may successfully replace traditional catalysts in a range of industrial applications carried out at low and moderate temperatures. Due to their singular traits, some cold-active hydrolases (i.e., lipases, amylases, and proteases,) isolated from cold-adapted yeasts have been studied since some decades, while some other, namely, xylanases, chitinases, pectinolytic enzymes, glycosidases, phytases, and invertases, have recently attracted a rising attention for their biotechnological potential from the academy and industry for both food and nonfood exploitations.

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10.1 Introduction

Cold ecosystems represent one of the largest biospheres of the globe: Arctic and Antarctica, non-polar cryosphere, and deep oceans cover 70% of the Earth's surface (Cavicchioli and Tortsen 2000; Buzzini and Margesin 2014a). Due to the highly selective pressure exerted by the environmental conditions occurring in cold habitats, highly specialized microbial communities have developed important adaptation strategies enabling them either to survive or even grow at very low temperatures. These adaptations include (Buzzini et al. 2012; Buzzini and Margesin 2014a; Alcaíno et al. 2015) (1) an increased membrane fluidity (e.g., obtained by shortening the chain of the fatty acids of the membrane triacylglycerols by increasing their unsaturation degree and by altering methyl branching and changes in their isomeric distribution), (2) the synthesis of cold-shock (csp) and antifreeze (afp) proteins that protect the microbial cell against damage caused by low temperatures, and (3) last, but not lesser important, the production of cold-active enzymes. Current literature reports that cold-active enzymes exhibit a number of advantages than their mesophilic and thermophilic homologues. Accordingly, they may successfully replace traditional catalysts in a range of industrial applications carried out at low temperatures (Buzzini et al. 2012; Białkowska and Turkiewicz 2014; Szczesna-Antczak et al. 2014).

The impact of yeast biotechnology on both traditional fermented foods and beverages (e.g., wine, beer, bread, etc.) as well as on the synthesis (de novo or via biocatalytic route) of valuable fine and bulk chemicals has been extensively documented (Buzzini and Vaughan-Martini 2006; Johnson and Echavarri-Erasun 2011; Steensels et al. 2014). The increasing demand for producing novel molecules has increased the number of studies on yeasts exhibiting original or improved metabolic properties. Among them, the use of yeasts as source of enzymes represents undoubtedly one of the most ancient and renowned yeast-associated biotechnologies (Buzzini and Vaughan-Martini 2006; Johnson and Echavarri-Erasun 2011). However, the impact of cold-active enzymes synthesized by cold-adapted yeasts has been discovered only in recent decades and is nowadays

characterized by a rising biotechnological interest (Buzzini et al. 2012; Białkowska and Turkiewicz 2014; Szczesna-Antczak et al. 2014).

10.2 Fundamentals of Cold-Adapted Yeasts

Although the correctness of using maximum growth temperatures as the principal indicator of the thermal affinity of microorganisms is still an open question (Sabri et al. 2000; Margesin 2009; Rossi et al. 2009), the classical definition of psychrophiles, mesophiles, and thermophiles is based on thermal dependence of their growth kinetic limits (Morita 1975; Cavicchioli and Tortsen 2000). Conventionally the organisms that tolerate low temperatures can be divided in psychrophiles (frequently labeled as obligate psychrophiles) and psychrotolerants (facultative psychrophiles). Psychrophiles exhibit a minimum growth temperature around 0 °C, an optimum of about 15 °C (or lower), and an upper growth temperature $\leq$20 °C. On the contrary, psychrotolerant organisms grow at about 0 °C, but have an optimal growth >20 °C (Cavicchioli and Tortsen 2000; Raspor and Zupan 2006; Vishniac 2006; Buzzini and Margesin 2014b).

An apparent relationship between the psychrophilic/psychrotolerant aptitude of a given organism and its conventional habitat was also observed (Sabri et al. 2000; Margesin 2009; Rossi et al. 2009). Psychrophiles are common in permanently cold habitats, while psychrotolerants are dominant in habitats exhibiting fluctuating low temperatures (Cavicchioli and Tortsen 2000; Buzzini and Margesin 2014a). On the basis of above definitions, psychrotolerant organisms evolved their physiology to tolerate cold, but they are not so specialized as psychrophiles, whose ability to exhibit a slow growth at low temperatures may be even considered an advantage in oligotrophic habitats, where a rapid exhaustion of available nutrients (in particular carbon, nitrogen, and phosphorous) would rapidly lead to cell starvation (Russell 1997; Rossi et al. 2009; Buzzini and Margesin 2014b). Cold habitats are often characterized by a mixture of stressing factors such as cold, low water activity, nutrient availability, etc. that make them inhospitable for life. Accordingly, current literature frequently considers psychrophiles as true extremophiles because they exhibit adaptation not only to cold but often also to a mix of other limiting conditions (Friedmann 1982; Yayanos 1995; Margesin and Schinner 2009; Staley and Gosink 1999; Cavicchioli and Tortsen 2000; Buzzini and Margesin 2014a).

10.3 Fundamentals of Cold-Active Enzymes

Several yeasts are known since decades as enzyme producers (Buzzini and Vaughan-Martini 2006; Johnson and Echavarri-Erasun 2011). However, the enzymes produced from mesophilic yeasts sometimes exhibit quite poor performances at low temperatures. The industrial demand of cold-active enzymes is rapidly increasing: some hydrolases (i.e., lipases, amylases, proteases, and xylanases) represent well-known examples of cold-active enzymes produced by

both psychrophilic and psychrotolerant yeasts (Feller and Gerday 1997, 2003; Gerday et al. 1997; Shivaji and Prasad 2009; Buzzini et al. 2012; Białkowska and Turkiewicz 2014; Szczesna-Antczak et al. 2014). These enzymes play a key role in some food and nonfood catalysis carried out at low temperature through substantial energy savings and allowing the transformation (or refinement) of heat-sensitive molecules (Margesin and Schinner 2009; Feller and Gerday 2003). Moreover, because such enzymes are easily inactivated at mild temperature, they may be exploited in those processes requiring fast, efficient, and selective enzyme denaturation after the treatment (Gerday et al. 1997, 2000; Feller and Gerday 2003).

The superior activity of cold-active enzymes at low temperature and their heat lability in comparison with their mesophilic homologues are closely correlated with an increased plasticity and flexibility of their 3D structural components involved in the catalysis. This is probably due to the selective pressure of temperature fluctuations, which affects their specific activity, but not their stability factors, thus suggesting the weakening of intra-molecular forces, which results in reduced stability toward denaturing agents (Gerday et al. 1997, 2000; Feller and Gerday 2003; Gerday 2014).

The hypothesis of a close association between the high activity expressed by cold-active enzymes at low temperatures and a modification of their active sites has been contradicted by the evidence that all amino acid residues involved in the reaction mechanism are strictly conserved in both cold-active and mesophilic enzymes, thus suggesting that the molecular basis of cold adaptation should be found in different places and/or mechanisms. Structural comparisons of cold-active enzymes with their mesophilic and thermophilic homologues confirmed that only minor structural modifications are apparently required to convert a mesophilic or thermophilic enzyme into a corresponding cold-active homologue, including a decrease of the density of charges at the enzyme surface and an increase of the density of hydrophobic residues exposed to the solvent (Gerday et al. 1997, 2000; Feller and Gerday 2003; Gerday 2014). Based on current literature, the improvement of catalytic efficiency of cold-active enzymes at low temperatures could be the result of different strategies (Gerday et al. 1997, 2000; Feller and Gerday 2003; Gerday 2014): (1) an increased specific activity (overall catalytic rate $= k_{cat}$); (2) an improvement of substrate affinity, namely, a decrease of Michaelis constant (K_m); and (3) a simultaneous modification of both parameters. As above stated, this is most probably achieved through an increase in the flexibility of either a selected part or the whole protein 3D structure that enhances its ability to reach the conformational changes required for optimizing the catalysis at low temperatures (Feller and Gerday 2003; Gerday 2014).

Such structural modifications lead to a higher thermosensitivity in comparison with the mesophilic and thermophilic homologues, which is due to the following adaptations (Gerday et al. 1997, 2000; Feller and Gerday 2003; Gerday 2014): (1) a general decrease in the stability of the protein led to an increased overall plasticity of the molecule, which is probably required to accommodate macromolecular substrates; (2) a specific domain of the protein evolves into a highly flexible unit, whereas another domain remains much more rigid: this led to an appropriate K_m of the protein for small substrates (Feller and Gerday 2003; Gerday 2014).

10.4 Cold-Active Yeast Lipases and Other Esterases

Conventionally, lipases include some esterases that, under thermodynamically favorable conditions, catalyze the reactions of hydrolysis, acidolysis, alcoholysis, and aminolysis of esters. Their chemo-, regio-, and stereoselectivity make possible their broad application (Szczesna-Antczak et al. 2014). Among them the typical lipases, i.e., triacylglycerol lipases, which catalyze the hydrolysis of triacylglycerols to free fatty acids and glycerol, are regarded as the key biocatalysts for a plethora of food and chemical industries, biomedical sciences, detergent production, and environmental exploitations. A consistent part of the global enzyme market is covered by lipases (Szczesna-Antczak et al. 2014). Among them the cold-active lipases are considered an appealing biotechnology showing a significantly rising potential. Both psychrophilic and psychrotolerant yeasts are becoming a source of cold-active lipases for biotechnological exploitations. Due to their high activity at very low temperatures, which is a favorable property for the production of relatively heat-sensitive molecules, these lipases have recently attracted a remarkable attention because of their rising use as powerful and flexible catalytic solutions for organic chemists, pharmacists, biophysicists, biochemical and process engineers, biotechnologists, microbiologists, and biochemists (Joseph et al. 2008).

The ability to produce cold-active lipases was found to be very widespread among yeasts inhabiting worldwide cold environments (Buzzini et al. 2012; Szczesna-Antczak et al. 2014). Advanced studies on cold-active lipases A and B (labeled as CALA and CALB) isolated from *Candida antarctica* (currently *Moesziomyces antarcticus*), purified to homogeneity and characterized, were carried out since the 1990s (Patkar et al. 1993; Dominguez de Maria et al. 2005). CALA and CALB are the first two examples of patented cold-active enzymes from psychrophilic yeasts. They are at present included inside the portfolio of commercial enzymes sold by Novozymes (Denmark). CALA, sold under the commercial name Novocor® AD L, exhibits an activity range from 30 to 60 °C at pH from 5.0 to 9.0 and higher thermal stability (90 °C) than other lipases (Liese et al. 2000; Faber 2004; Patel 2004; Dominguez de Maria et al. 2005; Gotor-Fernandéz et al. 2006; Szczesna-Antczak et al. 2014). On the contrary CALB, synthesized using a recombinant fungal strain of *Aspergillus niger*, is sold under the commercial names Lipozyme® CALB L (in soluble form) and Novozym® 435 (in immobilized form) (Margesin and Feller 2010; Szczesna-Antczak et al. 2014). The structural features and catalytic activity of both CALA and CALB, as well as their heterologous expression in suitable hosts, have been broadly investigated (Kirk and Christensen 2002; Krishna et al. 2002; Blank et al. 2006; Chen et al. 2008; Ericsson et al. 2008; Miletić and Loos 2009; Emond et al. 2010; Forde et al. 2010; Juhl et al. 2010; Gutarra et al. 2011; Habeych et al. 2011; Gruber and Pleiss 2012; Kahveci and Xu 2012).

Other cold-active lipases from psychrophilic yeasts were studied. Rashid et al. (2010) found the production of cold-active lipases by *Leucosporidium antarcticum* (now *Glaciozyma antarctica*), while Zaliha et al. (2012) patented a bifunctional lipase isolated and purified from the same species: this lipase exhibited an optimal

temperature of 20 °C. Interestingly, at 5 °C the enzyme retained 60% of its maximum activity. More recently, a cold-active lipase from an Antarctic strain of *Mrakia blollopis* was isolated, purified to homogeneity and characterized (Tsuji et al. 2013).

The production of cold-active lipases from psychrophilic and psychrotolerant yeasts isolated from worldwide cold ecosystems has been recently reviewed (Buzzini et al. 2012; Szczesna-Antczak et al. 2014).

In the industrial enzymes market, the esterases have a growing potential to be used in food, textile, detergent, agrochemical industries, and in bioremediation as well as in environmentally friendly exploitations. Microbial sterol esterases could be used as diagnostic reagents for measuring cholesterol in human blood serum and in paper pulp industry to decrease (or eliminate) the lipid residues (Vaquero et al. 2016). On the other hand, microbial cold-active esterases such as carboxylesterases could be used for chemical synthesis due to their regio- and stereospecific mode of action that is necessary for the production of optically pure fine chemicals (Tutino et al. 2010). A cold-active monomeric glycosylated 86 kDa benzoyl esterase isolated from *Rhodotorula mucilaginosa* showed optimum activity at pH 7.5 and at 45 °C, but retained 20% of residual activity at 0 °C. This characteristic makes it a promising candidate to be used in the synthesis of benzoylated compounds at low temperatures for pharmaceutical or fine chemical applications (Zimmer et al. 2006). The list of cold-active lipases produced by psychrophilic and psychrotolerant yeasts is reported in Table 10.1.

10.5 Cold-Active Yeast Amylases

Amylolytic enzymes exhibiting the ability to efficiently hydrolyze starch at low temperatures have a high potential to be applied in food, detergent, laundry, textile, and food industries, in wastewater treatment, bioremediations in cold climates, and in molecular biology applications (Janeček et al. 2014). Starch is one of the most abundant carbohydrates in nature; for this reason, it is highly desired to be used as a substrate for fuel generation. The complete degradation of raw starch is fundamental for an efficient industrial microbial production of biofuels, which currently is accomplished by the supplementation of α-amylase and glucoamylase during the fermentative process (El-Fallal et al. 2012; van Zyl et al. 2012). The amylolytic enzymes synthesized by microorganisms are variable: among them, the α-amylases, which catalyze the hydrolysis of α-1,4-glucosidic bonds in starch and related α-glucans, are the most characterized and used in baking and textile industries and in starch saccharification (Kuddus et al. 2011; Janeček et al. 2014). The α-amylases commercially available have limited activity at low pHs and temperatures and display Ca^{2+} dependency for their activity (Sharma and Satyanarayana 2013). In this context, the market is evolving toward the discovery of novel microbial sources of amylases showing high activity at low temperatures and displaying specific properties that make them compatible with their application

Table 10.1 Production of cold-active enzymes from cold-adapted yeasts

Cold-active enzymes	Species (synonyms)	Industrial exploitation	References
Lipases	**Basidiomycota** *Cystobasidium laryngis* (*Rhodotorula laryngis*), *Cystobasidium macerans* (*Cryptococcus macerans*) *Dioszegia crocea*, *Dioszegia fristigensis* *Glaciozyma antarctica* (*Leucosporidium antarcticum*) *Goeffeauzyma gilvescens* (*Cryptococcus gilvescens*), *Goeffeauzyma gastrica* (*Cryptococcus gastricus*) *Leucosporidium creatinivorum* (*Leucosporidiella creatinivora*), *Leucosporidium drummii*; *Leucosporidium fragarium* (*Leucosporidiella fragaria*) *Moesziomyces antarcticus* (*Candida antarctica*, *Pseudozyma antarctica*) *Mrakia blollopis*, *Mrakia gelida*, *Mrakia robertii* *Papiliotrema laurentii* (*Cryptococcus laurentii*) *Phenoliferia glacialis* (*Rhodotorula glacialis*) *Rhodosporidiobolus colostri* (*Rhodotorula colostri*) *Rhodotorula mucilaginosa* *Solicoccozyma terricola* (*Cryptococcus terricola*) *Sporobolomyces ruberrimus*, *Sporobolomyces salmonicolor* (*Sporidiobolus salmonicolor*) *Vishniacozyma victoriae* (*Cryptococcus victoriae*)	Production of food and beverages, chemicals, and detergents	Patkar et al. (1993), Dominguez de Maria et al. (2005), Zimmer et al. (2006), Brizzio et al. (2007), Joseph et al. (2008), Juhl et al. (2010), Margesin and Feller (2010), Rashid et al. (2010), Carrasco et al. (2012), Zaliha et al. (2012), Tsuji et al. (2013)
Amylases	**Ascomycota** *Clavispora lusitaniae* *Geotrichum*: *Geotrichum* sp. **Basidiomycota** *Cystobasidium laryngis* (*Rhodotorula laryngis*), *Cystobasidium macerans* (*Cryptococcus macerans*) *Dioszegia crocea*, *Dioszegia fristingensis* *Goeffeauzyma gilvescens* (*Cryptococcus gilvescens*), *Goeffeauzyma gastrica* (*Cryptococcus gastricus*)	Production of food and beverages, chemicals, detergents, and pharmaceuticals; paper manufacturing	De Mot and Verachtert (1987), Wanderley et al. (2004), Brizzio et al. (2007), Li et al. (2007a, b), Galdino et al. (2011), Carrasco et al. (2012), de García et al. (2012), Divya and Naga Padma (2014), Singh et al. (2014), Carrasco et al. (2016), Ranjan et al. (2016)

(continued)

Table 10.1 (continued)

Cold-active enzymes	Species (synonyms)	Industrial exploitation	References
	Holtermanniella wattica (*Cryptococcus watticus*) *Leucosporidium creatinivorum* (*Leucosporidiella creatinivora*) *Moesziomyces antarcticus* (*Candida antarctica*, *Pseudozyma antarctica*) *Mrakia blollopis*, *Mrakia gelida*, *Mrakia robertii* *Papiliotrema laurentii* (*Cryptococcus laurentii*) *Phenoliferia glacialis* (*Rhodotorula glacialis*) *Rhodotorula svalbardensis* *Saitozyma flava* (*Cryptococcus flavus*) *Solicoccozyma terricola* (*Cryptococcus terricola*) *Sporobolomyces ruberrimus* **Dimorphic yeast-like fungi** *Aureobasidium pullulans*		
Proteases	**Basidiomycota** *Cystobasidium laryngis* (*Rhodotorula laryngis*), *Cystobasidium macerans* (*Cryptococcus macerans*) *Glaciozyma antarctica* *Goeffeauzyma gilvescens* (*Cryptococcus gilvescens*) *Itersonilia pannonicus* (*Udeniomyces pannonicus*) *Leucosporidium creatinivorum* (*Leucosporidiella creatinivora*), *Leucosporidium fragarium* (*Leucosporidiella fragaria*) *Mrakia frigida* (*Leucosporidium frigidum*), *Mrakia gelida* *Naganishia adeliensis* (*Cryptococcus adeliensis*) *Papiliotrema laurentii* (*Cryptococcus laurentii*) *Rhodotorula mucilaginosa*, *Rhodotorula toruloides* (*Rhodosporidium toruloides*) *Solicoccozyma terricola* (*Cryptococcus terricola*) *Sporobolomyces salmonicolor* (*Sporidiobolus salmonicolor*) *Udeniomyces megalosporus*, *U. pyricola* *Vanrija humicola* (*Candida humicola*)	Production of food and beverages	Ray et al. (1992), Ogrydziak (1993), Pazgier et al. (2003), Turkiewicz et al. (2003), Brizzio et al. (2007), Huston (2008), Carrasco et al. (2012), de García et al. (2012), Duarte et al. (2013), Alias et al. (2014), Lario et al. (2015), Chaud et al. (2016)

Xylanases	**Basidiomycota** *Dioszegia fristigensis* *Naganishia adeliensis* (*Cryptococcus adeliensis*), *Naganishia albida* (*Cryptococcus albidus*) *Papiliotrema laurentii* (*Cryptococcus laurentii*) *Pseudozyma brasiliensis* *Tausonia pullulans* (*Guehomyces pullulans*)	Production of biofuels and solvents; paper and waste manufacturing	Amoresano et al. (2000), Gomes et al. (2000), Petrescu et al. (2000), Scorzetti et al. (2000), Duarte et al. (2013), Borges et al. (2014)
Chitinases	**Ascomycota** *Metschnikowia* sp. **Basidiomycota** *Dioszegia fristigensis* *Glaciozyma antarctica* *Mrakia psychrophila* *Sporobolomyces salmonicolor* (*Sporidiobolus salmonicolor*)	Degradation of chitin-rich wastes, control of phytopathogens, and microbial spoilage of foodstuffs	Ramli et al. (2011), Carrasco et al. (2012)
Pectinases	**Ascomycota** *Metschnikowia pulcherrima* **Basidiomycota** *Cystobasidium laryngis* (*Rhodotorula laryngis*), *Cystobasidium macerans* (*Cryptococcus macerans*) *Cystofilobasidium capitatum* (*Rhodosporidium capitatum*), *Cystofilobasidium lari-marini* *Dioszegia crocea*, *Dioszegia fristigensis* *Leucosporidium drummii* *Mrakia aquatica* (*Mrakiella aquatica*), *Mrakia frigida* (*Leucosporidium frigidum*), *Mrakia psychrophila*, *Mrakia robertii* *Phenoliferia glacialis* (*Rhodotorula glacialis*) *Piskurozyma cylindrica* (*Cryptococcus cylindricus*) *Rhodotorula mucilaginosa* *Sporobolomyces salmonicolor* (*Sporidiobolus salmonicolor*)	Manufacturing of fruit and vegetables	Nakagawa et al. (2002, 2004, 2005a, b), Birgisson et al. (2003), Margesin et al. (2005), Carrasco et al. (2012), de García et al. (2012), Sahay et al. (2013), Belda et al. (2016)

(continued)

Table 10.1 (continued)

Cold-active enzymes	Species (synonyms)	Industrial exploitation	References
Glycosidases	**Ascomycota** *Hanseniaspora uvarum* *Pichia membranifaciens* **Basidiomycota** *Cystofilobasidium capitatum* (*Rhodosporidium capitatum*) *Glaciozyma antarctica* (*Leucosporidium antarcticum*) *Naganishia albida* (*Cryptococcus albidus*) *Rhodotorula mucilaginosa* *Tausonia pullulans* (*Guehomyces pullulans*)	Production of food and beverages, chemicals, and pharmaceuticals	Pavlova et al. (2002), Turkiewicz et al. (2005), Nakagawa et al. (2006), Song et al. (2010), Hamid et al. (2013), Hu et al. (2016)
Phytases	**Ascomycota** *Candida carpophila*, *Candida tropicalis*, *Candida parapsilosis* *Hanseniaspora uvarum* *Kodamaea ohmeri* *Pichia kudriavzevii* (*Issatchenkia orientalis*, *Candida krusei*) *Saccharomyces cerevisiae*, *Saccharomyces pastorianus* *Yarrowia lipolytica* **Basidiomycota** *Papiliotrema laurentii* (*Cryptococcus laurentii*) *Rhodotorula mucilaginosa*	Production of feeds	Hirimuthugoda et al. (2006), Pavlova et al. (2008), Nuobariene et al. (2011), Ranjan and Sahay (2013), Yu et al. (2015)
β-Fructofuranosidase (invertase)	**Basidiomycota** *Glaciozyma antarctica* *Xanthophyllomyces dendrorhous*	Food and beverages	Turkiewicz et al. (2005), Linde et al. (2009)

in different fields, such as acid stability for bio-pulping and compatibility with detergent components.

Starch-degrading activity was detected in psychrophilic and psychrotolerant yeasts isolated from soils of King George Island (subantarctic region). Isolates were cultured in media supplemented with both glucose and soluble starch. When the activity was analyzed using the cell-free protein extracts, the majority of the yeasts exhibited the highest amylase activity at pH 5.4–6.2 and from 30 to 37 °C, with the sole exception of *Rhodotorula glacialis* (now *Phenoliferia glacialis*) that showed the highest activity from 10 to 22 °C. The highest starch-degrading activity was expressed at pH 5.4 and 30 °C by a fungal isolate identified as *Tetracladium* sp., which produced an approximately 70 kDa protein that was identified as an α-amylase according to peptide mass fingerprinting analysis (Carrasco et al. 2016). Although the temperature allowing the highest amylolytic activity of yeasts was apparently quite high, if compared with that of cold environments from which they were isolated, it was lower than those reported for amylases produced by mesophilic yeasts, such as a 75 kDa amylase synthesized by a strain of *Cryptococcus flavus* (now *Saitozyma flava*) isolated from Brazilian fruits, which displayed its highest activity at 50 °C (Wanderley et al. 2004).

The strain N13d of the dimorphic yeast-like fungi *Aureobasidium pullulans* was isolated from marginal seas of the Pacific Ocean and produced a glucoamylase when cultured in seawater medium supplemented with 1% peptone and 1% soluble starch, pH 4.0. The optimal production was at 28 °C (Li et al. 2007b). This enzyme hydrolyzed potato starch granules, but poorly digested raw corn and sweet potato starch. After hydrolysis, only glucose was detected as product, indicating that the crude enzyme could hydrolyze both -1,4 and -1,6 linkages (Li et al. 2007a). Divya and Naga Padma (2014) studied yeasts isolated from diverse sources of cold-stored spoilt fruits and vegetables, mountain soils, and Himalayan soils. An isolate, identified as *Geotrichum* sp., grew well at 20–25 °C and displayed a promising starch-degrading activity at 5 °C, but no further characterization was performed. A strain of *Clavispora lusitaniae* isolated from vegetables in a Bhopal market (India) was reported to produce an amylase showing the highest activity at pH 11.0 and 42 °C; however, it maintained 42% of this activity at 4 °C. Interestingly, this enzyme did not show Ca^{2+} dependency for activity and stability and retained nearly 80% of activity after exposure to various detergents (sodium dodecyl sulfate, Tween-80, and Triton X-100), oxidizing (H_2O_2) and bleaching ($NaClO_3$) agents for 2 h. This noteworthy stability turns this enzyme into a good candidate to be used in detergent formulations (Ranjan et al. 2016). The list of cold-active amylases produced by psychrophilic and psychrotolerant yeasts is reported in Table 10.1.

10.6 Cold-Active Yeast Proteases

Cold-active proteases have a wide range of industrial and technological applications. They are considered as key enzymes in food industry (in particular in low-temperature and low-moisture cheeses ripening as a microbiological

alternative to rennet), in seafood processing for removing both scales and skin from fish as well as for extracting carotenes from shellfish, and in meat processing to give tenderness to refrigerated products without loss of flavors (Huston 2008).

A few cold-active proteases have been isolated from both psychrophilic and psychrotolerant yeasts, purified to homogeneity and characterized. They were often characterized by maximum activity in environments with relatively low pH (3.0–4.5). The discovery of a cold-active aspartyl proteinases (molecular mass = 36 kDa) from an Antarctic strain of *Cryptococcus humicola* (now *Vanrija humicola*) dated back to the early 1990s. This enzyme was active at temperatures ranging from 0 to 45 °C, with an optimum at 37 °C, and retained about 15% of its maximum activity at 0 °C (Ray et al. 1992).

The species *G. antarctica* was shown to be an active producer of cold-active proteases, in particular of a few subtilases belonging to the proteinase K subfamily (subtilisin family). The purified enzymes exhibited very high specific activity, a wide specificity, with the greatest affinity to substrates characteristic of chymotrypsin and chymotrypsin-like enzymes. Such high specific activity exhibited by *G. antarctica* subtilases is similar to that found in most other proteases produced by bacteria as well as by a few mesophilic yeasts and dimorphic yeast-like fungi (Tobe et al. 1976; Ogrydziak 1993; Białkowska and Turkiewicz 2014).

An extracellular serine proteinase was isolated from an Antarctic strain of *L. antarcticum* (now *G. antarctica*), purified to homogeneity and characterized. The sequence of its 35 N-terminal amino acid residues exhibited 31% identity to that found in proteinase K (Pazgier et al. 2003). Likewise, Turkiewicz et al. (2003) characterized a glycosylated serine proteinase (molecular mass = 34.4 kDa) produced by a strain of the same species: a quite low optimal temperature (25 °C), a poor thermal stability, and a high catalytic efficiency from 0 to 25 °C were found.

Alias et al. (2014) isolated the PI12 protease gene from genomic and mRNA sequences of *G. antarctica* and amplified it by rapid amplification of cDNA ends strategy. The recombinant PI12 protease (molecular mass = 99 kDa) obtained using *Pichia pastoris* (now *Komagataella pastoris*) host at 20 °C exhibited over 40% identity with the subtilisin-like protease synthesized by *Rhodosporidium toruloides* (now *Rhodotorula toruloides*), but no homology with other cold-active proteases. On the contrary, Lario et al. (2015) isolated an extracellular protease from an Antarctic strain of *R. mucilaginosa*. The enzyme was purified to homogeneity and characterized. The purified protease presented optimal catalytic activity at pH 5.0 and 50 °C and high stability in the presence of high concentrations of NaCl.

The ability of psychrophilic and psychrotolerant yeasts to produce cold-active proteases has been extensively reviewed (de García et al. 2012; Zaliha et al. 2012; Duarte et al. 2013; Białkowska and Turkiewicz 2014; Chaud et al. 2016). The list of cold-active proteases produced by psychrophilic and psychrotolerant yeasts is summarized in Table 10.1.

10.7 Cold-Active Yeast Xylanases

Xylanases catalyze the hydrolysis of hemicellulose (the main component of plant cell walls) in xylose. These enzymes are used in biotechnological processes, in particular in biofuel and paper industries (Amoresano et al. 2000; Borges et al. 2014). Cold-active xylanases may be applied in low-temperature digestion of farm, industrial, and sewage wastes (Shivaji and Prasad 2009).

A few cold-active xylanases from psychrophilic and psychrotolerant yeasts have been studied. A glycosylated xylanase synthesized by an Antarctic strain of *Cryptococcus albidus* (now *Naganishia albida*) was purified to homogeneity and characterized. MS analysis revealed a primary sequence of 338 amino acids and the occurrence of *N*-glycosylation only at Asn254, which was modified by high-mannose structure (Amoresano et al. 2000). Similarly, a cold-active glycosylated xylanase produced by an Antarctic strain of *Naganishia adeliensis* (synonyms: *Cryptococcus adeliensis*, *Cryptococcus adeliae*) was investigated as well. Due to peculiar changes in the 3D structure, the enzyme exhibited a high catalytic efficiency between 0 and 20 °C (Petrescu et al. 2000). Finally, xylanases were isolated from *Pseudozyma brasiliensis* (now *Kalmanozyma brasiliensis*), purified to homogeneity and characterized. The molecular mass was 24 kDa, and the optimal pH and temperature were 4.0 and 55 °C, respectively. The xylanase exhibited a predominantly ß-sheets secondary structure and a high specific activity (Borges et al. 2014). The ability of psychrophilic and psychrotolerant yeasts to produce cold-active xylanases has been reviewed (Gomes et al. 2000; Duarte et al. 2013; Białkowska and Turkiewicz 2014). The list of cold-active xylanases produced by psychrophilic and psychrotolerant yeasts is reported in Table 10.1.

10.8 Cold-Active Yeast Chitinases

Chitin is an insoluble homopolysaccharide (containing ß-1,4-linked N-acetylglucosamine) and is the second most abundant renewable biopolymer found in nature, mainly in marine invertebrates, insects, fungi, and algae. The defense mechanisms of some plants and vertebrates involve the production of chitinases, which are also produced by some microorganisms that have important roles in chitin recycling in nature. Chitinases are important for nutrition and parasitism in bacteria and are involved in the morphogenesis of protozoa, invertebrates, and fungi (Kumar 2000; Patil et al. 2000; Souza et al. 2011). Chitinases can be grouped into two broad categories: (1) endochitinases that cleave chitin randomly at internal sites and (2) exochitinases, which in turn are divided into chitobiosidases and β-1,4 *N*-acetyl glucosaminidases that cleave the oligomeric products of endochitinases and chitobiosidases, generating monomers of *N*-acetylglucosamine (GlcNAc) (Dahiya et al. 2006). Chitinases are applied in several fields, in particular in basic sciences, medicine, and biological control of plant diseases (Zhu et al. 2008; Guttmann et al. 2014). The current interest in cold active chitinases falls into their use in biodegradation of chitin-rich wastes at low

temperatures and biocontrol of phytopathogens in cold environments (Bai et al. 2008).

Generally, it has been observed that the addition of chitin to the culture medium of chitinolytic microorganisms enhances chitinase yields; this induction effect depends on the different types of chitin sources, being the colloidal chitin as the best inducer of chitinase enzyme production (Nampoothiri et al. 2004; Sandhya et al. 2004; Sharaf 2005; Saima et al. 2013). Although the production of a chitinase of 63 kDa that showed the highest activity at 40–45 °C and at pH 7.0 was described in actinomycetes belonging to the species *Streptomyces rimosus* (Brzezinska et al. 2013), there are few reports so far on the synthesis of chitinases by fungi, especially by psychrophilic and psychrotolerant yeasts. In the Antarctic fungus *Lecanicillium muscarium*, two chitinases of 61 and 25 kDa with optimal activities at pH 5.5 and 4.5, and at 45 and 40 °C, respectively, were isolated and characterized. Both were cold-active chitinases because they retained high activity at 5 °C (Barghini et al. 2013).

Extracellular chitinase activity was found in some Antarctic yeast strains belonging to *Dioszegia fristingensis*, *Metschnikowia* sp., and *Sporidiobolus salmonicolor* (now *Sporobolomyces salmonicolor*), but not further characterization was performed (Carrasco et al. 2012). On the contrary, the production of cold-active chitinase active at 15 °C and at pH 4.0 was described in a strain of the species *G. antarctica*; the corresponding gene was expressed in *P. pastoris* (*K. pastoris*). The recombinant chitinase secreted into the medium was active at 5–25 °C and remained stable between pH 3.0 and 4.5. Its activity was higher in the presence of K^+, Mn^{2+}, and Co^{2+}, and the preferred substrate was colloidal chitin (Ramli et al. 2011). The list of cold-active chitinases produced by psychrophilic and psychrotolerant yeasts is reported in Table 10.1.

10.9 Cold-Active Yeast Pectinolytic Enzymes

Pectinases are among the most used groups of enzymes in fruit processing: they contribute to increase the yield and production of juices by hydrolyzing pectin occurring of plant tissues. In this way, these enzymes are very useful for clarification of fruit juices and wine (Tapre and Jain 2014; Kumar and Suneetha 2016). The pectinolytic enzymes (otherwise labeled as pectic enzymes) are divided into three broader groups according to their mode of action and substrate: (1) protopectinases that degrade protopectin, (2) esterases that remove methoxyl esters from pectin (de-esterification), and (3) depolymerases that catalyze the hydrolytic cleavage of α-1,4-glycosidic bonds in the D-galacturonic acid moieties of the pectic substances (Pedrolli et al. 2009). Although pectinases are produced by a number of different microorganisms, industrial pectinases are obtained mainly from mesophilic filamentous fungi, in particular from *Aspergillus* species. These pectinases are active until 35 °C, but below this temperature, the commercial preparations exhibit poor performances (Gummadi and Kumar 2005). Because of some processes that need to be carried out at lower temperatures (below 15 °C) to

prevent growth of contaminant microorganisms, to retain labile and volatile flavor compounds, and for energy saving, there is a rising market requirement of cold-active pectinases. Furthermore, pectinases exhibiting good activity between pH 2.5 and 3.5 are necessary in the case of acidic grape must and fruit juices (Pulicherla et al. 2011; Adapa et al. 2014).

Pectinase production has been reported in cold-adapted microorganisms such as bacteria, fungi, and yeast (Birgisson et al. 2003; Nakagawa et al. 2004; Cabeza et al. 2011; Carrasco et al. 2012). The strain PPY-1 of the psychrophilic species *Cystofilobasidium capitatum*, isolated from Japanese soil samples, was able to grow using pectin as a sole carbon source; the cell-free extracts displayed pectin degradation at 5 °C (Nakagawa et al. 2002). Other strains isolated from the same locality and belonging to the species *Cryptococcus cylindricus* (now *Piskurozyma cylindrica*) and *Mrakia frigida* exhibited similar pectinolytic ability (Nakagawa et al. 2004).

Sahay et al. (2013) studied the yeasts capability of utilizing pectin as sole carbon source at low temperatures and identified two yeast strains of *R. mucilaginosa* and *C. capitatum* as good candidates to be used in the wine industry; they exhibited the ability to secrete pectinolytic enzymes active from 6 to 12 °C and pH 3.0–5.0. In cold wine fermentations, the use of *Metschnikowia pulcherrima* (as a source of pectinolytic enzymes) in combination with *Saccharomyces cerevisiae* improved the results in the terms of filterability, turbidity, color increment, anthocyanin, and polyphenol content when compared to fermentations performed using only *S. cerevisiae* (Belda et al. 2016). Naga Padma et al. (2011) screened yeasts isolated from cold-stored spoiled fruits, vegetables, and cold soils for their ability to hydrolyze pectin at 5 and 25 °C and selected a strain of *Saccharomyces* sp. exhibiting the highest polygalacturonase activity at 5 °C: pectinase activity was higher using several pectin sources, namely, orange, apple, and mango peel (21, 20, and 19 U/ml, respectively). The list of cold-active pectinases produced by psychrophilic and psychrotolerant yeasts is reported in Table 10.1.

10.10 Cold-Active Yeast Glycosidases

Oligo- and polysaccharides play a fundamental role in numerous biological processes. In addition, they are applied in many industries such as food ingredients and pharmacological supplements (Stephen 1995; Patel and Goyal 2011). Accordingly, there is a strong interest in synthesizing these polymers at both laboratory and industrial scale. To accomplish this task, glycosidases are pivotal tools, because these enzymes are quite efficient in the reversible hydrolysis of very stable glycosidic bonds in oligo- and polysaccharides (Cerqueira et al. 2012; Divakar 2013). Glycosidases and glycosyltransferases are currently used as biocatalysts in the enzymatic synthesis of oligo- and polysaccharides, as they catalyze the release of free and new glycosides through their hydrolysis and their polymerization via transglycosylation, respectively (Henrissat et al. 2008). For instance, β-glycosidases exhibiting the advantage to work at room temperature, neutral pH

and atmospheric pressure have been patented for synthesizing alkyl-glycosides, which are molecules used in personal care products (O'Lenick and O'Lenick 2006). However, the production costs of these enzymes remains high (Rather and Mishra 2013).

In winemaking, the use of β-glucosidases represents a great contribution through the release of free monoterpenes from their glycosylated form and the formation of a fruit-like flavor (Maicas and Mateo 2016). High β-glucosidase activity was reported in strains of *Hanseniaspora uvarum*, *Pichia membranifaciens*, and *R. mucilaginosa* isolated from some regions of China. A strain of *H. uvarum* showed the highest activity when assayed under winemaking conditions, exhibiting catalytic specificity for volatile glycosides of C13-norisoprenoids and some terpenes, thus improving the fresh floral, sweet, berry, and nutty aroma characteristics in wine (Hu et al. 2016).

Compared to the number of studies on enzymes degrading polysaccharides, the study of enzymes that hydrolyzes di- and oligosaccharides, which are valuable compounds for food processing (in particular for the synthesis of probiotic oligosaccharides), is far to be fully studied (Asraf and Gunasekaran 2010). Among them, cold-active β-galactosidases that hydrolyze the lactose at low temperatures even during refrigerated storage of milk are achieving a rising great interest (Sheridan and Brenchley 2000; Husain 2010; Horner et al. 2011). Antarctic strains of the species *L. antarcticum* (now *G. antarctica*) exhibited the production of both intra- and extracellular glucosidases; even though these strains showed an optimal growth temperature of 15 °C, the maximal production of the enzymes was reached at 5 °C. The extracellular glucosidase showed the highest activity at 35 °C and from pH 6.7 to 7.5, while its activity was stable for 30 min at 20 °C (Turkiewicz et al. 2005). The list of cold-active glycosidases produced by psychrophilic and psychrotolerant yeasts is summarized in Table 10.1.

10.11 Cold-Active Yeast Phytases

Phosphorus is present in cereal grains, legumes, and oilseeds mainly as phytic acid (inositol hexakisphosphate) or in its salt form (phytate). The presence of phytate in mammalian diets has adverse effects on mineral uptake and in protein, lipid, and carbohydrate utilization (Bohn et al. 2008; Kumar et al. 2010). Therefore, a prerequisite to improve the vegetal nutritional value is the dephosphorylation of phytate to decrease its adverse effects and, in turn, to increase the availability of phosphate. The enzyme phytase (myo-inositol hexakisphosphate phosphohydrolase) catalyzes the release of phosphate from phytate, having great importance for the improvement of nutritional value of vegetables in animal feed (Pandey et al. 2001; Lei et al. 2013).

Currently, cheap microbial sources of phytases at the lower cost possible are desirable to be applied in great scale. Phytases from bacteria, including cold-active phytases (Huang 2009; Park and Cho 2011; Kumar et al. 2013; Jain et al. 2016), and from yeasts isolated from gastrointestinal animal, fishes, spoiled vegetables, and

from fermentations (Hirimuthugoda et al. 2007; Banerjee and Ghosh 2014; Das and Ghosh 2014; Nuobariene et al. 2014) have been reported. A high level of extracellular phytase was found in strains of *Saccharomyces pastorianus*, *S. cerevisiae*, and *Candida krusei* (syn. *Issatchenkia orientalis*, now *Pichia kudriavzevii*) isolated from grain-based food and beer (Nuobariene et al. 2011). Marine yeasts capable of producing extracellular or cell-bound phytases were described for the first time in the 2000s. These yeasts were isolated from the gut of several fishes and were identified as belonging to the species *H. uvarum*, *Yarrowia lipolytica*, *Kodamaea ohmeri*, *Candida carpophila*, *I. orientalis* (now *P. kudriavzevii*), and *Candida tropicalis* (Hirimuthugoda et al. 2006). Likewise, yeasts isolated from the intestine of the freshwater fishes *Cirrhinus mrigala* and *Oreochromis niloticus* produced phytases showing activities from 0.7 to 1.0 U/ml (Banerjee and Ghosh 2014). Finally, an Antarctic strain of *R. mucilaginosa* produced a cold-active phytase of 63 kDa, which was most active at 50 °C and pH 5.0. Nonetheless, the enzyme retained 85% of its activity at 37 °C and was stable at pH 3.0–7.0. Interestingly, this enzyme showed a lower K_m (247 mM) and higher k_{cat} (1394 s^{-1}) when compared to mesophilic homologues; its resistance to pepsin and trypsin degradation make it useful for feed applications (Yu et al. 2015).

A strain of *Cryptococcus laurentii* (now *Papiliotrema laurentii*) isolated from Antarctica showed intracellular phytase activity; the supplementation of the medium with 5 mg/l of phosphorus (added as KH_2PO_4) determined an increased (76%) activity compared to the same yeast cultured in control medium. Interestingly, higher concentrations of phosphorous determined a decrease of enzyme production, while biomass production remained stable. Maximum production of phytase by *P. laurentii* was observed at 24 °C, while the optimal phytase activity was found at 40 °C and pH 4.8 (Pavlova et al. 2008).

A strain of *Candida parapsilosis* was selected as the best phytase producer among some yeasts isolated from spoiled vegetables, fruits, leaves, and seeds. Under optimal culture conditions, the phytase activity by *C. parapsilosis* was 112 U/ml, and the enzyme displayed an optimal activity at 37 °C and pH 3.0, but it tolerated temperatures from 25 to 45 °C and pH values from 2.0 to 6.8. Accordingly, this phytase could be applied in animal feed, such as aquaculture and poultry, to improve feed nutritional value and to reduce environmental phosphorus pollution (Ranjan and Sahay 2013). The list of cold-active phytases produced by psychrophilic and psychrotolerant yeasts is summarized in Table 10.1.

10.12 Cold-Active Yeast β-Fructofuranosidase (Invertase)

β-Fructofuranosidases catalyze the release of fructose from the nonreducing termini of various β-D-fructofuranoside oligomers. In particular, in the case of hydrolysis of the disaccharide sucrose (dextrorotatory), the release of equimolar concentrations of glucose (slightly dextrorotatory) and fructose (strongly levorotatory) determines an inversion of the rotation of the plane of polarized light: accordingly, β-fructofuranosidases are also commonly labeled as invertases (Nadeem et al.

2015). Invertases are quite appealing for biotechnological purposes as the resulting mixture of glucose and fructose (otherwise called "inverted sugar syrup") is sweeter than sucrose itself due to the higher sweetness of fructose. Currently, inverted sugar syrup is used in the production of soft-centered candies, jams, and artificial honey, among others. On the other hand, some invertases produced by fungi or yeasts may also have fructosyltransferase activity producing short-chain fructooligosaccharides (FOS) by linking one to three fructosyl moieties to a sucrose skeleton by different glycosidic bonds depending on the source (Linde et al. 2009; Kadowaki et al. 2013). FOS are also biotechnologically attractive as they are used as components of functional foods. Besides, due to their nature of calorie-free and noncariogenic sweeteners, they can act as prebiotic compounds and exert beneficial effects on human health contributing to the prevention of colon cancer and reducing cholesterol, phospholipid, and triglyceride levels in serum (Maiorano et al. 2008).

Although invertases have been found in animals, plants, filamentous fungi, yeasts, and bacteria, microbial invertases are highly desirable for industry, also in view of their higher stability than their plant and animal homologues (Nadeem et al. 2015). However, even though several studies on invertase production by microorganisms have been published, studies on the potential production of invertase by psychrophilic and psychrotolerant yeasts are still limited.

Turkiewicz et al. (2005) characterized a cold-active invertase from the psychrophilic yeast *G. antarctica*. Due to the low invertase activity found in cell-free culture supernatants, the authors suggested that the enzyme was probably bound either to cell membranes or to the cell wall. In addition, the purified enzyme was extremely unstable, losing its activity within 24 h at 0 °C. The invertase synthesized by *G. antarctica* displayed the highest activity between 25 and 35 °C, with maximum activity at 30 °C and retaining 20% of the maximum activity at 0 °C. The enzyme also showed a weak thermal stability, being completely inactivated after 30 min incubation at 40 °C (Turkiewicz et al. 2005).

More recently, invertase activity was checked on several yeasts isolated from soil samples collected both in South Shetland Islands and Antarctic Peninsula (Troncoso et al. 2017) and from sedimentary rocks of Union Glacier in Antarctica (Barahona et al. 2016). Troncoso et al. (2017) found that 13 isolates displayed invertase activity when grown in agar plates with YM medium supplemented with 2% sucrose, but only two repeated the same results when cell-free supernatants were used. Similarly, in the study of Barahona et al. (2016), only two out of ten yeast isolates exhibited invertase activity using cell-free supernatants. This could be due to a strict association between enzyme and cell membrane or cell wall, as previously suggested for *G. antarctica* (Turkiewicz et al. 2005).

Invertase activity was also found in the basidiomycetous carotenogenic yeast *Xanthophyllomyces dendrorhous* (Linde et al. 2009). This is a psychrotolerant yeast exhibiting an optimum growth between 20 and 22 °C (Andrewes et al. 1976; An et al. 1989) and a maximum for growth between 23 and 25 °C (Golubev 1995). Interestingly, strains of *X. dendrorhous* have been isolated in subantarctic soil samples (Contreras et al. 2015). A noteworthy invertase activity was found in

cell-free culture supernatants of several *X. dendrorhous* strains. The purified enzyme exhibited maximum activity at 65–70 °C, which is above the optimum temperature displayed by most yeast invertases, which is generally close to 40–50 °C. However, the *X. dendrorhous* invertase still represents an attractive candidate for biotechnological purposes as it was demonstrated that this enzyme also catalyzes the production of FOS, mainly neokestose, which has prebiotic effects that may be superior in comparison to those produced by commercial FOS (Kritzinger et al. 2003; Linde et al. 2009). The list of cold-active invertases produced by psychrophilic and psychrotolerant yeasts is summarized in Table 10.1.

10.13 Conclusions

Psychrophilic and psychrotolerant organisms (including yeasts) that colonize permanently or temporary cold habitats have developed a number of physiological adaptations to overcome the adverse effect of low temperatures. Among them, the synthesis of cold-active enzymes represents undoubtedly a key feature of this adaptation. Their high specific activity at low and moderate temperatures, which is higher than those exhibited by their mesophilic homologues under the same conditions, reflects the improvement of its structural flexibility. This is induced by rather discrete structural modifications that generally lead to a higher thermal instability of these enzymes, frequently associated with an even faster heat inactivation.

The study of psychrophilic and psychrotolerant yeasts as source of cold-active enzymes represents unquestionably an attractive field of research characterized by a rising biotechnological interest (Buzzini et al. 2012; Białkowska and Turkiewicz 2014; Szczesna-Antczak et al. 2014). Although current research on useful cold-active enzymes has been so far mainly limited to hydrolases (in particular lipases and proteases for producing high-value fine chemicals, pharmaceuticals, foods, polymers, detergents, biodiesel, etc.), other classes of enzymes could be equally important in the future for both laboratory and industrial applications. Among them, some redox enzymes could play a rising essential role in biotransformations carried out in both aqueous and organic solvents (Straathof et al. 2002; Burton 2003; Goretti et al. 2009, 2011; Hamada et al. 2011; Solano et al. 2012; Białkowska and Turkiewicz 2014; Forti et al. 2015).

Due to the vast array of engineering techniques currently available, the recombinant DNA technology (or even metagenomic approaches to discover new enzymes from unculturable yeasts) could be profitably used for obtaining heterologous expression of cold-active enzymes in suitable hosts. It seems worthwhile to pursue commercial production of recombinant enzymes in heterologous hosts and their modification by chemical or protein engineering methods in view to obtain higher activity levels and greater catalytic robustness. However, an alternative approach can be offered by worldwide yeast diversity as source of novel cold-active enzymes produced by psychrophilic and psychrotolerant yeasts. In recent years, the isolation of novel strains from extreme habitats enhanced the general

knowledge on both taxonomic and metabolic diversity of non-*Saccharomyces* yeasts. Since the market is evolving toward the use of biological approaches to replace synthetic chemical routes, the availability of new useful enzymes from yeasts should be considered strategic for developing the future of biotech-oriented industry. In this context, the availability of large amounts of yeast strains preserved in public repositories for screening surveys can be considered an important way of disclosing metabolic diversity of biotechnological interest (Boundy-Mills et al. 2016).

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References

Adapa V, Ramya LN, Pulicherla KK, Rao KR (2014) Cold active pectinases: advancing the food industry to the next generation. Appl Biochem Biotechnol 172:2324–2337

Alcaíno J, Cifuentes V, Baeza M (2015) Physiological adaptations of yeasts living in cold environments and their potential applications. World J Microbiol Biotechnol 31:1467–1473

Alias N, Ahmad Mazian M, Salleh AB, Basri M, Rahman RN (2014) Molecular cloning and optimization for high level expression of cold-adapted serine protease from Antarctic yeast *Glaciozyma antarctica* PI12. Enzym Res 2014:1–20

Amoresano A, Andolfo A, Corsaro MM, Zocchi I, Petrescu I, Gerday C, Marino G (2000) Structural characterization of a xylanase from psychrophilic yeast by mass spectrometry. Glycobiology 10:451–458

An GH, Schuman DB, Johnson EA (1989) Isolation of *Phaffia rhodozyma* mutants with increased astaxanthin content. Appl Environ Microbiol 55:116–124

Andrewes AG, Phaff HJ, Starr MP (1976) Carotenoids of *Phaffia rhodozyma*, a red-pigmented fermenting yeast. Phytochemistry 15:1003–1007

Asraf SS, Gunasekaran P (2010) Current trends of ß-galactosidase research and application. In: Mendez-Vilas A (ed) Current research, technology and education topics in applied microbiology and microbial biotechnology. Formatex, Badajoz, pp 880–890

Bai Z, Jin B, Li Y, Chen J, Li Z (2008) Utilization of winery wastes for *Trichoderma viride* biocontrol agent production by solid state fermentation. J Environ Sci (China) 20:353–358

Banerjee S, Ghosh K (2014) Enumeration of gut associated extracellular enzyme-producing yeasts in some freshwater fishes. J Appl Ichthyol 30:986–993

Barahona S, Yuivar Y, Socias G, Alcaíno J, Cifuentes V, Baeza M (2016) Identification and characterization of yeasts isolated from sedimentary rocks of Union Glacier at the Antarctica. Extremophiles 20:479–491

Barghini P, Moscatelli D, Garzillo AM, Crognale S, Fenice M (2013) High production of cold-tolerant chitinases on shrimp wastes in bench-top bioreactor by the Antarctic fungus *Lecanicillium muscarium* CCFEE 5003: bioprocess optimization and characterization of two main enzymes. Enzym Microb Technol 53:331–338

Belda I, Conchillo LB, Ruiz J, Navascués E, Marquina D, Santos A (2016) Selection and use of pectinolytic yeasts for improving clarification and phenolic extraction in winemaking. Int J Food Microbiol 223:1–8

Białkowska A, Turkiewicz M (2014) Miscellaneous cold-active yeast enzymes of industrial importance. In: Buzzini P, Margesin R (eds) Cold-adapted yeasts: biodiversity, adaptation strategies and biotechnological significance. Springer, Heidelberg, pp 377–396

Birgisson H, Delgado O, García Arroyo L, Hatti-Kaul R, Mattiasson B (2003) Cold-adapted yeasts as producers of cold-active polygalacturonases. Extremophiles 7:185–193

Blank K, Morfill J, Gumpp H, Gaub HE (2006) Functional expression of *Candida antarctica* lipase B in *Escherichia coli*. J Biotechnol 125:474–483

Bohn L, Meyer AS, Rasmussen SK (2008) Phytate: impact on environment and human nutrition. A challenge for molecular breeding. J Zhejiang Univ Sci B 9:165–191

Borges TA, de Souza AT, Squina FM, Riaño-Pachón DM, Corrêa dos Santos RA, Machado E, Velasco de Castro Oliveira J, Damásio ARL, Goldman GH (2014) Biochemical characterization of an endoxylanase from *Pseudozyma brasiliensis* sp. nov. strain GHG001 isolated from the intestinal tract of *Chrysomelidae* larvae associated to sugarcane roots. Proc Biochem 49:77–83

Boundy-Mills K, Glantschnig E, Roberts IN, Yurkov A, Casaregola S, Daniel HM, Groenewald M, Turchetti B (2016) Yeast culture collections in the twenty-first century: new opportunities and challenges. Yeast 33:243–260

Brizzio S, Turchetti B, de Garcìa V, Libkind D, Buzzini P, van Broock M (2007) Extracellular enzymatic activities (EEA) of basidiomycetous yeasts isolated from glacial and subglacial waters of northwest Patagonia (Argentina). Can J Microbiol 53:519–525

Brzezinska MS, Jankiewicz U, Walczak M (2013) Biodegradation of chitinous substances and chitinase production by the soil actinomycete *Streptomyces rimosus*. Int Biodeter Biodegr 84:104–110

Burton SG (2003) Oxidizing enzymes as biocatalyst. Trends Biotechnol 21:543–549

Buzzini P, Margesin R (2014a) Cold-adapted yeasts: biodiversity, adaptation strategies and biotechnological significance. Springer, Heidelberg

Buzzini P, Margesin R (2014b) Cold-adapted Yeasts: a lesson from the cold and a challenge for the XXI century. In: Buzzini P, Margesin R (eds) Cold-adapted yeasts: biodiversity, adaptation strategies and biotechnological significance. Springer, Heidelberg, pp 3–22

Buzzini P, Vaughan-Martini A (2006) Yeast biodiversity and biotechnology. In: Rosa CA, Peter G (eds) Biodiversity and ecophysiology of yeasts. Springer, Heidelberg, pp 533–559

Buzzini P, Branda E, Goretti M, Turchetti B (2012) Psychrophilic yeasts from worldwide glacial habitats: diversity, adaptation strategies and biotechnological potential. FEMS Microbiol Ecol 82:217–241

Cabeza MS, Baca FL, Puntes EM, Loto F, Baigorí MD, Morata VI (2011) Selection of psychrotolerant microorganisms producing cold-active pectinases for biotechnological processes at low temperature. Food Technol Biotechnol 49:187–195

Carrasco M, Rozas JM, Barahona S, Alcaíno J, Cifuentes V, Baeza M (2012) Diversity and extracellular enzymatic activities of yeasts isolated from King George Island, the sub-Antarctic region. BMC Microbiol 12:251

Carrasco M, Villarreal P, Barahona S, Alcaíno J, Cifuentes V, Baeza M (2016) Screening and characterization of amylase and cellulase activities in psychrotolerant yeasts. BMC Microbiol 16:21

Cavicchioli R, Tortsen T (2000) Extremophilic. In: Lederberg J (ed) Encyclopaedia of microbiology, vol 2, 2nd edn. Academic, London, pp 317–337

Cerqueira N, Brs N, Joo M, Alexandrino P (2012) Glycosidases – a mechanistic overview. In: Chang CF (ed) Carbohydrates – comprehensive studies on glycobiology and glycotechnology. InTech, Rijeka, pp 117–134

Chaud LC, Lario LD, Bonugli-Santos RC, Sette LD, Pessoa Junior A, Felipe MD (2016) Improvement in extracellular protease production by the marine Antarctic yeast *Rhodotorula mucilaginosa* L7. N Biotechnol 33:807–814

Chen B, Hu J, Miller EM, Xie W, Cai M, Gross RA (2008) *Candida antarctica* lipase B chemically immobilized on epoxy-activated micro- and nanobeads: catalysts for polyester synthesis. Biomacromolecules 9:463–471

Contreras G, Barahona S, Sepúlveda D, Baeza M, Cifuentes V, Alcaíno J (2015) Identification and analysis of metabolite production with biotechnological potential in *Xanthophyllomyces dendrorhous* isolates. World J Microbiol Biotechnol 31:517–526

Dahiya N, Tewari R, Hoondal GS (2006) Biotechnological aspects of chitinolytic enzymes: a review. Appl Microbiol Biotechnol 71:773–782

Das P, Ghosh K (2014) The presence of phytase in yeasts isolated from the gastrointestinal tract of four major carps [*Labeo rohita* (Hamilton, 1822), *Catla catla* (Hamilton, 1822), *Cirrhinus mrigala* (Hamilton, 1822), *Hypophthalmichthys molitrix* (Valenciennes, 1844)], climbing perch [*Anabas testudineus* (Bloch, 1792)] and *Mozambique tilapia* [*Oreochromis mossambicus* (Linnaeus, 1758)]. J Appl Ichthyol 30:403–407

de García V, Brizzio S, van Broock M (2012) Yeasts from glacial ice of Patagonian Andes, Argentina. FEMS Microbiol Ecol 82:540–550

De Mot R, Verachtert H (1987) Purification and characterization of extracellular α-amylase and glucoamylase from the yeast *Candida antarctica* CBS 6678. Eur J Biochem 164:643–654

Divakar S (2013) Enzymatic transformation. Springer, New Delhi

Divya K, Naga Padma P (2014) Yeast isolates from diverse sources for cold-active polygalacturonase and amylase production. Int J Sci Technol Res 3:145–148

Dominguez de Maria P, Carboni-Oerlemans C, Tuin B, Bergeman G, Meer A, Gemert R (2005) Biotechnological applications of *Candida antarctica* lipase A: state-of-the-art. J Mol Catal B 37:36–46

Duarte AW, Dayo-Owoyemi I, Nobre FS, Pagnocca FC, Chaud LC, Pessoa A, Felipe MG, Sette LD (2013) Taxonomic assessment and enzymes production by yeasts isolated from marine and terrestrial Antarctic samples. Extremophiles 17:1023–1035

El-Fallal A, Abou M, El-Sayed A, Omar N (2012) Starch and microbial α-amylases: from concepts to biotechnological applications. In: Chang CF (ed) Carbohydrates – comprehensive studies on glycobiology and glycotechnology. InTech, Rijeka, pp 459–488

Emond S, Montanier C, Nicaud JM, Marty A, Monsan P, Andre I, Remaud-Simeon M (2010) New efficient recombinant expression system to engineer *Candida antarctica* lipase B. Appl Environ Microbiol 76:2684–2687

Ericsson DJ, Kasrayan A, Johansson P, Berqfors T, Sandstrom AG, Bäckvall JE, Mowbray SL (2008) X-ray structure of *Candida antarctica* lipase B shows a novel lid structure and a likely mode of interfacial activation. J Mol Biol 376:109–119

Faber K (2004) Biotransformations in organic chemistry. A textbook, 5th edn. Springer, Heidelberg

Feller G, Gerday C (1997) Psychrophilic enzymes: molecular basis of cold adaptation. Cell Mol Life Sci 53:830–841

Feller G, Gerday C (2003) Psychrophilic enzymes: hot topics in cold adaptation. Nat Rev Microbiol 1:200–208

Forde J, Vakurov A, Gibson TD, Millner P, Whelehan M, Marisin IW, Ó'Fágáin C (2010) Chemical modification and immobilisation of lipase B from *Candida antarctica* onto mesoporous silicates. J Mol Cat B 66:203–209

Forti L, Di Mauro S, Cramarossa MR, Filippucci S, Turchetti B, Buzzini P (2015) Non-conventional yeasts whole cells as efficient biocatalysts for the production of flavors and fragrances. Molecules 20:10377–10398

Friedmann EI (1982) Endolithic microorganisms in the Antarctic cold desert. Science 215:1045–1053

Galdino AS, Silva RN, Lottermann MT, Alvares AC, de Moraes LM, Torres FA, de Freitas SM, Ulhoa CJ (2011) Biochemical and structural characterization of amy1: an alpha-amylase from *Cryptococcus flavus* expressed in *Saccharomyces cerevisiae*. Enzym Res 30:157294

Gerday C (2014) Fundamentals of cold-active enzymes. In: Buzzini P, Margesin R (eds) Cold-adapted yeasts: biodiversity, adaptation strategies and biotechnological significance. Springer, Heidelberg, pp 325–350

Gerday C, Aittaleb M, Arpigny JL, Baise E, Chessa JP, Garsoux G, Petrescu I, Feller G (1997) Psychrophilic enzymes: a thermodynamic challenge. Biochim Biophys Acta 1342:119–131

Gerday C, Aittaleb M, Bentahir M, Chessa JP, Claverie P, Collins T, D'Amico S, Dumont J, Garsoux G, Georlette D, Hoyoux A, Lonhienne T, Meuwis MA, Feller G (2000) Cold-adapted enzymes: from fundamentals to biotechnology. Trend Biotechnol 18:103–107

Golubev WI (1995) Perfect state of *Rhodomyces dendrorhous* (*Phaffia rhodozyma*). Yeast 11:101–110

Gomes J, Gomes I, Steiner W (2000) Thermolabile xylanase of the Antarctic yeast *Cryptococcus adeliae*: production and properties. Extremophiles 4:227–235

Goretti M, Ponzoni C, Caselli E, Marchegiani E, Cramarossa MR, Turchetti B, Buzzini P, Forti L (2009) Biotransformation of electron-poor alkenes by yeasts: asymmetric reduction of (*4S*)-(+)-carvone by yeast enoate reductases. Enzym Microb Technol 45:463–468

Goretti M, Ponzoni C, Caselli E, Marchegiani E, Cramarossa MR, Turchetti B, Forti L, Buzzini P (2011) Bioreduction of α,β-unsaturated ketones and aldehydes by non-conventional yeast (NCY) whole-cells. Biores Technol 102:3993–3998

Gotor-Fernandéz V, Busto E, Gotor V (2006) *Candida antarctica* lipase B: an ideal biocatalyst for the preparation of nitrogenated organic compounds. Adv Synth Catal 348:797–812

Gruber CC, Pleiss J (2012) Lipase B from *Candida antarctica* binds to hydrophobic substrate – water interfaces via hydrophobic anchors surrounding the active site entrance. J Mol Catal B 84:48–54

Gummadi SN, Kumar DS (2005) Microbial pectic transeliminases. Biotechnol Lett 27:451–458

Gutarra MLE, Romero O, Abian O, Torres FAG, Freire DMG, Castro AM, Guisan JM, Palomo JM (2011) Enzyme surface glycosylation in the solid phase: improved activity and selectivity of *Candida antarctica* lipase B. Chem Cat Chem 3:1902–1910

Guttmann S, Neumann S, Ringer L, Losche K, Helmke E (2014) Use of enzymes to preserve baked products. Bak Biscuit 2:70–73

Habeych DI, Juhl PB, Pleiss J, Venegas D, Eggink G, Boeriu CG (2011) Biocatalytic synthesis of polyesters from sugar-based building blocks using immobilized *Candida antarctica* lipase B. J Mol Catal B 71:1–9

Hamada S, Seike Y, Tanimori S, Sakamoto T, Kishida M (2011) Characterization of D-galacturonate reductase purified from the psychrophilic yeast species *Cryptococcus diffluens*. J Biosci Bioeng 111:518–521

Hamid B, Singh P, Mohiddin FA, Sahay S (2013) Partial characterization of cold-active β-galactosidase activity produced by *Cystophallobaidium capatitum* SPY11 and *Rodotorella musloganosa* PT1. Endocytobiosis Cell Res 24:23–26

Henrissat B, Sulzenbacher G, Bourne Y (2008) Glycosyltransferases, glycoside hydrolases: surprise, surprise. Curr Opin Struct Biol 18:527–533

Hirimuthugoda NY, Chi ZM, Li XY, Wang L, Wu LF (2006) Diversity of phytase-producing marine yeasts. Cienc Mar 32:673–682

Hirimuthugoda NY, Chi Z, Wu L (2007) Probiotic yeasts with phytase activity identified from the gastrointestinal tract of sea cucumbers. SPC Beche de Mer Inf Bull 26:31–33

Horner TW, Dunn ML, Eggett DL, Ogden LV (2011) β-Galactosidase activity of commercial lactase samples in raw and pasteurized milk at refrigerated temperatures. J Dairy Sci 94:3242–3249

Hu K, Qin Y, Tao YS, Zhu XL, Peng CT, Ullah N (2016) Potential of glycosidase from non-*Saccharomyces* isolates for enhancement of wine aroma. J Food Sci 81:M935–M943

Huang H (2009) Novel low-temperature-active phytase from *Erwinia carotovora* var. *carotovota* ACCC 10276. J Microbiol Biotechnol 19:1085–1091

Husain Q (2010) Beta galactosidases and their potential applications: a review. Crit Rev Biotechnol 30:41–62

Huston AL (2008) Biotechnological aspects of cold-adapted enzymes. In: Margesin R, Schinner F, Marx JC, Gerday C (eds) Psychrophiles: from biodiversity to biotechnology. Springer, Heidelberg, pp 347–363

Jain J, Sapna J, Singh B (2016) Characteristics and biotechnological applications of bacterial phytases. Proc Biochem 51:159–169

Janeček Š, Svensson B, MacGregor EA (2014) α-Amylase: an enzyme specificity found in various families of glycoside hydrolases. Cell Mol Life Sci 71:1149–1170

Johnson EA, Echavarri-Erasun C (2011) Yeast biotechnology. In: Kurtzman CP, Fell JW, Boekhout T (eds) The yeasts. A taxonomy study, vol 1. Elsevier, New York, pp 21–44
Joseph B, Ramteke PW, Thomas G (2008) Cold active microbial lipases: some hot issues and recent developments. Biotechnol Adv 26:457–470
Juhl PB, Doderer K, Hollmann F, Thum O, Pleiss J (2010) Engineering of *Candida antarctica* lipase B for hydrolysis of bulky carboxylic acid esters. J Biotechnol 150:474–480
Kadowaki MK, de Cassia GR, da Conceicao Silva SL, Aoki C (2013) Biotechnological advances in fungal invertases. In: Rai M (ed) Fungal enzymes. CRC Press, Boca Raton, FL, pp 1–30
Kahveci D, Xu X (2012) Bioimprinted immobilization of *Candida antarctica* lipase A for concentration of omega-3 polyunsaturated fatty acids. J Am Oil Chem Soc 89:1839–1845
Kirk O, Christensen MW (2002) Lipases from *Candida antarctica*: unique biocatalysts from a unique origin. Org Process Res Dev 6:446–451
Krishna H, Persson MM, Bornscheuer UT (2002) Enantioselective transesterification of a tertiary alcohol by lipase a from *Candida antarctica*. Tetrahedron Asymmetry 13:2693–2696
Kritzinger SM, Kilian SG, Potgieter MA, du Preez JC (2003) The effect of production parameters on the synthesis of the prebiotic trisaccharide, neokestose, by *Xanthophyllomyces dendrorhous* (*Phaffia rhodozyma*). Enzym Microb Technol 32:728–737
Kuddus M, Roohi AJM, Ramteke PW (2011) An overview of cold-active microbial α-amylase: adaptation strategies and biotechnological potentials. Biotechnology 10:246–258
Kumar MNVR (2000) A review of chitin and chitosan applications. React Funct Polym 46:1–27
Kumar P, Suneetha V (2016) Microbial pectinases: wonderful enzymes in fruit juice clarification. Int J MediPharm Res 2:119–127
Kumar V, Sinha AK, Makkar HPS, Becker K (2010) Dietary roles of phytate and phytase in human nutrition: a review. Food Chem 120:945–959
Kumar D, Rajesh S, Balashanmugam P, Rebecca LJ, Kalaichelvan PT (2013) Screening, optimization and application of extracellular phytase from *Bacillus megaterium* isolated from poultry waste. J Mod Biotechnol 2:46–52
Lario LD, Chaud L, Almeida M, Converti A, Sette LD, Pessoa A Jr (2015) Production, purification, and characterization of an extracellular acid protease from the marine Antarctic yeast *Rhodotorula mucilaginosa* L7. Fungal Biol 119:1129–1136
Lei XG, Weaver JD, Mullaney E, Ullah AH, Azain MJ (2013) Phytase, a new life for an "old" enzyme. Annu Rev Anim Biosci 1:283–309
Li H, Chi Z, Duan X, Wang L, Sheng J, Wu L (2007a) Glucoamylase production by the marine yeast *Aureobasidium pullulans* N13d and hydrolysis of potato starch granules by the enzyme. Proc Biochem 42:462–465
Li H, Chi Z, Wang X, Ma C (2007b) Amylase production by the marine yeast *Aureobasidium pullulans* N13d. J Ocean Univ China 6:60–65
Liese A, Weelbach K, Wandrey C (2000) Industrial BioTransformations, 2nd edn. Wiley, Weinheim
Linde D, Macias I, Fernández-Arrojo L, Plou FJ, Jiménez A, Fernández-Lobato M (2009) Molecular and biochemical characterization of a β-fructofuranosidase from *Xanthophyllomyces dendrorhous*. Appl Environ Microbiol 75:1065–1073
Maicas S, Mateo J (2016) Microbial glycosidases for wine production. Beverages 2:20
Maiorano AE, Piccoli RM, da Silva ES, de Andrade Rodrigues MF (2008) Microbial production of fructosyltransferases for synthesis of pre-biotics. Biotechnol Lett 30:1867–1877
Margesin R (2009) Effect of temperature on growth parameters of psychrophilic bacteria and yeasts. Extremophiles 13:257–262
Margesin R, Feller G (2010) Biotechnological applications of psychrophiles. Environ Technol 31:835–844
Margesin R, Schinner F (2009) Biotechnological applications of cold-adapted organisms. Springer, Heidelberg
Margesin R, Fauster V, Fonteyne PA (2005) Characterization of cold-active pectate lyases from psychrophilic *Mrakia frigida*. Lett Appl Microbiol 40:453–459

Miletić N, Loos K (2009) Over-stabilization of chemically modified and cross-linked *Candida antarctica* lipase B using various epoxides and diepoxides. Aust J Chem 62:799–805

Morita RY (1975) Psychrophilic bacteria. Bacteriol Rev 39:144–167

Nadeem H, Rashid MH, Siddique MH, Azeem F, Muzammil S, Javed MR, Ali MA, Rasul I, Riaz M (2015) Microbial invertases: a review on kinetics, thermodynamics, physiochemical properties. Proc Biochem 50:1202–1210

Naga Padma P, Anuradha K, Reddy G (2011) Pectinolytic yeast isolates for cold-active polygalacturonase production. Innovative Food Sci Emerg Technol 12:178–181

Nakagawa T, Yamada K, Miyaji T, Tomizuka N (2002) Cold-active pectinolytic activity of psychrophilic-basidiomycetous yeast *Cystofilobasidium capitatum* strain PPY-1. J Biosci Bioeng 94:175–177

Nakagawa T, Nagaoka T, Taniguchi S, Miyaji T, Tomizuka N (2004) Isolation and characterization of psychrophilic yeasts producing cold-adapted pectinolytic enzymes. Lett Appl Microbiol 38:383–387

Nakagawa T, Nagaoka T, Miyaji T, Tomizuka N (2005a) Cold-active polygalacturonase from psychrophilic basidiomycetous yeast *Cystofilobasidium capitatum* strain PPY-1. Biosci Biotechnol Biochem 69:419–421

Nakagawa T, Nagaoka T, Miyaji T, Tomizuka N (2005b) A cold-active pectin lyase from the psychrophilic and basidiomycetous yeast *Cystofilobasidium capitatum* strain PPY-1. Biotechnol Appl Biochem 42:193–196

Nakagawa T, Ikehata R, Uchino M (2006) Cold-active acid β-galactosidase activity of isolated psychrophilic-basidiomycetous yeast *Guehomyces pullulans*. Microbiol Res 161:75–79

Nampoothiri KM, Baiju TV, Sandhya C, Sabu A, Szakacs G, Pandey A (2004) Process optimization for antifungal chitinase production by *Trichoderma harzianum*. Proc Biochem 39:1583–1590

Nuobariene L, Hansen AS, Jespersen L, Arneborg N (2011) Phytase-active yeasts from grain-based food and beer: phytase-active yeasts. J Appl Microbiol 110:1370–1380

Nuobariene L, Arneborg N, Hansen ÅS (2014) Phytase active yeasts isolated from bakery sourdoughs. In: Proceedings of 9th baltic conference on food science and technology, FOODBALT 2014. Jelgava, Latvia, p 223

O'Lenick AJ Jr, O'Lenick KA (2006) Personal care applications of surfactants based upon alkyl p lyglycosides. US Patent US 7045506 B1

Ogrydziak DM (1993) Yeast extracellular proteases. Crit Rev Biotechnol 13:1–55

Pandey A, Szakacs G, Soccol CR, Rodriguez-Leon JA, Soccol VT (2001) Production, purification and properties of microbial phytases. Biores Technol 77:203–214

Park I, Cho J (2011) The phytase from Antarctic bacterial isolate, *Pseudomonas* sp. JPK1 as a potential tool for animal agriculture to reduce manure phosphorus excretion. Afr J Agric Res 6:1398–1406

Patel RN (2004) Biocatalytic synthesis of chiral pharmaceutical intermediates. Food Technol Biotechnol 42:305–325

Patel S, Goyal A (2011) Functional oligosaccharides: production, properties and applications. World J Microbiol Biotechnol 27:1119–1128

Patil RS, Ghormade V, Deshpande MV (2000) Chitinolytic enzymes: an exploration. Enzym Microb Technol 26:473–483

Patkar SA, Björkling F, Zundel M, Schulein M, Svendsen A, Heldt-Hansen HP, Gormsen E (1993) Purification of two lipases from *Candida antarctica* and their inhibition by various inhibitors. Ind J Chem Sect B 32:76–80

Pavlova K, Angelova G, Savova I, Grigorova D, Kupenov L (2002) Studies of Antarctic yeast for β-glucosidase production. World J Microbiol Biotechnol 18:569–573

Pavlova K, Gargova S, Hristozova T, Tankova Z (2008) Phytase from Antarctic yeast strain *Cryptococcus laurentii* ALn. Folia Microbiol 53:29–34

Pazgier M, Turkiewicz M, Kalinowska H, Bielecki S (2003) The unique cold-adapted extracellular subtilase from psychrophilic yeast *Leucosporidium antarcticum*. J Mol Catal B Enzym 21:39–42

Pedrolli DB, Monteiro AC, Gomes E, Carmona EC (2009) Pectin and pectinases: production, characterization and industrial application of microbial pectinolytic enzymes. Open Biotechnol J 3:9–18

Petrescu I, Lamotte-Brasseur J, Chessa J-P, Ntarima P, Clayessens M, Devreese B, Marino G, Gerday C (2000) Xylanase from the psychrophilic yeast *Cryptococcus adeliae*. Extremophiles 4:137–144

Pulicherla KK, Ghosh M, Kumar PS, Rao KRSS (2011) Psychrozymes – the next generation industrial enzymes. J Mar Sci Res Dev 1:102

Ramli AN, Mahadi NM, Rabu A, Murad AM, Bakar FD, Illias RM (2011) Molecular cloning, expression and biochemical characterisation of a cold-adapted novel recombinant chitinase from *Glaciozyma antarctica* PI12. Microb Cell Fact 10:94

Ranjan K, Sahay S (2013) Identification of phytase producing yeast and optimization and characterization of extracellular phytase from *Candida parapsilosis*. Int J Sci Nat 4:583–590

Ranjan K, Lone MA, Sahay S (2016) Detergent compatible cold-active alkaline amylases from *Clavispora lusitaniae* CB13. J Microbiol Biotechnol Food Sci 5:306–310

Rashid FAA, Rahim RA, Ibrahim D (2010) Identification of lipase-producing psychrophilic yeast, *Leucosporidium* sp. Int J Microbiol 9(1). doi:10.5580/1215

Raspor P, Zupan J (2006) Yeasts in extreme environments. In: Rosa CA, Peter G (eds) Biodiversity and ecophysiology of yeasts. Springer, Heidelberg, pp 371–417

Rather M, Mishra S (2013) β-Glycosidases: an alternative enzyme based method for synthesis of alkyl-glycosides. Sustain Chem Process 1:7

Ray MK, Devi KU, Kumar GS, Shivaji S (1992) Extracellular protease from the Antarctic yeast *Candida humicola*. Appl Environ Microbiol 58:1918–1923

Rossi M, Buzzini P, Cordisco L, Amaretti A, Sala M, Raimondi S, Ponzoni C, Pagnoni UM, Matteuzzi D (2009) Growth, lipid accumulation and fatty acid composition in obligate psychrophilic, facultative psychrophilic, and mesophilic yeasts. FEMS Microbiol Ecol 69:363–372

Russell NJ (1997) Psychrophilic bacteria: molecular adaptations of membrane lipids. Comp Biochem Phys A 118:489–493

Sabri A, Jacques P, Wekkers F, Bare G, Hiligsmann S, Moussaïf M, Thonart DP (2000) Effect of temperature on growth of psychrophilic and psychrotrophic members of *Rhodotorula aurantiaca*. Appl Biochem Biotechnol 84–86:391–399

Sahay H, Babu BK, Singh S, Kaushik R, Saxena AK, Arora DK (2013) Cold-active hydrolases producing bacteria from two different sub-glacial Himalayan lakes. J Basic Microbiol 53:703–714

Saima KM, Roohi M, Ahmad IZ (2013) Isolation of novel chitinolytic bacteria and production optimization of extracellular chitinase. J Gen Eng Biotechnol 11:39–46

Sandhya C, Adapa LK, Nampoothiri KM, Binod P, Szakacs G, Pandey A (2004) Extracellular chitinase production by *Trichoderma harzianum* in submerged fermentation. J Basic Microbiol 44:49–58

Scorzetti G, Petrescu I, Yarrow D, Fell JW (2000) *Cryptococcus adeliensis sp. nov*., a xylanase producing basidiomycetous yeast from Antarctica. Antonie Van Leeuwenhoek 77:153–157

Sharaf EF (2005) A potent chitinolytic activity of *Alternaria alternata* isolated from Egyptian black sand. Polish J Microbiol 54:145–151

Sharma A, Satyanarayana T (2013) Microbial acid-stable α-amylases: characteristics, genetic engineering and applications. Proc Biochem 48:201–211

Sheridan PP, Brenchley JE (2000) Characterization of a salt-tolerant family 42 β-galactosidase from a psychrophilic Antarctic *Planococcus* isolate. Appl Environ Microbiol 66:2438–2444

Shivaji S, Prasad GS (2009) Antarctic yeasts: biodiversity and potential applications. In: Satyanarayana T, Kunze G (eds) Yeast biotechnology: diversity and applications. Springer, Berlin, pp 3–16

Singh P, Singh MV, Tsuji M, Prasad GS, Hoshino T (2014) *Rhodotorula svalbardensis* sp. nov., a novel yeast species isolated from cryoconite holes of Ny-Ålesund, Arctic. Cryobiology 68:122–128

Solano DM, Hoyos P, Hernáiz MJ, Alcántara AR, Sánchez-Montero JM (2012) Industrial biotransformations in the synthesis of building blocks leading to enantiopure drugs. Biores Technol 115:196–207

Song C, Chi Z, Li J, Wang X (2010) β-Galactosidase production by the psychrotolerant yeast *Guehomyces pullulans* 17–1 isolated from sea sediment in Antarctica and lactose hydrolysis. Bioprocess Biosyst Eng 33:1025–1031

Souza CP, Almeida BC, Colwell RR, Rivera IN (2011) The importance of chitin in the marine environment. Mar Biotechnol (NY) 13:823–830

Staley JT, Gosink JJ (1999) Poles apart: biodiversity and biogeography of sea ice bacteria. Annu Rev Microbiol 53:189–215

Steensels J, Snoek T, Meersman E, Picca Nicolino M, Voordeckers K, Verstrepen KJ (2014) Improving industrial yeast strains: exploiting natural and artificial diversity. FEMS Microbiol Rev 38:947–995

Stephen AM (1995) Food polysaccharides and their applications, 2nd edn. CRC Press, Boca Raton, FL

Straathof AJJ, Panke S, Schmidt A (2002) The production of fine chemicals by biotransformations. Curr Opin Biotechnol 13:548–556

Szczesna-Antczak M, Kaminska J, Florczak T, Turkiewicz M (2014) Cold-active yeast lipases: recent issues and future prospects. In: Buzzini P, Margesin R (eds) Cold-adapted yeasts: biodiversity, adaptation strategies and biotechnological significance. Springer, Heidelberg

Tapre AR, Jain RK (2014) Pectinases: enzymes for fruit processing industry. Int Food Res J 21:447–453

Tobe S, Takami T, Ikeda S, Mitsugi K (1976) Production and some enzymatic properties of alkaline proteinase by *Candida lipolytica*. Agric Biol Chem 40:1087–1092

Troncoso E, Barahona S, Carrasco M, Villarreal P, Alcaíno J, Cifuentes V, Baeza M (2017) Identification and characterization of yeasts isolated from the South Shetland Islands and the Antarctic peninsula. Polar Biol 40:649–658

Tsuji M, Yokota Y, Shimohara K, Kudoh S, Hoshino T (2013) An application of wastewater treatment in a cold environment and stable lipase production of Antarctic basidiomycetous yeast *Mrakia blollopis*. PLoS One 8:e59376

Turkiewicz M, Pazgier M, Kalinowska H, Bielecki S (2003) A cold-adapted extracellular serine proteinase of the yeast *Leucosporidium antarcticum*. Extremophiles 7:435–442

Turkiewicz M, Pazgier M, Donachie SP, Kalinowska H (2005) Invertase and α-glucosidase production by the endemic Antarctic marine yeast *Leucosporidium antarcticum*. Polish Polar Res 26:125–136

Tutino ML, Parrilli E, De Santi C, Giuliani M, Marino G, de Pascale D (2010) Cold-adapted esterases and lipases: a biodiversity still under-exploited. Curr Chem Biol 4:74–83

van Zyl WH, Bloom M, Viktor MJ (2012) Engineering yeasts for raw starch conversion. Appl Microbiol Biotechnol 95:1377–1388

Vaquero ME, Barriuso J, Martínez MJ, Prieto A (2016) Properties, structure, and applications of microbial sterol esterases. Appl Microbiol Biotechnol 100:2047–2061

Vishniac HS (2006) Yeast biodiversity in the Antarctic. In: Rosa CA, Peter G (eds) Biodiversity and ecophysiology of yeasts. Springer, Berlin, pp 420–440

Wanderley KJ, Torres FAG, Moraes LÄMP, Ulhoa CJ (2004) Biochemical characterization of α-amylase from the yeast. FEMS Microbiol Lett 231:165–169

Yayanos AA (1995) Microbiology to 10,500 m in the deep sea. Annu Rev Microbiol 49:777–805

Yu P, Wang X-T, Liu J-W (2015) Purification and characterization of a novel cold-adapted phytase from strain JMUY14 isolated from Antarctic: characterization of a novel cold-adapted phytase. J Basic Microbiol 55:1029–1039

Zaliha RN, Salleh AB, Basri M, Mohamad Ali MSB (2012) Cold active enzyme and method thereof. US Patent 2012/0058514 A1

Zhu Y, Pan J, Qiu J, Guan X (2008) Isolation and characterization of a chitinase gene from entomopathogenic fungus *Verticillium lecanii*. Braz J Microbiol 39:314–320

Zimmer C, Platz T, Cadez N, Giffhorn F, Kohring GW (2006) A cold active (2R,3R)-(-)-di-O-benzoyl-tartrate hydrolyzing esterase from *Rhodotorula mucilaginosa*. Appl Microbiol Biotechnol 73:132–140

Methylotrophic Yeasts as Producers of Recombinant Proteins

11

Oleh Stasyk

Abstract

Production of food and feed, industrial proteins, and biopharmaceuticals requires cost-efficient protocols and rationally chosen heterologous production hosts. Among established production platforms, ranging from bacteria to mammalian cells, yeasts combine the advantages of unicellular organisms, such as fast biomass growth and relatively easy genetic manipulations, with eukaryotic features such as correct posttranslational modifications of recombinant proteins and efficient secretory pathway. Simultaneously, unlike more complex eukaryotic organisms, yeast expression systems are more economical, and unlike bacterial hosts, they do not contain pyrogenic or viral inclusions. Yeasts also offer high diversity of metabolic and physiological adaptations that can be exploited in biotechnology, including recombinant protein production. Herein, we overview alternative expression platforms for production of heterologous proteins based on methylotrophic yeasts, mainly *Pichia pastoris* and *Hansenula polymorpha*.

Contents

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11.1 Introduction

Production of recombinant proteins is nowadays a sizable multibillion-dollar market. As natural sources of proteins of interest are often limited or native protein concentration is too low to allow for its efficient extraction, production of such proteins from genetically engineered heterologous hosts provides an alternative. This option became possible with advent of molecular or recombinant DNA techniques, and since the 1980s the number of recombinant proteins expressed and a number of suitable expression hosts have been steadily increasing. Among host organisms, yeasts occupy a special important place because they offer a combination of very useful traits. First of all, yeasts are eukaryotic unicellular microorganisms that share with metazoans such important features as correct protein folding, processing, and co-translational and posttranslational modifications and secretions. This in most cases allows for the synthesis of biologically active protein products. Importantly, yeast cells are well amendable to genetic manipulations owing to availability of a well-developed set of expression vectors, carbon source-regulated gene promoters, and full genome sequences. Additionally, the personalized design of protein production process can be implemented in yeast hosts to improve the product yield. Conversely, yeasts do not exhibit several limitation characteristics of traditional bacterial expression hosts, such as *Escherichia coli* or *Bacillus subtitis*, namely, contamination with pyrogenic compounds or sensitivity to viral infection (overviewed in Kim et al. 2015; Meehl and Stadheim 2014; Corchero et al. 2013; Mattanovich et al. 2012; Celik and Calik 2012).

As a rule, yeasts offer a set of characteristic that make them the host organisms of choice for production of particular groups of proteins. For instance, monoclonal antibodies (mAbs) and antibody fragments are today one of the most important biopharmaceutical products. Despite the ability of mammalian cells to N-glycosylate full length antibodies, they have important limitations with regard to bioprocessing and scaling-up, what leads to elevated production costs. However, antibody fragments that exhibit antigen-binding properties can be efficiently

produced in yeasts, taking into account also the availability of yeast hosts with humanized glycosylation patterns (Spadiut et al. 2014). Some features of particular yeast species make them specifically useful, for instance, for production of difficult to overexpress recombinant membrane proteins for structural studies or required for food and feed production, lignocellulosic enzymes, or biopharmaceuticals like recombinant human lysosomal enzymes (Emmerstorfer et al. 2014; Bill 2015; Ergün and Çalık 2016; Mellitzer et al. 2012; Spohner et al. 2015).

The design of the optimal expression system for a given recombinant protein involves several steps among which selecting the appropriate host organism and strain is the most crucial. For instance, when proper folding and posttranslational modifications are required for physiological activity of a protein product, expression host must be chosen among eukaryotic organisms. This is accompanied and followed with selection of the following:

1. A suitable vector (integrative or episomal) with an appropriate promoter (repressible/inducible or constitutive) and selectable marker (dominant antibiotic resistance or auxotrophic) that allows single or multicopy integration of the expression cassette
2. Deciding whether there will be necessary codon optimization of the recombinant gene
3. Fusion of the gene to an epitope tag necessary for more efficient affinity purification
4. Fusion to the signal sequence to target the protein product to a specific intracellular compartment (e.g., peroxisomes) or provide secretion into extracellular medium

Expression host optimization may also involve the possibility to introduce further genetic alterations, such as gene deletion of vacuolar proteases to prevent the proteolytic cleavage of the product, or manipulation with genes that facilitate secretion, such as co-expression of chaperones. Optimization of an expression system, depending on the chosen host, may further involve designing the optimal fermentation mode and medium (as manipulation with carbon and nitrogen sources), optimizing induction conditions, and incubating parameters (temperature, pH, aeration conditions, etc.).

Of note, no single yeast expression system can provide all of the desired properties for a given recombinant protein production. This is especially true when chemical and physical parameters and the chosen feeding strategy may critically influence the yield of the product or its functional characteristics.

11.2 Overview of Yeast Host Species Commonly Used for Recombinant Protein Production

Saccharomyces cerevisiae, a traditional baker's yeast, is the first and best characterized yeast expression system, developed yet in the 1980s. It was the first eukaryote for which full genome sequence was established and has been used as a major host for the production of recombinant proteins. However, its limitations as production host, such as hyperglycosylation of proteins and glycosylation terminated via α-1,3-linked mannose residues, which are considered to be allergenic, low protein yields, along with cases of plasmid instability, soon became apparent and have limited the number of marketed protein products from *S. cerevisiae* (Gellissen et al. 2005a; Darby et al. 2012). Current industrial popularity of *S. cerevisiae* continues mostly due to the accumulated in-depth knowledge on its physiology, genetics, and fermentation techniques and the fact that it has been generally recognized as safe (GRAS) species. Also, *S. cerevisiae* is possibly the organism with the most comprehensive experimental dataset available to date (Petranovic et al. 2010).

Nevertheless, in the last years, the number of published reports on utilization of alternative yeasts for recombinant protein production exceeds that of *S. cerevisiae*. Also, in the few systematic comparisons between different yeast production systems, clear advantages of the so-called nonconventional species over *S. cerevisiae*, especially for the production of secreted proteins, have been found (Mack et al. 2009; Dragosits et al. 2011; Darby et al. 2012). For instance, the production of the anticoagulant peptide hirudin in different expression systems demonstrated the significantly higher comparative productivities achieved with *P. pastoris* and *H. polymorpha* (Demain and Vaishnav 2009). Therefore, the folding and secretion pathways are the major sources of advantages of alternative yeasts, since strong and regulatable promoters and other genetic tools are readily available for these expression systems (Mattanovich et al. 2012).

Several nonconventional yeast species including methylotrophs *Hansenula polymorpha* and *Pichia pastoris*, two dimorphic yeast *Arxula adeninivorans* and *Yarrowia lipolytica*, the budding yeast *Kluyveromyces lactis*, and the fission yeast *Schizosaccharomyces pombe* have been proposed and used to a different extent as recombinant protein expression platforms.

In this review, we focus on characterization of the alternative methylotrophic yeast species as hosts for recombinant protein production. In contrast to *S. cerevisiae*, they belong to the group of so-called Crabtree-negative yeasts which do not ferment and produce ethanol under fully aerobic conditions. This enables these yeasts to grow to very high cell densities in bioreactor cultures that result in turn in higher product yields (Cereghino and Cregg 2000). Methylotrophic yeasts are characterized by a unique enzymatic machinery involved in utilization of toxic methanol. Many enzymes, especially those of peroxisomal nature, are highly overexpressed and strictly regulated by carbon sources and have been used, therefore, to drive heterologous protein production (Fig. 11.1). For comparative characterization of non-methylotrophic alternative yeast expression platforms, readers are

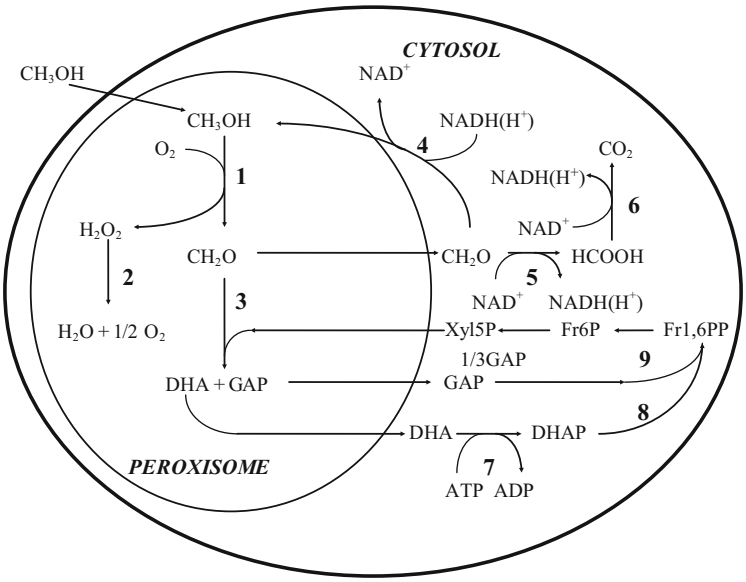

Fig. 11.1 Simplified schematic representation of the metabolic pathways of methanol oxidation and assimilation in the methylotrophic yeast cell. Main enzymes are numbered, and names of the corresponding gene promoters previously used to drive recombinant protein expression in different methylotrophic yeasts are listed in the legend : *1* alcohol (methanol) oxidase (*H. polymorpha* MOX, *P. pastoris* AOX1, *P. methanolica* MOD1/AUG1, *C. boidinii* AOD1); *2* catalase; *3* dihydroxyacetone synthase; *4* formaldehyde reductase; *5* formaldehyde dehydrogenase (*H. polymorpha* FLD, *P. pastoris* FLD1); *6* formate dehydrogenase (*H. polymorpha* FMD); *7* dihydroxyacetone kinase; *8* fructose-1,6-diphosphate aldolase; *9* fructose-1,6-diphosphatase. Abbreviations for intermediates: *GAP* glyceraldehyde, *DHAP* dihydroxyacetone (phosphate), *Fr1,6PP* fructose-1,6-diphosphate, *Fr6P* fructose-6-phosphate, *Xyl5P* xylulose-5-phosphate

referred to recent reviews on the subject (Gellissen et al. 2005b; Celik and Calık 2012; Corchero et al. 2013).

11.3 *Pichia (Komagataella) pastoris*

Of note, *Pichia pastoris* has been reclassified (Yamada et al. 1995), and the new species names such as *Komagataella pastoris* or *Komagataella phaffii* can be found in the recent literature (for further details, please see Kurtzman 2009; Sturmberger et al. 2016).

Since the unsuccessful introduction by Phillips Petroleum more than 40 years ago for the commercial production of single-cell protein (SCP), the methylotrophic yeast *P. pastoris* has become nowadays the most frequently used yeast host for heterologous protein production. This yeast is distinguished for its growth to very high cell densities, availability of strong and tightly regulated gene promoters, very efficient protein secretion, and ability to produce recombinant proteins in amounts

of gram per liter of culture (for reviews, see Cereghino and Cregg 2000; Daly and Hearn 2005; Gasser et al. 2013; Ahmad et al. 2014; Spohner et al. 2015). In basic research, *P. pastoris* has been a very popular yeast model in research on molecular mechanisms of peroxisome biogenesis and degradation (Dunn et al. 2005).

P. pastoris is a homothallic haploid yeast species able of mating, forming diploid cells, and producing ascospores under nitrogen limitation. Usually haploid auxotrophic strains or those harboring antibiotic resistance complementary markers that allow for selective growth of resulting diploid cells are used in genetic crossings in strain construction (Cregg et al. 1985, 2009).

As a methylotrophic organism, *P. pastoris* can use single-carbon compound methanol as its sole carbon and energy source in the absence of a repressing carbon source, such as glucose. Since *P. pastoris* is an obligate aerobe and prefers a respiratory mode of growth, fermentation products such as ethanol and acetic acid do not build up in the culture enabling them to reach very high cell densities of up to 200 g L^{-1} dry weight (Cereghino et al. 2002). Simultaneously, *P. pastoris* is the efficient secreting host for recombinant proteins because few of its own proteins are found in extracellular medium, which allows simple purification of the secreted products. Another advantage of *P. pastoris* is its less extensive hypermannosylation as compared to *S. cerevisiae* and the absence of α-1,3-linked mannosyltransferase, which produces highly immunogenic terminal α-1,3-linked mannosyl linkages in *S. cerevisiae* (Gemmill and Trimble 1999). Of note, yeasts glycosylate recombinant protein products adding the core (Man)8-(GlcNAc)2- groups, thus performing only some steps common in metazoans.

11.4 Toolbox of *P. pastoris* Expression Platform

11.4.1 Expression Strains

All *P. pastoris* expression strains are derivative of the wild-type strains NRRL-Y 11430 from the Agriculture Research Service Culture Collection (Peoria IL, USA) and NRRL Y-48124 (X-33, Invitrogen expression kit strains, Carlsbad CA, USA) (Kurtzman 2009) and are now identified as *Komagataella phaffii* (Sturmberger et al. 2016). In particular, GS115 his4 auxotrophic mutant lacking the functional gene encoding the histidinol dehydrogenase (Cereghino and Cregg 2000), KM71 Mut S strain that exhibits slow growth on methanol (see below), and protease-deficient strains (e.g., SMD1163, SMD1165, SMD1168) are commonly used as classical hosts for heterologous protein expression. The SMD1163 strain (his4, pep4, prb1) lacks both proteinase A which activates other endogenous *P. pastoris* proteases (pep4) and proteinase B (prb1) activities, whereas SMD1165 and SMD1168 are deficient in prb1 and pep4, respectively. Therefore, strain SMD1163 has been demonstrated to be the most efficient as a host for synthesis of recombinant proteins sensitive to proteolytic degradation (Cereghino and Cregg 2000). Depending on the protein of interest and further predicted modifications of the protein production process, all abovementioned strains provide certain useful features.

11.4.2 *P. pastoris* Vectors

The standard *P. pastoris* vectors used in biotechnological applications are shuttle vectors which can be replicated in *E. coli* and maintained in *P. pastoris* using a selectable marker, such as auxotrophic genetic markers (e.g., *HIS4*, *ARG4*, *MET2*, *ADE1*, *URA3*). A set of plasmids for intracellular and secretory protein expression in *P. pastoris* based on different auxotrophic genetic markers has been developed (Lin Cereghino et al. 2001). Selection in *P. pastoris* can also be provided by the heterologous genes conferring resistance to drugs, e.g., Geneticin (G418) and Zeocin.

The distinctive feature of *P. pastoris* expression system [patented by Research Corporation Technologies (Tucson, AZ, USA)] is that the basic expression kit with standard vector systems for intracellular and secretory expression is commercially available for research use from Invitrogen Corporation (Carlsbad, CA, USA). It also provides *P. pastoris* expression vectors and strains for humanized glycosylation of target proteins (Vogl and Glieder 2013) and can be purchased from BioGrammatics (Carlsbad, CA, USA).

Of note, unlike in *S. cerevisiae*, where homologous recombination predominates, in *P. pastoris* nonhomologous recombination usually occurs upon transformation with a linearized vector. Also, homologous recombination in *P. pastoris* requires elongated homologous regions flanking heterologous DNA in the expression cassettes (Cereghino and Cregg 2000). Several different transformation methods and protocols can be applied in *P. pastoris* to integrate vector DNA into its genome. The most commonly used transformation method is electroporation, yielding up to 10^5 transformants/μg of DNA (Cregg and Russell 1998). For other up-to-date tools of *P. pastoris* expression system, readers are referred to recent comprehensive review on the subject by Gasser et al. (2013).

11.4.3 Gene Promoters for Governing Recombinant Protein Expression

Promoter of the gene of alcohol oxidase, *AOX1* gene, encoding a key peroxisomal enzyme of methanol utilization pathway is known as one of the strongest and tightly regulated yeast promoters. The classical *P. pastoris* expression system has been developed based on this promoter (Cregg et al. 1985). The *AOX1* promoter is repressed in the presence of glucose or glycerol but strongly upregulated in the presence of methanol, making it tightly controlled by carbon source expression system. The advantage of catabolite regulation of the *AOX1* promoter, especially important in case a toxic recombinant product is to be expressed, is that the high biomass growth can be achieved under repressing conditions and afterward the expression can be induced. The transcriptional regulation of this promoter has been extensively studied, and cis-acting elements and transacting factors were characterized (Hartner and Glieder 2006). A wide range of proteins have been expressed from control of this promoter, with claimed yields of recombinant protein

up to 20–30 g L^{-1} (Spohner et al. 2015). Of note, one of the recent studies suggested that protein expression from under *AOX1* promoter may occur during the preinduction phase in cultures grown in bioreactors contrary to shake flask cultures, tentatively indicating that the corresponding promoter may be leaky under certain conditions (Bawa et al. 2014).

However, methanol, the *AOX1* promoter inducer, is a potential fire hazard and a toxic compound, which may pose risks under large-scale fermentation conditions. Therefore, methanol non-utilizing strains have been constructed that utilize alternative carbon sources for growth but can be also cultivated on a controlled methanol concentration at a low growth rate or under mixed feed protocols (Cereghino et al. 2002). *P. pastoris* harbors in its genome two genes encoding for peroxisomal alcohol oxidase, major *AOX1* and auxiliary *AOX2*. Therefore, depending on these genes' status, there are three phenotypes for *P. pastoris*, with respect to methanol utilization: Mut+ (wild-type or intact methanol utilization), MutS (slow methanol utilization, disrupted *AOX1*, intact *AOX2*), and Mut− (deficient methanol utilization, both *AOX1* and AOX2 disrupted) (Cereghino and Cregg 2000).

Besides, new alternative promoters have also been identified in *P. pastoris* and characterized with regard to their feasibility for protein production. These include several constitutive promoters from housekeeping genes (e.g., *GAP1*, *TEF1*, *PGK1*, *TPI1*) and regulatable (inducible) promoters from particular biochemical pathways (e.g., *FLD1*, *PHO89*, *THI11*, *AOD*) (Prielhofer et al. 2013). Interestingly, application of the *P. pastoris FLD1* promoter of the formaldehyde dehydrogenase gene allows induction of recombinant protein expression with methylamine instead of methanol and repression by glucose (Resina et al. 2005).

GAP promoter provides high-level constitutive expression of foreign proteins on glycerol and glucose-containing media. This feature is particularly useful for protein production in large-scale fermenters, since it does not require flammable and therefore hazardous methanol in the process. In some cases, a constitutive expression from under *GAP* promoter yields even higher protein levels relative to that controlled by the *AOX1* promoter (Waterham et al. 1997). Therefore, when recombinant proteins are not toxic for the yeast host, as in the case of most enzymes of food industry, constitutive promoters are applicable and are even favorable because they support more productive continuous modes of fermentation.

For a detailed and comprehensive description of the available *P. pastoris* promoters, the readers are referred to the review by Vogl and Glieder (2013). Several *P. pastoris* transcriptional factors have been identified and studied with respect to their control of host physiology and fermentation-relevant traits. These include Hac1 involved in the control of unfolded protein response (UPR), Fep1 in iron uptake, and Yap1 in oxidative stress response (see Gasser et al. 2013; Puxbaum et al. 2015; and references thereof). Understanding of these transcriptional regulatory networks together with synthetic biology and metabolic engineering approaches forms a solid basis that enables the design of tailor-made hosts for production of recombinant proteins.

11.4.4 Other Tools

Genes of interest are often codon and GC content optimized for expression in *P. pastoris*. Since the comparison with the non-codon optimized gene is not usually reported, what difference this makes to the yield of a given protein is not clear. However, in the case of membrane proteins, as demonstrated in one of the studies with mouse P-glycoprotein, expression from a codon-optimized *P. pastoris* gene not only yielded substantially more protein than expression from the wild-type gene but also concomitantly correlated with better product stability (Bai et al. 2011). A positive effect of codon optimization in *P. pastoris* has been also reported for endochitinase (Yu et al. 2013), inulinase (He et al. 2014), keratinase (Hu et al. 2013), lignocellulolytic enzymes (Mellitzer et al. 2012), and some other enzymes.

The *P. pastoris* vectors usually integrate into the host cell genome producing stably expressing clones. The number of copies [up to 30 in *P. pastoris* (Cregg et al. 2009)] which are integrated cannot be precisely controlled, so the optimal clone must be experimentally identified. Copy number can be roughly estimated via colony screening in the presence of increasing concentrations of antibiotic when a corresponding resistance gene is included in the vector as a selectable marker or by other methods, such as real-time PCR. It has to be stressed that the higher copy numbers of the integrated vector do not necessarily correlate with higher expression of protein product, so it is usually necessary to follow copy number analysis with the direct assessment of protein expression levels. The main approaches of how to achieve high-expression cassette copy numbers are summarized in Gasser et al. (2013).

Although untagged recombinant proteins have been successfully isolated from *P. pastoris* based on their individual properties, it is more usual to produce tagged versions of the target proteins to facilitate both their detection and isolation. A range of different tags is compatible with expression in *P. pastoris*. The vast majority of recombinant proteins can and have been expressed as His-tagged constructs to allow one-step purification by affinity chromatography. Besides, FLAG tags have also been used both for protein analysis and their isolation. GFP tags can be incorporated on expression vectors to assist in protein visualization. A number of different protease-cleavage sites have also been employed to successfully remove tags after product purification if required.

For efficient secretion, *P. pastoris* expression vectors can incorporate the *S. cerevisiae* alpha-mating factor (α-MF) pre-pro leader sequence signal upstream of the heterologous gene of interest. The alpha-mating factor should be cleaved following expression by the native Kex2 protease. Recently a novel *P. pastoris* strain with increased Kex2 copy number which alters Kex2 endogenous level and increases production of a secreted protein has been described (Yang et al. 2013). Recently, some modifications of the MFα pre-pro leader to further enhance secretion in *P. pastoris* have been proposed (Lin-Cereghino et al. 2013).

Better understanding of the *P. pastoris* genetics, promoter regulation, and physiology was greatly facilitated after full genome sequences have become available (Küberl et al. 2011). Readers are referred to a recent publication of

Sturmberger et al. (2016) on *P. pastoris* genome for more up-to-date details. Genome Browser database of *P. pastoris* genome sequences is publicly available (Sturmberger et al. 2016). Also, new achievements in strain improvement were recently reported. These concerned intracellular trafficking, protein folding, glycosylation, and proteolytic degradation (Puxbaum et al. 2015; Kim et al. 2004; Yu et al. 2015).

An expanding toolbox of genetic elements and *P. pastoris* strains for the improvement of recombinant protein production is being generated, including novel promoters, gene copy number-enhancing vectors, and host strains adapted for efficient secretion. One of the recent reviews highlights established tools for protein expression in *P. pastoris* and novel developments in expression vector design, host strain engineering, and screening for high-level expression strains (Gasser et al. 2013).

11.4.5 Industrial, Pharmaceutical, and Other Applications

In the last several years, a number of products based on *P. pastoris* platform got approval as biopharmaceuticals [for review, see Meehl and Stadheim (2014)]. Several products from *P. pastoris* like human serum albumin, insulin, interferon alpha, and hepatitis B vaccine are marketed in India and/or Japan (Shekhar 2008). The first therapeutic polypeptide, ecallantide, expressed in *P. pastoris* and used for the treatment of hereditary angioedema received approval from FDA in 2009 (Thompson 2010) and is produced by Dyax Corporation (Cambridge, MA). Successful genetic engineering to humanize N-glycosylation *in P. pastoris* has led to further improvement of this host as producer of pharmaceutical proteins dependent on glycosylation pattern for their activity (Puxbaum et al. 2015 and references thereof). This permitted efficient production of fully active human erythropoietin in *P. pastoris* (Hamilton et al. 2006). These features made *P. pastoris* a remarkably cost-efficient expression platform. The most important event for the usage of the *P. pastoris* expression system in food technology was receiving the generally recognized as safe (GRAS) status from the Food and Drug Administration (FDA) and the FDA approval of recombinant proteins (Ciofalo et al. 2006; Thompson 2010).

One important feature of the *P. pastoris* system is that it is highly suitable for large-scale growth and cultivation in bioreactors (Cereghino et al. 2002). Bioreactors provide precise control over culture parameters including temperature, pH, and feed rate as well as allow real-time monitoring of changes in cell optical density and dissolved oxygen. This allows that ultrahigh cell densities (>100 g/L dry cell weight, >500 OD600 units/mL) can be achieved (Cereghino et al. 2002). However, usually medium cell density cultures are utilized in bioreactors since this usually reduces cellular stress and proteolysis associated with high-density cultures (Mattanovich et al. 2004). This feature combined with inexpensive growth media makes *P. pastoris* a highly cost-effective expression system.

Depending on which promoter is used, different fermentation techniques are applied in *P. pastoris*. The standard fermentation procedure for the expression system based on the *AOX1* promoter is a three-stage fed-batch fermentation (Zhang et al. 2000). Usually the first stage, so-called growth phase, consists of batch fermentation with glycerol as a carbon source to get initial sufficient cell biomass. The second "transition" stage starts right after all glycerol is exhausted and is a fed-batch phase aimed at production of more biomass and at metabolic transition from the glycerol to methanol metabolism. In this phase, sole glycerol feed or a mixed feed of glycerol and methanol, where glycerol feed progressively decreases and methanol feed progressively increases, can be applied. For the "production phase," different feeding profiles can be applied, such as constant oxygen feeding, constant methanol concentration feeding, constant specific growth rate feeding, oxygen-limited fed batch, and temperature-limited fed batch (Potvin et al. 2012).

The fermentation strategy for recombinant protein production under control of constitutive *GAP* promoter differs significantly from the ones described above. In this case, no separate transition and production phases nor methanol induction is necessary, and the amount of produced protein depends solely on the cell mass. Therefore, the fed-batch and the continuous fermentation are the modes of choice for GAP promoter. An example for an efficient large-scale fed-batch protocol using the *GAP* promoter is the production of lipase from *Candida rugosa*, generating up to 500 g/L wet cell weight and stable lipase activity of 14,000 U/mL (Zhao et al. 2008).

For more details on fermentation process optimization and vector design for recombinant protein production in *P. pastoris*, readers are referred to recent specialized reviews on the subjects (Looser et al. 2015; Celik and Calık 2012; Potvin et al. 2012). For review on the industrial expression of food and feed enzymes in *Pichia pastoris*, see Spohner et al. (2015).

P. pastoris has been particularly successful as an expression platform for various eukaryotic membrane proteins, especially those required for structural biology studies. One of the key issues associated with such proteins is the difficulty in expressing the large amounts of the product. This is due to the fact that eukaryotic membrane proteins require specific co- and posttranslational processing and membrane lipid composition, meaning that bacterial expression systems are as a rule not suitable for their production. Although the higher eukaryotic cell-based systems (e.g., based on insect cells) have a good track record for production of membrane proteins, they are much more expensive and time-consuming as compared to microbial hosts. The yeast expression systems combine the advantages of low production costs with eukaryotic modifications and ease of manipulation. Although the bakers yeast *S. cerevisiae* has a good track record in production of membrane proteins for structural studies, much better success has been achieved with membrane proteins heterologously produced in *P. pastoris*. The recent reviews on this subject summarize successful examples of eukaryotic membrane proteins produced for structural studies in *P. pastoris* (Routledge et al. 2016; Byrne 2015; Emmerstorfer et al. 2014).

Several genome-scale metabolic models *for P. pastoris* have been created and recently updated a consensus model (Tomàs-Gamisans et al. 2016). This will provide a new efficient tool for manipulations of *P. pastoris* metabolic networks, constructing the new generation of host strains and in silico simulations for optimizing fermentation protocols for more efficient recombinant protein production (Nocon et al. 2014). Other applications of yeast synthetic biology for the production of recombinant therapeutic proteins are summarized in the review of Kim et al. (2015).

11.4.6 Expression System's Limitations

Not every protein of interest is produced, especially secreted, in *P. pastoris* at high titers. For complex proteins which are membrane bound, hetero-oligomers, or prone to proteolysis, yields are often lower than usually achieved. Protein folding, glycosylation, and secretion efficiency have been identified among major bottlenecks and limiting factors for this host, pinpointing major targets for strain optimization.

It is known that *P. pastoris* can hyperglycosylate recombinant products although to a lesser extent than what is observed in *S. cerevisiae*. Comparative analysis of 25 non-glycosylated recombinant G-protein-coupled receptors (GPCRs) expressed in *P. pastoris* revealed that loss of glycosylation resulted in many cases in reduced functional expression as compared to a glycosylated counterpart (Yurugi-Kobayashi et al. 2009). The data indicated that the effects of deviant glycosylation need to be assessed for each individual heterologous protein.

For the comprehensive recent review on the subject of the challenges of recombinant protein folding, glycosylation, and secretion in *P. pastoris* expression platform, see Puxbaum et al. (2015).

11.5 *Hansenula (Ogataea) polymorpha*

(Of note, *Hansenula polymorpha* has recently been reclassified, and new species name synonyms such as *Pichia angusta* or *Ogataea polymorpha* can be found in the recent literature (for further details, please see Yamada et al. 1994, 1995; Kurtzman 2009).)

H. polymorpha is another methylotrophic yeast successfully utilized as an expression platform for recombinant proteins. Peculiarity of this species is that it is a remarkably thermotolerant yeast able to grow up to 45° C and even higher and is resistant to heavy metals and oxidative stress (Gellissen 2002). Therefore, it is well suited for the production of thermostable enzymes and proteins. Another peculiarity is that it harbors a nitrate assimilation pathway introgressed from bacteria that is not present in other yeasts, including methylotrophs (Morales and Dujon 2012; Morales et al. 2013). *H. polymorpha* expression system is also known for remarkable stability of strains-producers, a feature very important for large-scale production (Kang and Gellissen 2004). The presence of excessive

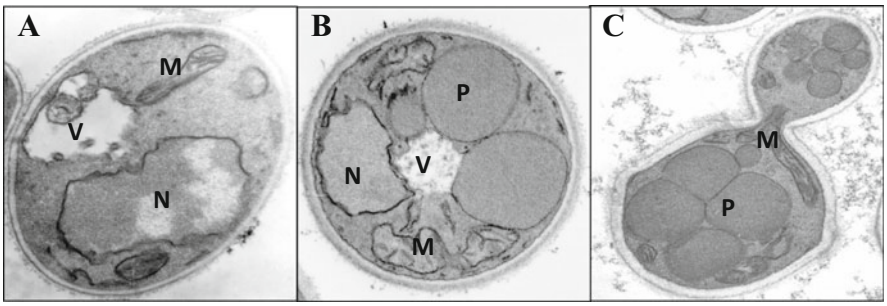

Fig. 11.2 Induction of peroxisomes in the cells of *H. polymorpha* (**a**) grown in glucose-supplemented batch culture, (**b**) grown in methanol medium in batch culture, (**c**) in chemostat culture on methanol to maximally induce alcohol oxidase (MOX) promoter. Big crystalloids of alcohol oxidase protein tightly packed in peroxisomes and the intracellular volume they occupy inform the strength of the MOX promoter. Abbreviations: *N* nucleus, *M* mitochondrion, *P* peroxisome, *V* vacuole [Photo "C" adapted from Gellissen et al. (2005b)]

peroxisomal membrane in *H. polymorpha* provides a convenient site for targeting heterologous membrane proteins when fused with an appropriate signal peptide of native peroxins (Leão and Kiel 2003) (Fig. 11.2). Similarly to *P. pastoris*, *H. polymorpha* is a Crabtree-negative yeast allowing for high dilution rates and high biomass yields in bioreactors. *H. polymorpha* efficiently secretes foreign proteins and exhibits a lesser extent of hypermannosylation and no terminal immunogenic α-1,3-linked mannose residues considered to be allergenic as compared to *S. cerevisiae* (Kang et al. 1998; Gellissen 2000; Kim et al. 2004). Several recombinant products derived from *H. polymorpha* expression platform have reached the market, including the hepatitis B vaccine Hepavax-Gene (Crucell, NL), insulin, and interferon alpha-2 (Rhein-Biotech, DE). In addition, *H. polymorpha* has received a GRAS status as producer of food enzymes (Gellissen et al. 2005a).

For additional information on *H. polymorpha* expression system, see Mattanovich et al. (2012), Gellissen et al. (2005b), Gellissen (2000), and Sudbery et al. (1988).

11.6 Toolbox of *H. polymorpha* Expression Platform

11.6.1 Expression Strains

There are three main strains of *H. polymorpha* known that have independent origins. For biotechnological applications, mostly strains derivative of CBS4732 and DL-1 are used, whereas strain NCYC495 is used mainly in fundamental studies. Recently DL-1 strain has been reclassified as *Hansenula (Ogataea) polymorpha* sister species—*Ogataea parapolymorpha* (Ravin et al. 2013; Kurtzman et al. 2011). All *H. polymorpha* isolates are homothallic, and reproduction occurs vegetatively by

budding. Methods of classic and molecular genetics are well developed for *H. polymorpha* (Gellissen 2002; Kang and Gellissen 2004).

11.6.2 *H. polymorpha* Vectors

There are many variants of *H. polymorpha* vectors that provide either replicative or integrative expression of heterologous genes in the host (Mattanovich et al. 2012). Among auxotrophic marker genes, the native and heterologous *LEU2* and *URA3* are the most frequently used. Recently, we successfully applied a *P. pastoris* heterologous gene marker *ADE1* for isolation of multicopy transformants and *H. polymorpha* native *FLD1* gene under shortened GAP promoter as a novel selectable marker in *H. polymorpha* (Krasovska et al. 2007, 2013). A wide-range yeast vector system (CoMedTM) has been constructed that provides a versatile transformation/expression tool for a range of yeasts, namely, *H. polymorpha*, *S. cerevisiae*, *P. pastoris*, *Arxula adeninivorans*, and *Kluyveromyces lactis*. In its basic form, the vector is composed of genetic modules that are functional in all mentioned yeasts, namely, a rDNA targeting sequence, an appropriate selection marker, and an expression cassette under control of a *TEF1* promoter from various sources (Steinborn et al. 2006).

Efficient strategies for gene replacements and marker recycling and introducing multiple heterologous genes are essential for a developed expression platforms. In addition to the counterselection strategy using the *URA3* marker that has been conventionally applied in yeasts, more advanced approaches such as the Cre/loxP and Flp/FRT recombinase-based systems have been also developed. These systems act via active excision of the marker gene fragments to recycle markers to allow for sequential rounds of deletions in several yeast species, including *H. polymorpha* (Qian et al. 2009).

Of note, the frequency of homologous versus nonhomologous recombination upon integration of foreign DNA fragments into *H. polymorpha* genome is much lower than in *S. crevisiae* (Kang and Gellissen 2004). It was shown in several yeasts, including *H. polymorpha*, that after deletion of KU70 or KU80 homologues involved in nonhomologous end joining, the frequency of homologous recombination was significantly enhanced (Kim et al. 2015).

Positive selection markers such as Geneticin- or Zeocin-resistant genes are routinely applied in *H. polymorpha* for obtaining multicopy transformants with different vectors harboring expression cassettes. However, it has become apparent that there is not always a linear correlation between expression cassette copy number and resulting protein expression level, particularly for the secretory recombinant proteins (Aw and Polizzi 2013). For example, a study involving secretory production of human serum albumin in *H. polymorpha* showed that the effect of gene dosage on expression was abolished, and a single copy of the optimized expression cassette integrated into the MOX locus was sufficient for maximal expression of human serum albumin (Kang et al. 2001).

11.6.3 Gene Promoters for Governing Recombinant Protein Expression

The *H. polymorpha* expression platform is based on strong, strictly regulated promoters of the genes of methanol utilization pathway, encoding for methanol oxidase (MOX) and formate dehydrogenase (FMD) (for a comprehensive review, see Hartner and Glieder 2006). Unlike the *P. pastoris AOX1* promoter-based expression system, which strictly requires methanol for induction, the *H. polymorpha MOX* promoter is highly derepressed under glucose exhaustion, thus supporting a methanol-free fermentation process. *H. polymorpha* is rather exceptional hosts among methylotrophic yeasts as its *FMD* promoter is more frequently employed for governing recombinant protein production (Kang and Gellissen 2004). For more details on the regulation of promoters of methanol utilization pathway in this yeast, see the review of Hartner and Glieder (2006). Besides methylotrophic promoters, native and heterologous constitutive promoters of glyceraldehyde-3-phosphate dehydrogenase (*GAP*) are used in *H. polymorpha* (Kang and Gellissen 2004).

We described the mutations in *H. polymorpha* putative glucose transporter with sensing function Gcr1 that affects catabolite repression of *MOX* promoter in *H. polymorpha* (Stasyk et al. 2004). The gcr1 mutants exhibited defects in MOX promoter regulation only by hexoses and xylose, but not by disaccharides or ethanol. With derivative gcr1 mutant strains as hosts, we developed a modified two-carbon source mode expression platform that utilizes convenient sugar substrates for growth (sucrose) and induction of recombinant protein expression (glucose or xylose). Efficient regulatable by sugar carbon sources expression of three recombinant proteins: a secreted glucose oxidase from the fungus *Aspergillus niger*, a secreted miniproinsulin, and an intracellular hepatitis B virus surface antigen was demonstrated in these mutant hosts (Krasovska et al. 2007, 2013). Thus, this modified expression platform preserved the favorable regulatable nature of MOX promoter without addition of methanol, making a convenient alternative to the traditional system.

11.6.4 Other Tools

H. polymorpha full genome sequence has been established and is available online for the strain NCYC495 at http://genome.jgi.doe.gov/Hanpo1/Hanpo1.home.html, which allows efficient screening for gene homologues and application of different system biology approaches with this species.

It was recently found that *H. polymorpha* differs from *S. cerevisiae* or *P. pastoris* in that it has a unique codon usage frequency. Even codon-optimized genes that are expressed well in *S. cerevisiae* and *P. pastoris* may be inefficiently expressed in *H. polymorpha*; thus, rare codons must be avoided when universal optimized gene versions are designed for expression in host panel that includes *H. polymorpha* (Liu et al. 2014).

In addition, similar to *P. pastoris*, initial studies in *H. polymorpha* have been conducted for host strain engineering to perform humanized recombinant protein glycosylation (Oh et al. 2008; Kim et al. 2004). These glycoengineering studies have yielded glycoproteins in *H. polymorpha* mainly containing trimannosyl core N-glycan (Man3GlcNAc2) (Oh et al. 2008). Also, an alternative strategy for reconstructing the biosynthetic pathway of initial lipid-linked oligosaccharide synthesis was developed based on deletion of the *H. polymorpha ALG3* gene coding for dolichyl-phosphate-mannose (Dol-P-Man)-dependent α-1,3 mannosyltransferase (Cheon et al. 2012).

It is known that correct protein folding in the yeast ER is very important as it determines whether the protein enters the recombinant secretory pathway or whether it is targeted for ER-associated degradation (ERAD), thus affecting the recombinant protein production. It was reported that an increase of calnexin gene dosage which controls folding and quality control of glycoproteins boosts the secretion of recombinant proteins in *H. polymorpha* (Klabunde et al. 2007). Recently, an improved processing of secretory proteins in *H. polymorpha* by sequence variation near the processing site of the alpha-mating factor pre-pro sequence was reported (Eilert et al. 2013).

H. polymorpha, similarly to *P. pastoris*, can reach high cell densities and efficiently secretes recombinant proteins, as exemplified by phytase production with the yield of 13.5 g L^{-1} (Mayer et al. 1999). Fermentation process development in *H. polymorpha* was recently reviewed by Stockmann et al. (2009) and Celik and Calik (2012). Similarly to *P. pastoris*, a defined minimal mineral medium composed of salts, vitamins, and trace elements is used to support growth. Based on the selected promoter (*FMD* or *MOX*), fermentation strategies are developed using either glucose or glycerol in the beginning of fermentation, followed by carbon source limitation in the second phase (Stockmann et al. 2009). Of note, one interesting example of comparative analysis of rotavirus VP6 production has been recently reported where *H. polymorpha* outperformed both *P. pastoris* and *E. coli* during bioreactor cultivation but not in shake flask cultures (Bredell et al. 2016).

11.6.5 Industrial, Pharmaceutical, and Other Applications

Although not that popular as *P. pastoris* has been, *H. polymorpha* was exploited for the production of a number of recombinant proteins, e.g., enzymes (glucose oxidase) (Hodgkins et al. 1993; Krasovska et al. 2007), phytase (Mayer et al. 1999), glycolate oxidase and catalase (Gellissen et al. 1996), isopenicillin-N synthase (Gidijala et al. 2008), novel fibrinolytic recombinant staphylokinase (Moussa et al. 2012), pharmaceuticals: IFNα-2a (Müller et al. 2002), interleukin-6 (Böer et al. 2007), and recombinant human serum albumin (Heo et al. 2008).

As mentioned, the recombinant hepatitis B vaccine produced in *H. polymorpha* (Seo et al. 2008) has been commercialized. The *H. polymorpha*-produced hepatitis B virus S antigen (HbsAg) was found to be assembled into yeast-derived lipid membranes. Other studies have indicated that this lipoprotein particle structure is

essential for the antigenicity of the HBsAg (Petre et al. 1992). The methanol induction, which is routinely used for high-level production of recombinant proteins in *H. polymorpha*, is a favorable condition for lipid membrane formation (e.g., peroxisomal membranes) and thus advantageous to produce the recombinant hepatitis B vaccine with desirable antigenicity. Indeed, a codon-adapted HPV 52 L1 gene encoding for recombinant human papillomavirus protein was recently successfully expressed in *H. polymorpha*, which is used as an industrial platform for economical hepatitis B surface antigen particle production in China. It was found that the recombinant proteins, similar to HBsAg, could form virus-like particles in this host (Liu et al. 2014, 2015).

H. polymorpha has recently become a new promising yeast factory for production of biofuel ethanol from pentose xylose (see Chap. 1 of this book). Several heterologous proteins have been successfully expressed in *H. polymorpha* strains and improved xylose fermenters, such as bacterial xylose isomerase (Voronovsky et al. 2005; Dmytruk et al. 2008), amylolytic enzymes alpha-amylase and glucoamylase from the yeast *Schwanniomyces occidentalis*, endoxylanase of the fungus *Trichoderma reesei*, as well as beta-xylosidase of the fungus *Aspergillus niger* (Voronovsky et al. 2009).

11.7 Other Methylotrophic Yeasts

11.7.1 *Candida boidinii*

The *Candida boidinii* expression system has been reviewed by Yurimoto and Sakai (2009).

Using this yeast, several cytosolic, peroxisomal, and extracellular recombinant proteins have been produced. They included cytosolic human α1-antitrypsin (Yurimoto and Sakai 2009), *Aspergillus niger* pectin methylesterase (Kawaguchi et al. 2014), secretory glucoamylase from *Rhizopus oryzae* (Sakai et al. 1996), bovine cathepsin C (Komeda et al. 2002a), and peroxisome-targeted fructosyl-amino acid oxidase (FAOD) from *Penicillium janthinellum* (Sakai et al. 1999). Thus, based on *C. boidinii* platform, a system has been elaborated for recombinant toxic protein production which are targeted to peroxisomes. It was also observed that propeptide of transglutaminase (TGase) from the actinomycete *Streptomyces mobaraensis* efficiently drives secretion in *C. boidiniii* (Yurimoto et al. 2004). The *C. boidinii*-based expression system also exhibits several characteristics that may be beneficial for certain application as compared with other methylotrophic yeasts because different carbon sources provide three different expression levels in the same *C. boidinii* system based on repressible alcohol oxidase (AOD1) promoter. This promoter exhibits a high level of expression in cells grown on methanol or methanol supplemented with glycerol, an intermediate expression level in cells grown on glycerol, and a repressed expression in cells grown on glucose or ethanol (Sakai et al. 1995). As in other methylotrophs, high recombinant protein expression levels can be obtained in *C. boidinii* using cheap synthetic salt-based media. In

addition, like in *P. pastoris*, a *C. boidinii* host strain is available in which vacuolar protease genes, encoding the proteinase A (PEP4) and proteinase B (PRB1), are disrupted (Komeda et al. 2002b).

11.7.2 *Pichia methanolica*

Only a few recombinant proteins have been expressed in methylotrophic yeast *Pichia methanolica* under the control of the promoter of its alcohol oxidase gene, AUG1. They included an isoform of human glutamate decarboxylase (Raymond et al. 1998), hepatitis B virus polymerase (HBV P protein) (Choi et al. 2002), secreted lignin peroxidase of *Phanerochaete chrysosporium* (Wang et al. 2004) and bovine lactoferricin (Wang et al. 2007), and recombinant laccase from *Trametes versicolor* (Guo et al. 2006). In Mayson et al. (2003), various methanol concentrations were investigated in mixed glucose/methanol fed-batch cultures of *P. methanolica* expressing the human transferrin N-lobe protein. Of note, lignin peroxidase expressed under the control of the AUG1 promoter which was followed by either the lignin peroxidase leader peptide of *P. chrysosporium* or the *S. cerevisiae* alpha-factor signal peptide was efficiently secreted (Wang et al. 2004).

It is known that the methylotrophic yeasts *P. pastoris* and *P. methanolica* share the same methanol utilization pathway and have two alcohol oxidase genes: AOX1and AOX2 in *P. pastoris* (Cereghino et al. 2000) and AUG1 and AUG2 *in P. methanolica* (Raymond et al. 1998). Some studies suggested that MutS strains may produce higher levels of recombinant proteins than wild-type (Mut+) strains in certain applications while utilizing substantially less methanol (Cereghino et al. 2000). Therefore, the system analogous to MutS and Mut− phenotypes has been also developed in *P. methanolica* (Raymond et al. 1998). AUG1 gene disruptions resulted in a slow methanol utilization phenotype (MutS); in contrast, a disruption of AUG2 did not markedly affect methanol-dependent growth, whereas the double-mutant strains were totally deficient in methanol utilization (Mut−) (Raymond et al. 1998). Of note, *P. methanolica* and *P. pastoris* have been shown to produce similar levels of a recombinant protein when transformed with the analogous expression cassettes (Raymond et al. 1998).

For additional details on *P. methanolica* as well as *C. boidinii* expression systems, please see the review of Gellissen (2000).

11.7.3 *Ogataea minuta*

Ogataea minuta (Yamada et al. 1994) is another novel methylotrophic yeast species recently used as a host for production of human antibodies. In yeast glycosylation studies, the vectors and host strains of this species were constructed, and the strain producing a Man5GlcNAc2 high mannose-type sugar chain as a prototype of a humanized sugar chain was obtained by disruption of the OmOCH1 gene and the introduction of the α-1,2-mannosidase gene (Kuroda et al. 2006).

Also, some strategies based on partial suppression of *O*-glycosylation by addition of specific inhibitors of this pathway (e.g., rhodanine-3-acetic acid) have been successfully tested in the *O. minuta*-producing IgG (Kuroda et al. 2008). Strains bearing the OCH1 disruption (thus eliminating the alpha-1,6-mannosyltransferase initiating the synthesis of the hypermannosylated glycan structures in yeast) and co-overexpressing one of the genes involved in mannosyl phosphorylation of N-glycans (e.g., MNN4, MNN6, and PNO1) have been constructed in *O. minuta* (Akeboshi et al. 2009). The usefulness of protease-deficient strains in the production of antibodies in the yeast *O. minuta* has been demonstrated as well (Kuroda et al. 2007). Also, production of recombinant beta-hexosaminidase A, a potential enzyme for replacement therapy for Tay-Sachs and Sandhoff diseases, has been achieved in *O. minuta* (Akeboshi et al. 2007). The therapeutic effect in mice of a recombinant human lysosomal β-hexosaminidase A (HexA) with terminal M6P residues on their N-glycans produced in the glycoengineered *O. minuta* strain has recently been shown (Tsuji et al. 2011). It remains to be seen whether application spectrum of alternative methylotrophic yeasts expression platforms will be broadened in the future.

11.8 Conclusions and Outlook

For production of recombinant proteins including biopharmaceuticals, food and feed enzymes, and proteins for structural studies, various yeast expression systems have been successfully used over the years. While *S. cerevisiae* is the best genetically characterized eukaryotic organism out of all, for quite some time, the most frequently used yeast species for heterologous protein expression is *P. pastoris* (Spohner et al. 2015). For *P. pastoris* and other methylotrophic yeasts, the availability of commercial expression kits and large strain collections, wide variety of integrative vectors and auxotrophic or dominant selectable markers that allow integration into the genomes in a controlled copy number, strictly regulated by carbon sources inducible and constitutive promoters, and efficient transformation protocols, combined with the well-elaborated large-scale fermentation techniques, make these expression platforms very attractive for biotechnological exploitation. In addition to such common yeast features as ability to perform complex posttranslational modifications and being neither pyrogenic nor pathogenic, what is relevant for pharmaceutical proteins, methylotrophic yeasts are also known to secrete recombinant proteins more efficiently than other yeasts. Some methylotrophic yeasts already possess a generally recognized as safe (GRAS) status for applications in food industry. Efforts to humanize the glycosylation pathway similar to that in *P. pastoris* will continue with other glycoengineered yeast hosts to further develop them as producers of biopharmaceuticals. The recent revolutionary advances in the next-generation DNA sequencing, systems and synthetic biology, and comprehensive mathematical genome-scale metabolic models (GEMs) are predicted to greatly intensify the works on new host construction and

optimization of high cell density fermentation in methylotrophic yeasts yet in the near future.

References

Ahmad M, Hirz M, Pichler H, Schwab H (2014) Protein expression in *Pichia pastoris*: recent achievements and perspectives for heterologous protein production. Appl Microbiol Biotechnol 98:5301–5317

Akeboshi H, Chiba Y, Kasahara Y, Takashiba M, Takaoka Y, Ohsawa M, Tajima Y, Kawashima I, Tsuji D, Itoh K, Sakuraba H, Jigami Y (2007) Production of recombinant beta-hexosaminidase A, a potential enzyme for replacement therapy for Tay-Sachs and Sandhoff diseases, in the methylotrophic yeast *Ogataea minuta*. Appl Environ Microbiol 73:4805–4812

Akeboshi H, Kasahara Y, Tsuji D, Itoh K, Sakuraba H, Chiba Y, Jigami Y (2009) Production of human beta-hexosaminidase A with highly phosphorylated N-glycans by the overexpression of the *Ogataea minuta* MNN4 gene. Glycobiology 19:1002–1009

Aw R, Polizzi KM (2013) Can too many copies spoil the broth? Microb Cell Fact 12:128

Bai J, Swartz DJ, Protasevich II, Brouillette CG, Harrell PM, Hildebrandt E, Gasser B, Mattanovich D, Ward A, Chang G et al (2011) A gene optimization strategy that enhances production of fully functional P-glycoprotein in *Pichia pastoris*. PLoS One 6:e22577

Bawa Z, Routledge SJ, Jamshad M, Clare M, Sarkar D, Dickerson I, Ganzlin M, Poyner DR, Bill RM (2014) Functional recombinant protein is present in the pre-induction phases of *Pichia pastoris* cultures when grown in bioreactors, but not shake-flasks. Microb Cell Fact 13:561

Bill RM (2015) Recombinant protein subunit vaccine synthesis in microbes: a role for yeast? J Pharm Pharmacol 67:319–328

Bredell H, Smith JJ, Prins WA, Görgens JF, van Zyl WH (2016) Expression of rotavirus VP6 protein: a comparison amongst *Escherichia coli*, *Pichia pastoris* and *Hansenula polymorpha*. FEMS Yeast Res 16:fow001

Böer E, Steinborn G, Gellissen G, Kunze G (2007) Production of interleukin-6 in *Arxula adeninivorans*, *Hansenula polymorpha* and *Saccharomyces cerevisiae* by applying a wide-range yeast vector (CoMed™) system to simultaneous comparative assessment. FEMS Yeast Res 7:1181–1187

Byrne B (2015) *Pichia pastoris* as an expression host for membrane protein structural biology. Curr Opin Struct Biol 32:9–17

Celik E, Calık P (2012) Production of recombinant proteins by yeast cells. Biotechnol Adv 30:1108–1118

Cereghino JL, Cregg JM (2000) Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. FEMS Microbiol Rev 24:45–66

Cereghino GPL, Cereghino JL, Ilgen C, Cregg JM (2002) Production of recombinant proteins in fermenter cultures of the yeast *Pichia pastoris*. Curr Opin Biotechnol 13:329–332

Cheon SA, Kim H, Oh DB, Kwon O, Kang HA (2012) Remodeling of the glycosylation pathway in themethylotrophic yeast *Hansenula polymorpha* to produce human hybrid-type N-glycans. J Microbiol 50:341–348

Choi J, Kim EE, Park YI, Han YS (2002) Expression of the active human and duck hepatitis B virus polymerases in heterologous system of *Pichia methanolica*. Antiviral Res 55:279–290

Ciofalo V, Barton N, Kreps J, Coats I, Shanahan D (2006) Safety evaluation of a lipase enzyme preparation, expressed in *Pichia pastoris*, intended for use in the degumming of edible vegetable oil. Regul Toxicol Pharmacol (ISRTP) 45:1–8

Corchero JL, Gasser B, Resina D, Smith W, Parrilli E, Vázquez F, Abasolo I, Giuliani M, Jäntti J, Ferrer P, Saloheimo M, Mattanovich D, Schwartz S Jr, Tutino ML, Villaverde A (2013) Unconventional microbial systems for the cost-efficient production of high-quality protein therapeutics. Biotechnol Adv 31:140–153

Cregg JM, Russell KA (1998) Transformation. Methods Mol Biol 103:27–39
Cregg JM, Barringer KJ, Hessler AY, Madden KR (1985) *Pichia pastoris* as a host system for transformations. Mol Cell Biol 5:3376–3385
Cregg JM, Tolstorukov I, Kusari A, Sunga J, Madden K, Chappell T (2009) Expression in the yeast *Pichia pastoris*. In: Burgess RR, Deutscher MP (eds) Methods in enzymology. Guide to protein purification, 2nd edn, vol 463. Academic, pp 169–89
Daly R, Hearn MT (2005) Expression of heterologous proteins in *Pichia pastoris*: a useful experimental tool in protein engineering and production. J Mol Recognit 18:119–138
Darby RA, Cartwright SP, Dilworth MV, Bill RM (2012) Which yeast species shall I choose? *Saccharomyces cerevisiae* versus *Pichia pastoris*. Methods Mol Biol 866:11–23
Demain AL, Vaishnav P (2009) Production of recombinant proteins by microbes and higher organisms. Biotechnol Adv 27:297–306
Dmytruk OV, Dmytruk KV, Abbas CA, Voronovsky AY, Sibirny AA (2008) Engineering of xylose reductase and overexpression of xylitol dehydrogenase and xylulokinase improves xylose alcoholic fermentation in the thermotolerant yeast *Hansenula polymorpha*. Microb Cell Fact 7:21
Dragosits M, Frascotti G, Bernard-Granger L, Vazquez F, Giuliani M, Baumann K et al (2011) Influence of growth temperature on the production of antibody Fab fragments in different microbes: a host comparative analysis. Biotechnol Prog 27:38–46
Dunn WA Jr, Cregg JM, Kiel JA, van der Klei IJ, Oku M, Sakai Y, Sibirny AA, Stasyk OV, Veenhuis M (2005) Pexophagy: the selective autophagy of peroxisomes. Autophagy 1:75–83
Eilert E, Rolf T, Heumaier A, Hollenberg CP, Piontek M, Suckow M (2013) Improved processing of secretory proteins in *Hansenula polymorpha* by sequence variation near the processing site of the alpha mating factor prepro sequence. J Biotechnol 167:94–100
Emmerstorfer A, Wriessnegger T, Hirz M, Pichler H (2014) Overexpression of membrane proteins from higher eukaryotes in yeasts. Appl Microbiol Biotechnol 98:7671–7698
Ergün BG, Çalık P (2016) Lignocellulose degrading extremozymes produced by *Pichia pastoris*: current status and future prospects. Bioprocess Biosyst Eng 39:1–36
Gasser B, Prielhofer R, Marx H, Maurer M, Nocon J, Steiger M, Puxbaum V, Sauer M, Mattanovich D (2013) *Pichia pastoris*: protein production host and model organism for biomedical research. Future Microbiol 8:191–208
Gellissen G, Piontek M, Dahlems U, Jenzelewski V, Gavagan JE, DiCosimo R (1996) Recombinant *Hansenula polymorpha* as a biocatalyst: coexpression of the spinach glycolate oxidase (GO) and the *S. cerevisiae* catalase T (CTT1) gene. Appl Microbiol Biotechnol 46:46–54
Gellissen G (2000) Heterologous protein production in methylotrophic yeasts. Appl Microbiol Biotechnol 54:741–750
Gellissen G (ed) (2002) *Hansenula polymorpha* – biology and applications. Wiley-VCH, Weinheim
Gellissen G, Strasser AWM, Suckow M (2005a) Key and criteria to the selection of an expression platform. In: Gellissen G (ed) Production of recombinant proteins — novel microbial and eukaryotic expression systems. Wiley-VCH, Weinheim, pp 1–5
Gellissen G, Kunze G, Gaillardin C, Cregg JM, Berardi E, Veenhuis M, van der Klei I (2005b) New yeast expression platforms based on methylotrophic *Hansenula polymorpha* and *Pichia pastoris* and on dimorphic *Arxula adeninivorans* and *Yarrowia lipolytica* – a comparison. FEMS Yeast Res 5:1079–1096
Gemmill TR, Trimble RB (1999) Overview of N- and O-linked oligosaccharide structures found in various yeast species. BBA-Gen Subjects 1426:227–237
Gidijala L, Bovenberg RAL, Klaassen P, van der Klei IJ, Veenhuis M, Kiel JAKW (2008) Production of functionally active *Penicillium chrysogenum* isopenicillin N synthase in the yeast *Hansenula polymorpha*. BMC Biotechnol 8:29
Guo M, Lu F, Du L, Pu J, Bai D (2006) Optimization of the expression of a laccase gene from *Trametes versicolor* in *Pichia methanolica*. Appl Microbiol Biotechnol 71:848–852

Hamilton SR, Davidson RC, Sethuraman N, Nett JH, Jiang Y, Rios S, Bobrowicz P, Stadheim TA, Li H, Choi B-K, Hopkins D, Wischnewski H, Roser J, Mitchell T, Strawbridge RR, Hoopes J, Wildt S, Gerngross TU (2006) Humanization of yeast to produce complex terminally sialylated glycoproteins. Science 313:1441–1443

Hartner FS, Glieder A (2006) Regulation of methanol utilisation pathway genes in yeasts. Microb Cell Fact 5:3

He M, Wu D, Wu J, Chen J (2014) Enhanced expression of endoinulinase from *Aspergillus niger* by codon optimization in *Pichia pastoris* and its application in inulooligosaccharide production. J Ind Microbiol Biotechnol 41:105–114

Heo JH, Ananin V, Kang HA, Rhee SK, Kim CH (2008) Feeding strategies for the enhanced production of recombinant human serum albumin in the fed-batch cultivation of *Hansenula polymorpha*. Process Biochem 43:918–924

Hodgkins M, Mead D, Ballance DJ, Goodey A, Sudbery P (1993) Expression of the glucose oxidase gene from *Aspergillus niger* in *Hansenula polymorpha* and its use as a reporter gene to isolate regulatory mutations. Yeast 9:625–635

Hu H, Gao J, He J, Yu B, Zheng P, Huang Z, Mao X, Yu J, Han G, Chen D (2013) Codon optimization significantly improves the expression level of a keratinase gene in *Pichia pastoris*. PLoS One 8(3):e58393

Kang HA, Sohn JH, Choi ES, Chung BH, Yu MH, Rhee SK (1998) Glycosylation of human alpha 1-antitrypsin in *Saccharomyces cerevisiae* and methylotrophic yeasts. Yeast 14:371–381

Kang HA, Kang W, Hong WK, Kim MW, Kim JY, Sohn JH, Choi ES, Choe KB, Rhee SK (2001) Development of expression systems for the production of recombinant human serum albumin using the MOX promoter in *Hansenula polymorpha* DL-1. Biotechnol Bioeng 76:175–185

Kang HA, Gellissen G (2004) Hansenula polymorpha. In: Gellissen G (ed) Production of recombinant proteins, novel microbial and eukaryotic expression systems, 1st edn. Wiley-VCH, Weinheim, pp 111–142

Kawaguchi K, Yurimoto H, Sakai Y (2014) Expression of a codon-optimized *Aspergillus niger* pectin methylesterase gene in the methylotrophic yeast *Candida boidinii*. Biosci Biotechnol Biochem 78:718–721

Kim MW, Rhee SK, Kim JY, Shimma Y, Chiba Y, Jigami Y, Kang HA (2004) Characterization of N-linked oligosaccharides assembled on secretory recombinant glucose oxidase and cell wall mannoproteins from the methylotrophic yeast *Hansenula polymorpha*. Glycobiology 14:243–251

Kim H, Yoo SJ, Kang HA (2015) Yeast synthetic biology for the production of recombinant therapeutic proteins. FEMS Yeast Res 15:1–16

Klabunde J, Kleebank S, Piontek M, Hollenberg CP, Hellwig S, Degelmann A (2007) Increase of calnexin gene dosage boosts the secretion of heterologous proteins by *Hansenula polymorpha*. FEMS Yeast Res 7:1168–1180

Komeda T, Tazumi K, Shimada H, Kano K, Hayashi T, Saito H, Tsumura H, Kato N, Sakai Y, Kondo K (2002a) Production of active bovine cathepsin C (dipeptidyl aminopeptidase I) in the methylotrophic yeast *Candida boidinii*. Appl Microbiol Biotechnol 59:252–258

Komeda T, Sakai Y, Kato N, Kondo K (2002b) Construction of protease-deficient *Candida boidinii* strains useful for recombinant protein production: cloning and disruption of proteinase A gene (PEP4) and proteinase B gene (PRBI). Biosci Biotechnol Biochem 66:628–631

Krasovska OS, Stasyk OV, Nahorny VO, Stasyk OV, Granovski N, Kordium VA, Vozianov OF, Sibirny AA (2007) Glucose-induced production of recombinant proteins in *Hansenula polymorpha* mutants deficient in catabolite repression. Biotechnol Bioeng 97:858–870

Krasovska OS, Stasyk OV, Sibirny AA (2013) Stable overproducer of hepatitis B surface antigen in the methylotrophic yeast *Hansenula polymorpha* due to multiple integration of heterologous auxotrophic selective markers and defect in peroxisome biogenesis. Appl Microbiol Biotechnol 97:9969–9979

Küberl A, Schneider J, Thallinger GG, Anderl I, Wibberg D, Hajek T, Jaenicke S, Brinkrolf K, Goesmann A, Szczepanowski R, Pühler A, Schwab H, Glieder A, Pichler H (2011) High-quality genome sequence of *Pichia pastoris* CBS7435. J Biotechnol 154:312

Kuroda K, Kobayashi K, Tsumura H, Komeda T, Chiba Y, Jigami Y (2006) Production of Man5GlcNAc2-type sugar chain by the methylotrophic yeast *Ogataea minuta*. FEMS Yeast Res 6:1052–1062

Kuroda K, Kitagawa Y, Kobayashi K, Tsumura H, Komeda T, Mori E, Motoki K, Kataoka S, Chiba Y, Jigami Y (2007) Antibody expression in protease-deficient strains of the methylotrophic yeast *Ogataea minuta*. FEMS Yeast Res 7:1307–1316

Kuroda K, Kobayashi K, Kitagawa Y, Nakagawa T, Tsumura H, Komeda T, Shinmi D, Mori E, Motoki K, Fuju K, Sakai T, Nonaka K, Suzuki T, Ichikawa K, Chiba Y, Jigami Y (2008) Efficient antibody production upon suppression of O mannosylation in the yeast *Ogataea minuta*. Appl Environ Microbiol 74:446–453

Kurtzman CP (2009) Biotechnological strains of *Komagataella (Pichia) pastoris* are *Komagataella phaffii* as determined from multigene sequence analysis. J Ind Microbiol Biotechnol 36:1435–1438

Kurtzman CP (2011) A new methanol assimilating yeast, *Ogataea parapolymorpha*, the ascosporic state of *Candida parapolymorpha*. Antonie Van Leeuwenhoek 100:455–462

Leão AN, Kiel JA (2003) Peroxisome homeostasis in *Hansenula polymorpha*. FEMS Yeast Res 4:131–139

Lin Cereghino GP, Lin Cereghino J, Sunga AJ, Johnson MA, Lim M, Gleeson MA, Cregg JM (2001) New selectable marker/auxotrophic host strain combinations for molecular genetic manipulation of *Pichia pastoris*. Gene 263:159–169

Lin-Cereghino GP, Stark CM, Kim D, Chang J, Shaheen N, Poerwanto H, Agari K, Moua P, Low LK, Tran N, Huang AD, Nattestad M, Oshiro KT, Chang JW, Chavan A, Tsai JW, Lin-Cereghino J (2013) The effect of alpha-mating factor secretion signal mutations on recombinant protein expression in *Pichia pastoris*. Gene 519:311–317

Liu C, Yang X, Yao Y, Huang W, Sun W, Ma Y (2014) Diverse expression levels of two codon-optimized genes that encode human papilloma virus type 16 major protein L1 in *Hansenula polymorpha*. Biotechnol Lett 36:937–945

Liu C, Yao Y, Yang X, Bai H, Huang W, Xia Y, Ma Y (2015) Production of recombinant human papillomavirus type 52 L1 protein in *Hansenula polymorpha* formed virus-like particles. J Microbiol Biotechnol 25:936–940

Looser V, Bruhlmann B, Bumbak F, Stenger C, Costa M, Camattari A, Fotiadis D, Kovar K (2015) Cultivation strategies to enhance productivity of *Pichia pastoris*: a review. Biotechnol Adv 33:1177–1193

Mack M, Wannemacher M, Hobl B, Pietschmann P, Hock B (2009) Comparison of two expression platforms in respect to protein yield and quality: *Pichia pastoris* versus *Pichia angusta*. Protein Expr Purif 66:165–171

Mayer AF, Hellmuth K, Schlieker H, Lopez-Ulibarri R, Oertel S, Dahlems U et al (1999) An expression system matures: a highly efficient and cost-effective process for phytase production by recombinant strains of *Hansenula polymorpha*. Biotechnol Bioeng 63:373–381

Mattanovich D, Gasser B, Hohenblum H, Sauer M (2004) Stress in recombinant protein producing yeasts. J Biotechnol 113:121–135

Mattanovich D, Branduardi P, Dato L, Gasser B, Sauer M, Porro D (2012) Recombinant protein production in yeasts. Methods Mol Biol 824:329–358

Mayson BE, Kilburn DG, Zamost BL, Raymond CK, Lesnicki GJ (2003) Effects of methanol concentration on expression levels of recombinant protein in fed-batch cultures of *Pichia methanolica*. Biotechnol Bioeng 81:291–298

Meehl MA, Stadheim TA (2014) Biopharmaceutical discovery and production in yeast. Curr Opin Biotechnol 30:120–127

Mellitzer A, Weis R, Glieder A, Flicker K (2012) Expression of lignocellulolytic enzymes in *Pichia pastoris*. Microb Cell Fact 11:61

Morales L, Dujon B (2012) Evolutionary role of interspecies hybridization and genetic exchanges in yeasts. Microbiol Mol Biol Rev 76:721–739

Morales L, Noel B, Porcel B, Marcet-Houben M, Hullo MF, Sacerdot C, Tekaia F, Leh-Louis V, Despons L, Khanna V, Aury JM, Barbe V, Couloux A, Labadie K, Pelletier E, Souciet JL, Boekhout T, Gabaldon T, Wincker P, Dujon B (2013) Complete DNA sequence of *Kuraishia capsulata* illustrates novel genomic features among budding yeasts (Saccharomycotina). Genome Biol Evol 5:2524–2539

Moussa M, Ibrahim M, El Ghazaly M, Rohde J, Gnoth S, Anton A, Kensy F, Mueller F (2012) Expression of recombinant staphylokinase in the methylotrophic yeast *Hansenula polymorpha*. BMC Biotechnol 12:96

Müller IIF, Tieke A, Waschk D, Mühle C, Müller FI, Seigelchifer M et al (2002) Production of IFNα-2a in *Hansenula polymorpha*. Process Biochem 38:15–25

Nocon J, Steiger MG, Pfeffer M, Sohn SB, Kim TY, Maurer M, Rußmayer H, Pflügl S, Ask M, Haberhauer-Troyer C, Ortmayr K, Hann S, Koellensperger G, Gasser B, Lee SY, Mattanovich D (2014) Model based engineering of *Pichia pastoris* central metabolism enhances recombinant protein production. Metab Eng 24:129–138

Oh DB, Park JS, Kim MW, Cheon SA, Kim EJ, Moon HY et al (2008) Glycoengineering of the methylotrophic yeast *Hansenula polymorpha* for the production of glycoproteins with trimannosyl core N-glycan by blocking core oligosaccharide assembly. Biotechnol J 3 (5):659–668

Petranovic D, Tyo K, Vemuri G, Nielsen J (2010) Prospects of yeast systems biology for human health: integrating lipid, protein and energy metabolism. FEMS Yeast Res 10:1046–1059

Petre J, Rutgers T, Hauser P (1992) Properties of a recombinant yeast-derived hepatitis B surface antigen containing S, preS2 and preS1 antigenic domains. Arch Virol Suppl 4:137–141

Potvin G, Ahmad A, Zhang Z (2012) Bioprocess engineering aspects of heterologous protein production in *Pichia pastoris*: a review. Biochem Eng J 64:91–105

Prielhofer R, Maurer M, Klein J, Wenger J, Kiziak C, Gasser B, Mattanovich D (2013) Induction without methanol: novel regulated promoters enable high-level expression in *Pichia pastoris*. Microb Cell Fact 12:5

Puxbaum V, Mattanovich D, Gasser B (2015) Quo vadis? The challenges of recombinant protein folding and secretion in *Pichia pastoris*. Appl Microbiol Biotechnol 99:2925–2938

Qian W, Song H, Liu Y, Zhang C, Niu Z, Wang H, Qiu B (2009) Improved gene disruption method and Cre-loxP mutant system for multiple gene disruptions in *Hansenula polymorpha*. J Microbiol Methods 79:253–259

Ravin NV, Eldarov MA, Kadnikov VV, Beletsky AV, Schneider J, Mardanova ES, Smekalova EM, Zvereva MI, Dontsova OA, Mardanov AV, Skryabin KG (2013) Genome sequence and analysis of methylotrophic yeast *Hansenula polymorpha* DL1. BMC Genomics 14:837

Raymond CK, Bukowski T, Holderman SD, Ching AFT, Vanaja E, Stamm MR (1998) Development of the methylotrophic yeast, *Pichia methanolica*, for the expression of the 65-kilodalton isoform of human glutamate decarboxylase. Yeast 14:11–23

Resina D, Cos O, Ferrer P, Valero F (2005) Developing high cell density fed-batch cultivation strategies for heterologous protein production in *Pichia pastoris* using the nitrogen source-regulated FLD1 promoter. Biotechnol Bioeng 91:760–767

Routledge SJ, Mikaliunaite L, Patel A, Clare M, Cartwright SP, Bawa Z, Wilks MD, Low F, Hardy D, Rothnie AJ, Bill RM (2016) The synthesis of recombinant membrane proteins in yeast for structural studies. Methods 95:26–37

Sakai Y, Rogi T, Takeuchi R, Kato N, Tani Y (1995) Expression of *Saccharomyces* adenylate kinase gene in *Candida boidinii* under the regulation of its alcohol oxidase promoter. Appl Microbiol Biotechnol 42:860–864

Sakai Y, Akiyama M, Kondoh H, Shibano Y, Kato N (1996) High-level secretion of fungal glucoamylase using the *Candida boidinii* gene expression system. Biochim Biophys Acta 1308:81–87

Sakai Y, Yoshida H, Yurimoto H, Yoshida N, Fukuya H, Takabe K, Kato N (1999) Production of fungal fructosyl amino acid oxidase useful for diabetic diagnosis in the peroxisome of *Candida boidinii*. FEBS Lett 459:233–237

Seo HS, Park JS, Han KY, Bae KD, Ahn SJ, Kang HA, Lee J (2008) Analysis and characterization of hepatitis B vaccine particles synthesized from *Hansenula polymorpha*. Vaccine 26:4138–4144

Shekhar C (2008) Pichia power: India's biotech industry puts unconventional yeast to work. Chem Biol 15:201–202

Spadiut O, Capone S, Krainer F, Glieder A, Herwig C (2014) Microbials for the production of monoclonal antibodies and antibody fragments. Trends Biotechnol 32:54–60

Spohner SC, Müller H, Quitmann H, Czermak P (2015) Expression of enzymes for the usage in food and feed industry with *Pichia pastoris*. J Biotechnol 202:118–134

Stasyk OV, Stasyk OG, Komduur J, Veenhuis M, Cregg JM, Sibirny AA (2004) A hexose transporter homologue controls glucose repression in the methylotrophic yeast *Hansenula polymorpha*. J Biol Chem 279:8116–8125

Steinborn G, Boer E, Scholz A, Tag K, Kunze G, Gellissen G (2006) Application of a wide-range yeast vector (Comed (TM)) system to recombinant protein production in dimorphic *Arxula adeninivorans*, methylotrophic *Hansenula polymorpha* and other yeasts. Microb Cell Fact 5:33

Stockmann C, Scheidle M, Klee D, Dittrich B, Merckelbach A, Hehmann G, Melmer G, Buchs J, Kang HA, Gellissen G (2009) Process development in *Hansenula polymorpha* and *Arxula adeninivorans*, a re-assessment. Microb Cell Fact 8:22–31

Sturmberger L, Chappell T, Geier M, Krainer F, Day KJ, Vide U, Trstenjak S, Schiefer A, Richardson T, Soriaga L, Darnhofer B, Birner-Gruenberger R, Glick BS, Tolstorukov I, Cregg J, Madden K, Glieder A (2016) Refined *Pichia pastoris* reference genome sequence. J Biotechnol 235:121–131

Sudbery PE, Gleeson MA, Veale RA, Ledeboer AM, Zoetmulder MC (1988) *Hansenula polymorpha* as a novel yeast system for the expression of heterologous genes. Biochem Soc Trans 16:1081–1083

Thompson CA (2010) FDA approves kallikrein inhibitor to treat hereditary angioedema. Am J Health-Syst Pharm 67:93

Tomàs-Gamisans M, Ferrer P, Albiol J (2016) Integration and validation of the genome-scale metabolic models of *Pichia pastoris*: a comprehensive update of protein glycosylation pathways, lipid and energy metabolism. PLoS One 11(1):e0148031

Tsuji D, Akeboshi H, Matsuoka K, Yasuoka H, Miyasaki E, Kasahara Y, Kawashima I, Chiba Y, Jigami Y, Taki T, Sakuraba H, Itoh K (2011) Highly phosphomannosylated enzyme replacement therapy for GM2 gangliosidosis. Ann Neurol 69:691–701

Vogl T, Glieder A (2013) Regulation of *Pichia pastoris* promoters and its consequences for protein production. N Biotechnol 30:385–404

Voronovsky AY, Ryabova OB, Verba OV, Ishchuk OP, Dmytruk KV, Sibirny AA (2005) Expression of xylA genes encoding xylose isomerases from *Escherichia coli* and *Streptomyces coelicolor* in the methylotrophic yeast *Hansenula polymorpha*. FEMS Yeast Res 5:1055–1062

Voronovsky AY, Rohulya OV, Abbas CA, Sibirny AA (2009) Development of strains of the thermotolerant yeast *Hansenula polymorpha* capable of alcoholic fermentation of starch and xylan. Metab Eng 11:234–242

Wang H, Lu F, Sun Y, Du L (2004) Heterologous expression of lignin peroxidase of *Phanerochaete chrysosporium* in *Pichia methanolica*. Biotechnol Lett 26:1569–1573

Wang H, Zhao X, Lu F (2007) Heterologous expression of bovine lactoferricin in *Pichia methanolica*. Biochemistry (Mosc) 72:640–643

Waterham HR, Digan ME, Koutz PJ, Lair SV, Cregg JM (1997) Isolation of the *Pichia pastoris* glyceraldehyde-3-phosphate dehydrogenase gene and regulation and use of its promoter. Gene 186:37–44

Yamada Y, Maeda K, Mikata K (1994) The phylogenetic relationships of the hat-shaped ascospore-forming, nitrate-assimilating Pichia species, formerly classified in the genus Hansenula

Sydow et Sydow, based on the partial sequences of 18S and 26Sribosomal RNAs (Saccharomycetaceae): the proposals of three new genera, Ogataea, Kuraishia, and Nakazawaea. Biosci Biotechnol Biochem 58:1245–1257

Yamada Y, Matsuda M, Maeda K, Mikata K (1995) The phylogenetic relationships of methanol-assimilating yeasts based on the partial sequences of 18S and 26S ribosomal RNAs: the proposal of *Komagataella* gen. nov. (*Saccharomycetaceae*). Biosci Biotechnol Biochem 59:439–444

Yang S, Kuang Y, Li H, Liu Y, Hui X, Li P, Jiang Z, Zhou Y, Wang Y, Xu A et al (2013) Enhanced production of recombinant secretory proteins in *Pichia pastoris* by optimizing Kex2 P10 site. PLoS One 8:e75347

Yu P, Yan Y, Gu Q, Wang X (2013) Codon optimisation improves the expression of *Trichoderma viride* sp. endochitinase in *Pichia pastoris*. Sci Rep 3:3043

Yu P, Zhu Q, Chen K, Lv X (2015) Improving the secretory production of the heterologous protein in *Pichia pastoris* by focusing on protein folding. Appl Biochem Biotechnol 175:535–548

Yurimoto H, Yamane M, Kikuchi Y, Matsui H, Kato N, Sakai Y (2004) The pro-peptide of *Streptomyces mobaraensis* transglutaminase functions in cis and in trans to mediate efficient secretion of active enzyme from methylotrophic yeasts. Biosci Biotechnol Biochem 68:2058–2069

Yurimoto H, Sakai Y (2009) Methanol-inducible gene expression and heterologous protein production in the methylotrophic yeast *Candida boidinii*. Biotechnol Appl Biochem 53:85–92

Yurugi-Kobayashi T, Asada H, Shiroishi M, Shimamura T, Funamoto S, Katsuta N, Ito K, Sugawara T, Tokuda N, Tsujimoto H, Murata T, Nomura N, Haga K, Haga T, Iwata S, Kobayashi T (2009) Comparison of functional non-glycosylated GPCRs expression in *Pichia pastoris*. Biochem Biophys Res Commun 380:271–276

Zhang W, Inan M, Meagher MM (2000) Fermentation strategies for recombinant protein expression in the methylotrophic yeast *Pichia pastoris*. Biotechnol Bioprocess Eng 5:275–287

Zhao W, Wang J, Deng R, Wang X (2008) Scale-up fermentation of recombinant *Candida rugosa* lipase expressed in *Pichia pastoris* using the GAP promoter. J Ind Microbiol Biotechnol 35:189–195

Biosensors Based on Yeast/Fungal Cells

12

Alexandre Chamas, Ha Thi Minh Pham, Kim Baronian, and Gotthard Kunze

Abstract

Yeast cells can be utilized as whole-cell biosensors for the detection of many target analytes. This is mainly because the latest molecular biology techniques allowed modification of the yeast genome to give the cells useful metabolic properties. The production of correctly folded recombinant proteins, and more particularly receptors, in yeast is nowadays very often successful, and the implementation of diverse reporting strategies is documented. A number of yeast whole-cell biosensors are now being used in many laboratories, with some of them available commercially. One of the major targets of these biosensors is the endocrine disruptors, a class of organic molecules which can disturb vertebrate endocrine system and therefore presents a potential threat to the environment. Recent research has focused on building new biosensors using other receptors such as the G protein-coupled receptors and the thyroid receptor. In this chapter, we will present a selection of newly developed biosensors and the different reporting strategies used to transduce the binding event into a measureable signal.

Contents

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12.1 Introduction

Measuring concentrations of molecule from the simplest gas to complicated organic molecules has been the goal of a number studies. The information they give will have application in diverse areas such as the environment, food production, and medical science. By analyzing the composition of rivers, the atmosphere, foods, or body fluids, for example, and by knowing the effective of threshold concentrations of molecules, this research has allowed the discovery of several compounds with negative impact on the ecosystem or on animal and human health. These results were in some cases used by governments to establish recommendations relative to the production, utilization, storage, or disposal of these compounds. Detection of natural or artificial compounds impacting on biological processes increasingly involves the use of biosensors. A biosensor has the particularity to use a biological partner, which can be strictly identical or derived from a natural occurring molecule produced by a living organism or an entire organism and is able to recognize a target analyte. The second part of a biosensor is the transducer, which should transform this biological interaction into physically detectable information. This information can be transported in several states (e.g., light, current, heat) to a detector who will transform, amplify, and process this information to give a numerical value.

In this chapter, only the latest advances in the development of transcription-related whole-cell yeast biosensors will be presented. Since the 1980s, other biosensors relying on non-transcriptional events have been presented, biosensors allowing the detection of sugars, alcohols, or lactate, for example (Adeniran et al. 2015; Riedel et al. 2006). These take advantage of yeast metabolic possibilities to transform these hardly detectable molecules into simple compounds. By monitoring parameters of the yeast culture such as the amount of dissolved oxygen (BOD) or the milieu pH, these biosensors can indirectly assess the concentration of the target analytes. However, few new biosensors relying on these strategies have been presented in the last decade, probably because analytical detection methods offer now much better sensitivity and reliability in the results. On the contrary, during the same period, publications concerning transcription-based yeast-cell biosensors increased dramatically, and some of these biosensors have been commercialized.

Their success is dependent on the fact that biosensors will assess the biological activity of compounds, a characteristic which cannot be obtained by analytical detection methods.

In the field of cell-based biosensors, yeast biosensors also present decisive advantages in comparison to bacteria-based biosensors or mammal cell suspensions. Firstly, it is because most of the target compounds of these biosensors are interacting with eukaryote organisms by binding to specific receptors or disturbing a specific signal pathway. Therefore yeast, as eukaryotic organisms, are more likely to mimic what will happen in higher eukaryotes than bacteria. Additionally, the latest advances in molecular biology tools allow a precise and easy modification of the yeast genome to either modulate the yeast own metabolism or insert new coding sequences for recombinant proteins, thus reducing the historical advantage of bacteria-based biosensors in terms of genetic modification easiness. Advanced genome editing techniques are nowadays a routine and allow greater control for knockouts or targeted gene integration. In comparison to mammalian cell culture, yeast-based biosensors present mostly practical advantages such as the fast and cheap propagation of the cells and the possibility to lyophilize cells for storage or shipment. However, there are applications in pathology where yeast-based biosensors cannot replicate the responses of mammalian cell cultures because of the large difference between the metabolism of lower and higher eukaryotes.

12.2 Utilization of Receptors

A key element for the development of a whole-cell yeast biosensor is the intracellular production of a protein able to bind the target analyte. Even though new in silico techniques strive to make theoretically possible the design of an artificial binding protein for every possible ligand, an easier way is to take advantage of the millions of years of vertebrate's evolution and the existence of specific binding proteins called receptors. These recombinant proteins can generally be produced in yeast, a topic which is discussed in other chapters of this book. Because the analyte is often presumed to have an impact on human health, utilization of recombinant human receptor appears as the natural choice although other vertebrate receptors are preferred in some biosensors. Among the receptors, a certain class—the nuclear receptors—is of particular interest because of its simple signaling pathway, which does not necessitate extra partner proteins to transform the binding into a transcriptional response. These nuclear receptors contain one domain responsible for ligand binding and act as a transcription factor by directly binding to specific DNA domain in the nucleus. Another large class of receptors, which is utilized for yeast-based biosensors, is the membrane-bound receptors, known as the G protein-coupled receptors. Because these receptors cannot directly translocate into the nucleus in order to induce the transcription of a reporter protein, the presence of a specific mobile G protein in the cell or of specific kinases is mandatory. While in some cases, the existing endogenous yeast G protein or kinases can be used for signal

transduction, the majority of these biosensors require the additional production of recombinant signaling proteins.

12.2.1 Steroid Receptors

The detection of molecules binding to steroid hormones receptors gained increased importance since the discovery that molecules called endocrine disruptors (EDCs) can interfere with vertebrate endocrine system and can lead to environmental or health hazards (Fent 2015; Kabir et al. 2015). Because of animal welfare and cost considerations in the determination of the potential endocrine disrupting activity of a molecule, pharmaceutical and cosmetic industries are attempting to replace classical animal testing. Many companies are now using yeast-cell biosensors to determine at a very early stage of development if a new compound is worth further development or if its endocrine disrupting properties make it unsuitable for further development. Initially receptor-based biosensors focused on the detection of estrogens, but new biosensors for the detection of androgens (Bhattacharjee and Khurana 2014; Gerlach et al. 2014), progesterone (Viswanath et al. 2008; Chamas et al. 2015), glucocorticoids (Bovee et al. 2011; Pham et al. 2016), and mineralocorticoids (Miller et al. 2010) were recently presented. Because a large number of research articles describing continuous optimization of the biosensors have been published in the last years, this chapter will only focus on the latest version of selected biosensors, and thus fundamental work, which paved the way for the development of the actual biosensors, will not be discussed. For a more complete overview of the steroid hormone yeast biosensors history, readers are invited to consider the following reference (Tag et al. 2006).

Two organisms, *S. cerevisiae* and *A. adeninivorans* have been mainly used for the development of steroid hormone yeast biosensors. However, a hormone receptor-based biosensor dedicated to the detection of endocrine disruptors has been recently engineered in *Y. lipolytica* (Cho et al. 2010). In all organisms, an integration of a human steroid receptor under the control of a constitutive promoter is the first step of the biosensor design. If a target ligand is present in the cultivation medium, it can enter the cell by passive diffusion through the membrane and bind to the receptor. This binding event induces a conformational change that allows the homodimerization of the receptor in the cytoplasm. The nuclear localization signal of the receptor will then allow translocation of the receptor homodimer into the nucleus to bind to its DNA response element, thus initiating transcription factor activity. This mechanism of hormone recognition is presented schematically in Fig. 12.1.

In *A. adeninivorans*, a modified version of promoter upstream of the reporter protein gene was constructed to introduce an inducible mechanism of the reporter gene. This consists of a modified glucoamylase promoter (*GAA*) containing two 15 bp sequences acting as the hormone response element (*HRE*) at position -107 of the promoter. *HRE* is a conserved DNA-binding site for the dimerized hormone receptor and only when this dimerized receptor is bound to the *HRE* can the

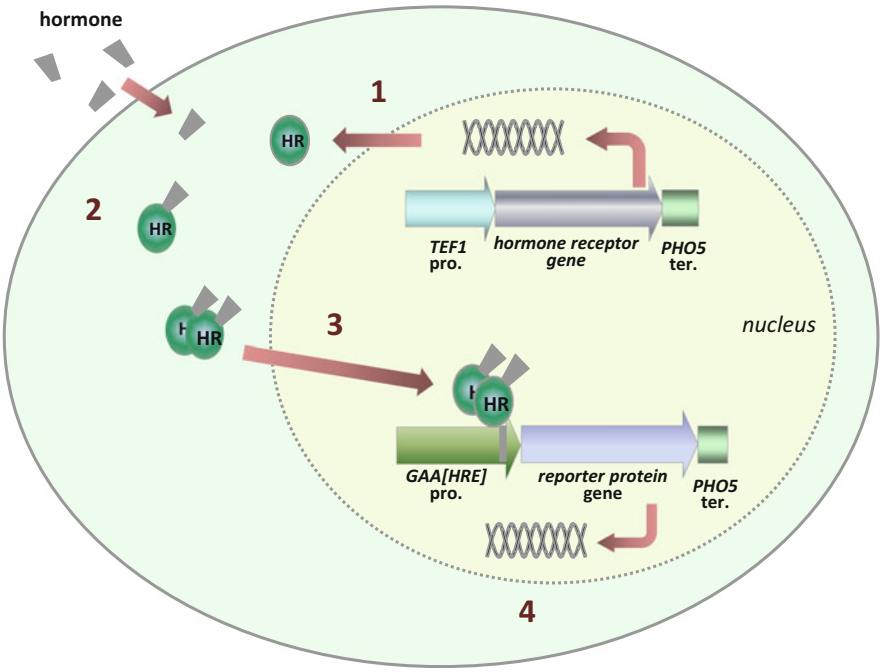

Fig. 12.1 Schematic representation of a modified yeast cell in steroid receptor-based biosensor. In the nucleus, a *human hormone receptor* gene is constitutively expressed, and the protein can be found in cytoplasm (1). When a binding hormone is present in the extracellular medium, it can cross yeast membrane and bind to the receptor (2). After binding-induced receptor dimerization, this dimer can retranslocate in the nucleus and bind to the *hormone response element* (*HRE*) (3), thus activating a promoter and allowing expression of the reporter protein gene (4). Modified after Chamas et al. (2017)

expression of the downstream gene occur. In the case of the estrogen-related assays, *HRE* was replaced by the slightly different estrogen response element (*ERE*) sequence as it was shown to have higher affinity to the estrogen receptor than *HRE* (Hahn et al. 2006). In *S. cerevisiae* biosensors, *PGK*, *GPD*, or *ADH1* promoters containing *HRE* or *ERE* sequences have been described (Leskinen et al. 2005; Sanseverino et al. 2005; Routledge and Sumpter 1996), while *ALK1*, *ICL1*, *RPS7*, or *TEF1* promoters have been used in *Y. lipolytica* (Cho et al. 2010). The reporter protein utilized will then lead to different variations of the assays with different sensitivity or practical needs, these aspects will be considered in the section "reporter proteins" of this chapter.

Besides the mechanism described above, additional research to enhance the performance of the biosensors has been described recently, notably the expression of accessory proteins and modification of the native hormone receptor. For example, the co-expression of the steroid receptor cofactor I (SRC1) has been implemented in the *S. cerevisiae* YES assay in order to increase the sensitivity of the biosensor (Chu et al. 2009). In human, SRC1 is known to bind to a number of

nuclear hormone receptors in the nucleus and helps stimulate transcriptional activity. Successful use of the β-isoform of the estrogen receptor (Lee et al. 2007) is also reported.

Another interesting research is the one performed by Rajasärkkä et al. in 2013 to develop a bisphenol A biosensor (Rajasärkkä et al. 2013). Bisphenol A is an artificial organic compound which possesses endocrine disrupting properties and which is extensively used in industry although it has been banned in products such as baby bottles in several countries. But despite its EDC activity, the yeast estrogen biosensor cannot detect low concentrations of bisphenol A, probably because the human estrogen receptor has a low affinity for this compound. Because no specific receptor for this pollutant has been found in nature, the bisphenol A biosensor used a human estrogen receptor with a sequence modified by targeted mutations to decrease its affinity toward most estrogens and to increase the affinity toward bisphenol A.

12.2.2 Other Nuclear Receptors

Newly developed yeast biosensors are also utilizing other nuclear receptors, which are not steroid receptors such as the human aryl hydrocarbon receptor (hAhR), the retinoid X receptor (RXR) and the thyroid receptor (TR). AhR-based biosensors were designed to detect dioxin-like compounds, which can accumulate in the environment, and some of their members are known to cause health hazards in humans. As for the development of steroid receptor-based yeast biosensors, most of the research in the field of AhR yeast biosensors were conducted in *S. cerevisiae* (Miller 1999; Kawanishi et al. 2013) although *A. adeninivorans* was also used to build a similar biosensor (Pham et al. 2015). The strategy to detect dioxin-like compounds is similar to the strategy used to detect steroid hormones albeit with some modifications. To detect dioxin-like compounds, two genes have to be integrated into the yeast genome to express constitutively hAhR and the human aryl hydrocarbon receptor nuclear translocator (hARNT). Only the hAhR protein can bind to dioxin-like compounds, but it has to form a heterodimer with hARNT in order to translocate into the nucleus. Additionally in *A. adeninivorans*, the cyp1A1-derived core sequence replaced the *HRE* sequence in the *GAA* promoter which allowed the interaction of the promoter and the hAhR-hARNT heterodimer.

The construction of a yeast biosensor using the binding properties of RXR is described in *S. cerevisiae* by Kabiersch et al. (2013). RXR can bind organotin compounds, a large family of molecules shown to have a negative impact on gastropods reproductive organs and on mammal endocrine system. They proposed a hybrid receptor consisting in the ligand-binding domain of the RXR and the DNA-binding domain of the estrogen receptor α to function as a transcription factor.

Recently, yeast biosensors dedicated to the detection of compounds interfering with the thyroid receptor (TR) were also presented (Li et al. 2008; Shiizaki et al. 2010). TR acts as a transcriptional factor and can be found as a homodimer or a

heterodimer together with RXR. Several molecules present in our environment such as PCBs, pesticides, or dioxin are believed to bind to the thyroid receptor and have a negative impact on an organisms' development.

12.2.3 GPCR

In vertebrates, the largest family of receptors is the family of G protein-coupled receptors (GPCR). These membrane-bound receptors specifically bind a diverse group of molecules from neurotransmitters to hormones and large proteins but, contrary to nuclear receptors, they cannot directly act as transcription factors. The binding event induces a conformational change of the receptor, which will release a multimeric protein known as protein G which will then be able to activate downstream effectors. Two GPCR pathways are known in yeast, but their target ligands are often not of interest for biosensing applications. Additionally, knockout of these two endogenous GPCRs is often required to avoid interference and improve biosensor performance. This leads to the fact that heterologous GPCR from vertebrate origin have to be integrated in yeast genome in order to detect the molecule of interest. In some cases, these heterologous receptors can interact with the endogenous yeast mating pheromone pathway (Bardwell 2005). In other cases, a heterologous expression of the mammalian protein G is mandatory, thus adding more complexity to the system.

Despite the large number of GPCRs and the potentially much larger number of target ligands than steroid nuclear receptors, only few yeast biosensors have been developed. Much preliminary work has been performed in order to develop such biosensors, mostly in *S. cerevisiae* (Ault and Broach 2006; Dong et al. 2010). The presence of a GPCR pathway in other yeast species remains obscure but could be a path to the development of these biosensors.

To explain the relative absence of GPCR-based yeast biosensors compared to steroid receptor-based biosensors, one reason may be that target ligands have not been identified as hazardous to human health and the environment the way that EDCs have been.

12.3 Transduction of Biological Signal: Reporter Proteins

The second fundamental part of a biosensor is the part responsible for the transformation of a biological interaction, the binding of a ligand to its receptor, into a measurable signal. For this, reporter proteins have to be integrated into yeast.

Two main types of reporter protein are used in yeast biosensors: enzymes and fluorescent proteins. Enzymes were the first to be integrated and are still part of a large number of yeast biosensors. However, their main drawback is that these proteins have to be transported in the extracellular milieu in order to perform enzymatic reactions. This implies either the breakdown of the cells or the implementation of an extracellular signal sequence in the reporter gene coding sequence.

Fluorescent proteins as reporters are relatively new and allow direct observation of the reporter production without extracellular localization or cell rupture.

12.3.1 Utilization of Enzymes as Reporter Proteins

ß-Galactosidase, phytase, and tannase are the most common reporter enzymes used in yeast biosensors. In *S. cerevisiae*, the use of *lacZ* and *luc* reporter genes responsible for the production of β-galactosidase and the firefly luciferase, respectively, is often reported (Leskinen et al. 2005; Bovee et al. 2004; Balsiger et al. 2010). In *A. adeninivorans*, the *Klebsiella*-derived *phytase K* gene (*phyK*) and the *A. adeninivorans*-derived *tannase* gene (*ATAN1*) were used (Kaiser et al. 2010; Gerlach et al. 2014). Both *phyK* and *ATAN1* genes produce an extracellular located enzyme with can be then used for enzymatic reaction. Transformation of the substrate into reaction product can be then followed either by colorimetry, amperometry, or fluorescence.

It is notable that *ATAN1* was solely used in a biosensor for estrogen detection (Kaiser et al. 2010), while phyK was then successfully used in other *A. adeninivorans* biosensors using enzymatic detection.

12.3.1.1 Colorimetric Detection

Colorimetric detection is generally considered as the easiest and less expensive method to follow an enzymatic reaction. Chromogenic substrates such as red-ß-D-galactopyranoside (CPRG) for β-galactosidase or *p*-nitrophenyl phosphate (*p*NPP) for phytase are used, and the appearance of the colored products is monitored using a spectrophotometer. To measure enzyme activity, the classical protocol necessitates either collecting the extracellular milieu or lysing the cells in order to obtain the reporter enzyme. The enzymatic reaction itself generally takes place in a second plate and is followed in a spectrophotometer. Increase of the reaction product is then directly linked to the concentration of hormone in the cultivation medium. This reporting system is used in the *S. cerevisiae* YES/YAS/YPS/YGS biosensors and in the *A. adeninivorans* A-YES, A-YAS, A-YPS, and A-YGS biosensors.

12.3.1.2 Fluorescence, Chemiluminescence, and Bioluminescence Detection

One of the major disadvantages of colorimetric detection method is often the relatively long time the enzymatic reaction requires, which is generally hours, and the possible estrogenic properties of both CPRG and its enzymatic product. To overcome this, other substrates have been selected producing chemiluminescence, bioluminescence, or fluorescence. There are nowadays a large number of commercial substrates of the β-galactosidase; the following section will only present some examples which are used in yeast-based biosensors.

The work of Balsiger et al. (2010) introduces the use of the commercial chemiluminescent compound, Tropix Gal-Screen, instead of CPRG. In

chemiluminescence, chemical energy is transformed into light, and the enzymatic reaction can then be followed in a luminometer. Several new chemiluminescent substrates are now commercially available, each of them presenting advantages and disadvantages in terms of sensitivity, duration of light emission, or compatibility with other reporter systems.

Besides chemiluminescence, it should be noted that substrates producing a fluorescent products are also used in some yeast biosensors, for example, 4-methylumbelliferyl-b-D-galactopyranoside (MUG), a compound which reacts with β-galactosidase to form 4-methylumbelliferone. In contrast to chemiluminescence, excitation at 366 nm is required to detect the blue fluorescence of this product. This substrate is used in the recently developed planar YES assay (p-YES), a variant of the YES assay combining a yeast biosensor and a thin-layer chromatography (Buchinger et al. 2013).

A third class of enzyme substrate is also described in the literature, namely, the bioluminescent substrates. An example is the firefly luciferase (luc) enzyme used as a reporter protein and integrated in the *S. cerevisiae* genome (Bovee et al. 2004). The luciferase can accept D-luciferin as substrate and emits in response light in the visible spectrum. A major drawback of this strategy is that the cells have to be lysed in order to allow the reaction between the D-luciferin and the luciferase.

12.3.1.3 Electrochemical Detection

Electrochemical yeast-based biosensors use an electrochemical transducer to produce a signal proportional to the analyte concentration. The electrochemical transducer eventually converts a biochemical reaction between the reporter protein and a substrate into a measurable signal such as current, potential, or conductance, which is then amplified and processed (Lei et al. 2006; Mulchandani and Rogers 1998; Riedel et al. 2002; Shantilatha et al. 2003; Turner et al. 1992). Depending on the type of transducer, electrochemical biosensors are divided into three types: amperometric, potentiometric, and conductometric.

Among electrochemical biosensors, the amperometric biosensor is the most commonly used. It operates at fixed potential with respect to the reference electrode. Historically, it has been one of the preferred detection methods used for the construction of BOD biosensors and copper-sensing assays (Riedel et al. 2006). An interesting new application of electrochemical detection is the development of the "EstraMonitor" device, which can determine hormonal activities in sewage treatment plants (Pham et al. 2012, 2013). The "EstraMonitor" was demonstrated to be a rapid detection method for estrogenic activity measurement in a semi-online, automated, and continuous format. In common with the A-YES assay, the "EstraMonitor" uses a modified *A. adeninivorans* constitutively producing the estrogen receptor and phytase K as reporter protein. But instead of analyzing the phytase K enzymatic reaction by colorimetry, it is monitored by amperometry. The substrate is *p*-aminophenyl phosphate (*p*APP), a compound which can be oxidized to *p*-aminophenol in the presence of phytase. This reaction produces electrons, which can be detected as a current in the EstraMonitor detection chamber.

Measured current has been shown to be proportional to the level of phytase activity, thus allowing to a correlation between estrogen concentration and the current.

12.3.2 Direct Fluorescence Measurement

A new development in yeast biosensor reporter strategies is to use reporter proteins which produce fluorescence or bioluminescence without the need of a substrate. The advantages are obvious in terms of handling because cell lysis or extra detection plate is required and cost because chemiluminescent and bioluminescent substrates are often expensive. Two main strategies have been followed in the past years to detect direct fluorescence: the utilization of bacterial luciferase (lux) and the utilization of self-fluorescing proteins such as GFP or DsRed.

12.3.2.1 The Lux System

The lux system is the basis of the *S. cerevisiae* BLYES and BLYAS biosensors, which aim to detect estrogens and androgens, respectively (Sanseverino et al. 2008). There are several different bacterial luciferases, which are heterodimers composed of two subunits, α and β, and which are encoded by two genes in an operon, *luxa* and *luxb*. This protein catalyzes the oxidation of fatty acids, a reaction, which produces light at a wavelength of 490 nm. Situated in the same operon, three additional genes, *luxC, luxd*, and *luxE*, which are responsible for the synthesis of three enzymes, are involved in the production and recycling of fatty acids. Having all five genes on the same operon allows bioluminescence without the need of an external substrate. In BLYES and BLYAS, these five genes have been inserted into the *S. cerevisiae* genome, and, with a suitable promoter, translocation of the nuclear receptor homodimer results in the expression of all five genes, thus producing bioluminescence.

12.3.2.2 Self-Fluorescing Proteins

Parallel to the development of the lux system, self-fluorescing proteins also found application in the development of yeast biosensors. For instance, GFP is a reporter protein integrated in the estrogen-sensing biosensor developed by Beck et al. (2005). GFP, which was first isolated from the jellyfish *Aequorea victoria* and which is nowadays one of the most commonly used proteins in biotechnology, has the property of undergoing an internal cyclization leading to fluorescence emission without the need of an external partner. The protein is relatively stable and reliable fluorescence measurement can be performed after 6 h of incubation.

Another self-fluorescing protein, DsRed, was chosen as reporter in *A. adeninivorans*-based biosensors dedicated to the detection of progesterone and glucocorticoids (Chamas et al. 2015; Pham et al. 2016). DsRed was isolated from *Discosoma* and fluoresces in the orange-red section of the visible spectrum. The maturation time of DsRed is known to be longer than for GFP, thus leading to delay in the signal measurement, but this maturation time can be influenced via targeted mutation in order to obtain more rapid maturation and more intense fluorescence.

A major advantage of self-fluorescing proteins over a lux system as reporter is the extreme simplicity of the reporting strategy necessitating the integration of only one heterologous protein. The fact that GFP and DsRed have been studied in-depth for biotechnological application also presents the advantage that several new variants of these proteins emitting fluorescence at different wavelengths have been created. Only few amino acid substitutions led to the creation of a large number of new self-fluorescing proteins detectable in the blue, yellow, orange, and the UV part of the light spectrum. Targeted mutagenesis also led to the creation of enhanced version of GFP in terms of fluorescence yield, which have been incorporated into some yeast biosensors (Bovee et al. 2004).

This diversity of self-fluorescing proteins led to the idea of creating a yeast biosensor able to simultaneously sense different classes of hormone (Chamas et al. 2017). The *A. adeninivorans* biosensor is based on three different strains, each of them producing a different steroid hormone receptor coupled with a different self-fluorescing protein as reporter. By incubating these three strains as a mix, the resulting biosensor can detect estrogenic, progesteronic, and androgenic activities in a sample. The assay requires three fluorescence scans at the emission wavelengths of the three reporter proteins to be performed.

This example highlights the potential of self-fluorescence proteins as reporter proteins. In comparison, the lux system needs the integration of a complete operon, and there are fewer options available to modify the emission wavelength produced by the luciferase in this system. One of the major limitations of self-fluorescing proteins as reporter remains the possibility that the sample itself fluoresces at the same wavelength, thus increasing the noise signal and lowering the sensitivity of the assay.

12.4 Applications of EDCs Targeting Yeast Biosensors

12.4.1 Sensitivity of the Biosensors

In Table 12.1, two important characteristics for the selection of a biosensor are indicated, the EC_{50} value and the limit of detection toward a known ligand. The EC_{50} value corresponds to the concentration of ligand responsible for half the maximum binding to the receptor, whereas the limit of detection (LoD) is the lowest concentration of ligand, which can be effectively distinguished from the noise signal and therefore the lowest concentration of ligand a biosensor can detect. EC_{50} calculation is usually not contentious, but the method to determine the LoD varies among researchers and can lead to confusion. For more information about this thematic, we refer to the article of Shrivastava and Gupta (2011), which gives an overview of the existing guidelines and recommendations to establish LoD. Comparison of LoD in different manuscripts is difficult because often the method of calculation is not clearly given and LoDs should be treated with caution. In Table 12.1, these values are presented as they given in their manuscript.

Table 12.1 Description of several estrogen, androgen, progestogen and glucocorticoid yeast biosensors

Target compound	Organism	Biosensor	Reporter gene	Detection method	EC_{50}	LoD	Source
Estrogens	*S. cerevisiae*	YES	*β-Galactosidase*	Colorimetry	17β-Estradiol: 30 ng/l	17β-Estradiol: 2.8 ng/l	Gehrmann et al. (2016)
		YES	*β-Galactosidase*	Luminescence	17β-Estradiol: 40 ng/l	n.i.	Balsiger et al. (2010)
		YES	*Luc*	Luminescence	17β-Estradiol: 54.5 ng/l	n.i.	Bovee et al. (2004)
		BLYES	*Lux*	Luminescence	17β-Estradiol: 108.8 ng/l	17β-Estradiol: 6.8 ng/l	Sanseverino et al. (2008)
		YES	*Gfp*	Fluorescence	17β-Estradiol: 163.2 ng/l	17β-Estradiol: 27.2 ng/l	Beck et al. (2005)
		p-YES	*β-Galactosidase*	Fluorescence	17β-Estradiol: 7.5 pg	17β-Estradiol: 1.3pg	Buchinger et al. (2013)
	A. adeninivorans	A-YES	*PhyK*	Colorimetry	17β-Estradiol: 17 ng/l	17β-Estradiol: 1.8 ng/l	Gehrmann et al. (2016)
		EstraMonitor	*PhyK*	Amperometric	17β-Estradiol: 54.6 ng/l	17β-Estradiol: 9.92 ng/l	Pham et al. (2013)
	Y. lipolytica		*β-Galactosidase*	Colorimetry	n.i.	17β-Estradiol: 27.2 ng/l	Cho et al. (2010)
Androgens	*S. cerevisiae*	YAS	*β-Galactosidase*	Colorimetry	Testosterone: 1043 ng/l	Testosterone: 189 ng/l	Gehrmann et al. (2016)
		YAS	*Luc*	Luminescence	5α-DHT: 1.6 µg/l	5α-DHT: 145 ng/l	Leskinen et al. (2005)
		BLYAS	*Lux*	Luminescence	5α-DHT: 3.2 µg/l	5α-DHT: 290 ng/l	Sanseverino et al. (2008)
	A. adeninivorans	A-YAS	*PhyK*	Biochemical	5α-DHT: 277 ng/l	5α-DHT: 56.5 ng/l	Gerlach et al. (2014)

Progestogens	*S. cerevisiae*	YPS	*Gfp*	Fluorescence	Progesterone: 314 ng/l	Progesterone: 31.4 ng/l	Chatterjee et al. (2008)
	A. adeninivorans	A-YPS	*PhyK*	Biochemical	Progesterone: 147 ng/l	Progesterone: 40.9 ng/l	Chamas et al. (2015)
		A-YPFS	*DsRED*	Fluorescence	Progesterone: 231 ng/l	Progesterone: 65.3 ng/l	Chamas et al. (2015)
Glucocorticoids	*S. cerevisiae*	YGS	*GFP*	Fluorescence	Dexamethasone: 120 µM	n.i.	Bovee et al. (2011)
	A. adeninivorans	A-YGS	*PhyK*	Biochemical	Dexamethasone: 0.81 µM	Dexamethasone: 0.29 µM	Pham et al. (2016)

12.4.2 Protocols

Historically, yeast biosensors were developed in a microtiter plate format for the detection of water samples. It is then not surprising to observe that the majority of the published works are presenting protocols utilizing this format. Generally, the yeast cells are undergoing a preliminary cultivation without sample to reach a certain cell density, which is a critical parameter in order to obtain repeatable results. Then the cells are incubated with the sample in an incubation plate for a certain time, and the detection is performed. Depending on the reporter protein used, the cell suspension is directly utilized without further processing, or an extra detection protocol is needed. An overview of these different protocols can be observed in Fig. 12.2.

Direct detection is made possible by the use of self-fluorescing proteins and lux reporter systems by directly scanning the incubation plate for fluorescence. When enzymes are used as reporters, these have to be extracted from the cell—either by

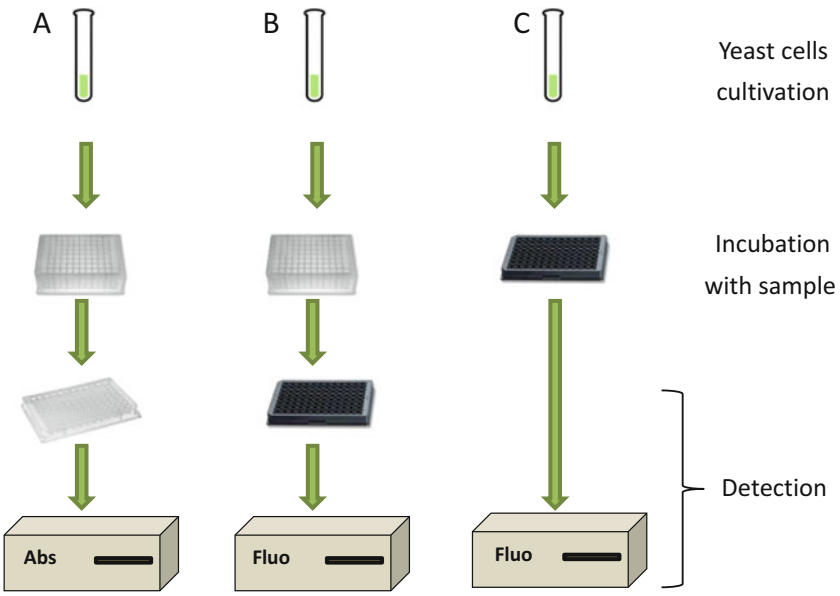

Fig. 12.2 Overview of three common microtiter protocols for the detection of hormones with yeast whole-cell biosensors. (**a**) General colorimetric detection protocol when an enzyme is the reporter protein. Incubation of cells with the sample takes place in multiwells incubation plates. For detection, cells are centrifuged, and the supernatant is transferred into new microtiter plate for enzyme assay. Detection occurs in spectrophotometer. (**b**) General fluorescence detection protocol when an enzyme is the reporter protein. Incubation of cells with the sample takes place in multiwells incubation plates. For detection, cells are centrifuged, and the supernatant is transferred into new black microtiter plate for enzyme assay. Detection occurs in fluorometer. (**c**) General fluorescence detection protocol when a fluorescence protein is the reporter protein. Incubation of cells with the sample and detection take place directly in black microtiter plate

cell breakdown or extracellular transport—and the enzyme solution is pipetted in a new microtiter plate for colorimetric or fluorescence measurement. Incubation parameters such as temperature, shaking, or duration of each step depend on the organism chosen and the nature of the reporter protein. When working with enzyme reporter in a microtiter plate format and besides the necessary devices for yeast incubation, the operator needs to have access to a spectrophotometer, a luminometer or a fluorometer, depending on the substrate they use. With self-fluorescing proteins as reporter proteins, only a fluorometer is needed to quantify the response.

A notable modification of the basic microtiter plate protocol can be seen in the planar yeast estrogen assay (Buchinger et al. 2013; Klingelhöfer and Morlock 2014). In this assay, the detection of estrogens is coupled with a prior separation of the sample on a thin-layer chromatography plate. The cells are directly incubated on the thin-layer chromatography plate, and the reporter activity, in this case β-galactosidase reaction with MUG, is followed via a fluorescence scan of the plate. For a complete identification of the potential endocrine disruptors, mass spectrometry can also be performed after the biosensor detection. An overview of this protocol can be seen in Fig. 12.3.

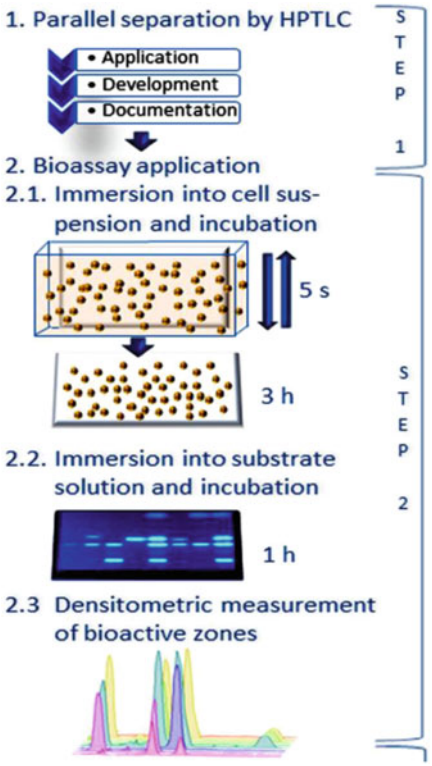

Fig. 12.3 Overview of planar YES assay (p-YES) first steps. In step one, components of the sample are separated with a high-performance thin-layer chromatography (HPTLC). Then the plate is shortly immersed in yeast cell suspension and allowed to incubate at 30 °C. After a second immersion in a substrate solution, the detection of fluorescent of compounds takes place in a densitometer. Modified after Klingelhöfer and Morlock (2015)

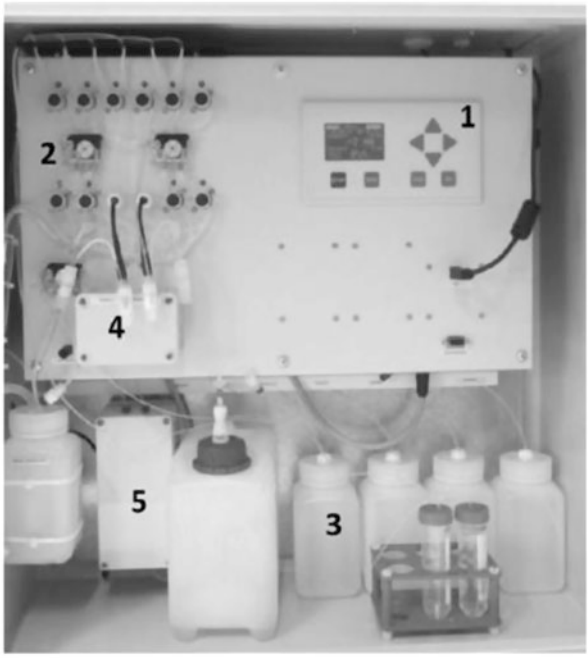

Fig. 12.4 Photography of the EstraMonitor device. (1) Amperometric transducer, (2) pump module for waste and nutrient exchange, (3) reservoir, (4) measuring chamber with immobilized yeast cells. Modified after Pham et al. (2012)

For amperometric detection, another protocol was designed which allows semi-automatic estrogen detection. For this, a device containing all the necessary fluidic, heating, and measuring elements was constructed (Fig. 12.4). The immobilized *A. adeninivorans* cells used in EstraMonitor are easily handled and can function in non-sterile conditions and can be reused up to 15 times without significant loss of sensitivity (Pham et al. 2012). Furthermore the use of immobilized cells and amperometric detection method makes EstraMonitor able to measure the total estrogenic activity in the sample in 4 h and 10 min. Interestingly, the immobilized *A. adeninivorans* cells are fully functional in a wide range of samples, including samples that contained up to 5% NaCl, and are able to determine the estrogenic effects in the environmental sample without the need for sterilization, extraction, or concentration (Pham et al. 2013). These results were also confirmed using GC-MS analyses.

12.4.3 Applications with Real Samples

The ultimate goal of yeast biosensors is to be trialed with real samples in order to identify potentially dangerous compounds in the environment or to monitor physiological level in humans and animals. A great majority of these real samples are wastewater samples because of their relative simplicity in comparison to body

fluids or solid samples, for example. Yeast biosensors are particularly valuable in analysis of wastewater samples because the cells are particularly robust.

The following table presents some of the researches applying yeast biosensor to real samples.

Real sample	Real sample type	Hormone activity	Organism	Detection	Source
Water based	Wastewater effluent/ influent	Estrogens	*S. cerevisiae*	Luminescence	Balsiger et al. (2010)
	Wastewater effluent/ influent	Estrogens	*A. adeninivorans*	Colorimetry	Kaiser et al. (2010)
	Wastewater effluent/ influent	Progesterone	*A. adeninivorans*	Colorimetry	Chamas et al. (2015)
	River water, wastewater effluent/ influent	Glucocorticoids	*A. adeninivorans*	Colorimetry	Pham et al. (2016)
	Surface water, wastewater effluent/ influent	Estrogens	*S. cerevisiae*	Fluorescence (p-YES)	Klingelhöfer and Morlock (2015)
	Reservoir water	Thyroids	*S. cerevisiae*	Colorimetry	Li et al. (2014)
Body fluids	Swine urine	Androgens	*A. adeninivorans*	Colorimetry	Gerlach et al. (2014)
	Swine urine	Estrogens	*A. adeninivorans*	Colorimetry	Kaiser et al. (2010)
	Monkey serum	Progesterone	*A. adeninivorans*	Fluorescence	Chamas et al. (2017)
	Human serum	Androgens	*S. cerevisiae*	Luminescence	Michelini et al. (2005)
Solid sample	Sediment	Organotins	*S. cerevisiae*	Luminescence	Kabierch et al. (2013)
	Moisturizing lotion	Estrogens	*S. cerevisiae*	Luminescence	Leskinen et al. (2005)
	Sunscreen, sediment	Estrogens	*S. cerevisiae*	Fluorescence (p-YES)	Buchinger et al. (2013)

12.5 Emerging Biosensors Strategies

The field of whole-cell biosensors is developing rapidly and many research teams around the world are proposing new strategies to establish innovative bioassays.

An example of an emerging strategy is the development of glucose sensing using yeast as the biocatalyst. In fact, because of the frequency of fluctuations in blood glucose levels, patients need to monitor their glucose levels several times a day

(Khadilkar et al. 2013; Hammond et al. 2016). Recently, Wang (Wang et al. 2015) reported a novel electrochemical glucose biosensor based on glucose oxidase (GOx) displayed on yeast surface. The entire *S. cerevisiae* cell catalyst (yeast-Gox) was immobilized with multiwalled carbon nanotubes on the electrodes, which allowed sensitive and selective glucose determination in a short time (Wang et al. 2015).

Another very interesting strategy is the one followed by Fukuda et al. (2010). They developed a yeast whole-cell biosensor able to detect organophosphorus compounds, which are in common use as pesticides. They constructed a *S. cerevisiae* strain that co-displays organophosphorus hydrolase (OPH) and enhanced green fluorescent protein (EGFP) on the cell surface using a Flo1p anchor system. Organophosphorus compound degradation by the hydrolase releases protons and causes a pH change. This pH change induces a conformational modification of EGFP, which triggers quenching of its fluorescence, thereby making this cell useful for visual detection of organophosphorus compounds presence.

12.6 Conclusions

Yeast whole-cell biosensors have proven themselves to be very useful for the detection of bioactive substances. Constant improvements in bioengineering and biochemistry have allowed the construction of robust biosensors, some of which have already been commercialized. Most of the yeast biosensors, which have been commercialized, are biosensors dedicated to the detection of vertebrate hormones. This is mainly due to the fact that endocrine disruptors gained wide notoriety because of their negative impact on environment and health. The range of possibilities is increasing as producing recombinant membrane G protein-coupled receptor is now not a technical obstacle. The different reporting strategies, which have been presented in this chapter, also allow the final user to select the most appropriate biosensor for their application. To choose a suitable biosensor, the user should not only consider the sensitivity of the assay toward one ligand but also take into account the time needed to perform the assay, which devices are necessary for the detection and for which the real sample assay have already been trialed with.

References

Adeniran A, Sherer M, Tyo KEJ (2015) Yeast-based biosensors: design and applications. FEMS Yeast Res 15:1–15

Ault AD, Broach JR (2006) Creation of GPCR-based chemical sensors by directed evolution in yeast. Protein Eng Des Sel 19:1–8

Balsiger HA, de la Torre R, Lee WY, Cox MB (2010) A four-hour yeast bioassay for the direct measure of estrogenic activity in wastewater without sample extraction, concentration, or sterilization. Sci Total Environ 408:1422–1429

Bardwell L (2005) A walk-through of the yeast mating pheromone response pathway. Peptides 26:339–350

Beck V, Pfitscher A, Jungbauer A (2005) GFP-reporter for high throughput assay to monitor estrogenic compounds. J Biochem Biophys Methods 64:19–37

Bhattacharjee GB, Khurana SMP (2014) In vitro reporter assays for screening of chemicals that disrupt androgen signaling. J Toxicol 2014:701752. doi:10.1155/2014/701752

Bovee TF, Helsdingen RJ, Hamers AR, Brouwer BA, Nielen MW (2011) Recombinant cell assay for the detection of (gluco)corticosteroids and endocrine-disrupting potencies of several environmental PCB contaminants. Anal Bioanal Chem 401:873–882

Bovee TF, Helsdingen RJR, Koks P, Kuiper HA, Hoogenboom RLAP, Keijer J (2004) Development of a rapid yeast estrogen bioassay, based on the expression of green fluorescent protein. Gene 325:187–200

Buchinger S, Spira D, Bröder K, Schlüsener M, Ternes T, Reifferscheid G (2013) Direct coupling of thin-layer chromatography with a bioassay for the detection of estrogenic compounds, application for effect-directed analysis. Anal Chem 85:7248–7256

Chamas A, Nieter A, Pham HTM, Giersberg M, Hettwer K, Uhlig S, Simon K, Baronian K, Kunze G (2015) Development of a recombinant *Arxula adeninivorans* cell bioassay for the detection of molecules with progesterone activity in wastewater. Anal Bioanal Chem 407:8109–8120

Chamas A, Pham HTM, Jähne M, Hettwer K, Uhlig S, Simon K, Einspanier A, Baronian K, Kunze G (2017) Simultaneous detection of three sex steroid hormone classes using a novel yeast-based biosensor. Biotechnol Bioeng. doi:10.1002/bit.26249

Chatterjee S, Kumar V, Majumder CB, Roy P (2008) Screening of some anti-progestin endocrine disruptors using a recombinant yeast based in vitro bioassay. Toxicol In Vitro 22:788–798

Cho EM, Lee HS, Eom CY, Ohta A (2010) Construction of high sensitive system for endocrine disruptors with yeast n-alkane –assimilating *Yarrowia lipolytica*. J Microbiol Biotechnol 20:1563–1570

Chu WL, Shiizaki K, Kawanishi M, Kondo M, Yagi T (2009) Validation of a new yeast-based reporter assay consisting of human estrogen receptors alpha/beta and coactivators SRC-1: application for detection of estrogenic activity in environmental samples. Environ Toxicol 24:513–529

Dong S, Rogan SC, Roth BL (2010) Directed molecular evolution of DREADDs: a generic approach to creating next-generation RASSLs. Nat Protoc 5:561–573

Fent K (2015) Progestins as endocrine disrupters in aquatic ecosystems: concentrations, effect and risk assessment. Environ Int 84:115–130

Fukuda T, Tsuchiya K, Makishima H, Tsuchiyama K, Mulchandani A, Kuroda K, Ueda M, Suye SI (2010) Organophosphorus compound detection on a cell chip with yeast coexpressing hydrolase and eGFP. Biotechnol J 5:515–519

Gehrmann L, Bielak H, Behr M, Itzel F, Lyko S, Simon A, Kunze G, Dopp E, Wagner M, Tuerk J (2016) (Anti-)estrogenic and (anti-)androgenic effects in wastewater during advanced treatment: comparison of three in vitro bioassays. Environ Sci Pollut Res. doi:10.1007/s11356-016-7165-7174

Gerlach TK, Kaiser C, Körner M, Hettwer K, Uhlig S, Simon K, Baronian K, Kunze G (2014) Development and assessment of a novel *Arxula adeninivorans* androgen screen (A-YAS) assay and its application in analysis of cattle urine. Sci Total Environ 490:1073–1081

Hahn T, Tag K, Riedel K, Uhlig S, Baronian K, Gellissen G, Kunze G (2006) A novel estrogen sensor based on recombinant *Arxula adeninivorans* cells. Biosens Bioelectron 21:2078–2085

Hammond JL, Formisano N, Estrela P, Carrara S, Tkac J (2016) Electrochemical biosensors and nanobiosensors. Essays Biochem 60:69–80

Kabiersch G, Rajasärkkä J, Tuomela M, Hatakka A, Virta M, Steffen K (2013) Bioluminescent yeast assay for detection of organotin compounds. Anal Chem 85:5740–5745

Kabir ER, Rahman MS, Rahman I (2015) A review on endocrine disruptors and their possible impacts on human health. Environ Toxicol Pharmacol 40:241–258

Kaiser C, Uhlig S, Gerlach T, Körner M, Simon K, Kunath K, Florschütz K, Baronian K, Kunze G (2010) Evaluation and validation of a novel *Arxula adeninivorans* estrogen screen (nAES)

assay and its application in analysis of wastewater, seawater, brackish water and urine. Sci Total Environ 408:6017–6026
Kawanishi M, Ohnisi K, Takigami H, Yagi T (2013) Simple and rapid yeast reporter bioassay for dioxin screening: evaluation of the dioxin-like compounds in industrial and municipal waste incineration plants. Environ Sci Pollut Res Int 20:2993–3002
Khadilkar KS, Bandgar T, Shivane V, Lila A, Shah N (2013) Current concepts in blood glucose monitoring. Indian J Endocrinol Metab 17(suppl 3):S643–S649
Klingelhöfer I, Morlock GE (2014) Sharp-bounded zones link to the effect in planar chromatography-bioassay-mass spectrometry. J Chromatogr A 1360:288–295
Klingelhöfer I, Morlock GE (2015) Bioprofiling of surface/wastewater and bioquantitation of discovered endocrine-active compounds by streamlined direct bioautography. Anal Chem 87:11098–11104
Lee HS, Cho EM, Jung JH, Ohta A (2007) Evaluation on antagonist activities of polycyclic aromatic hydrocarbons using the yeast two-hybrid detection system for endocrine disruptors. Environ Monit Assess 129:87–95
Lei Y, Chen W, Mulchandani A (2006) Microbial biosensors. Anal Chim Acta 566:200–210
Leskinen P, Michelini E, Picard D, Karp M, Virta M (2005) Bioluminescent yeast assay for detecting estrogenic and androgenic activity in different matrices. Chemosphere 61:259–266
Li J, Ma M, Wang Z (2008) A two-hybrid yeast assay to quantify the effects of xenobiotics on thyroid hormone-mediated gene expression. Environ Toxicol Chem 27:159–167
Li J, Ren S, Han S, Li N (2014) A yeast bioassay for direct measurement of thyroid hormone disrupting effects in water without sample extraction, concentration, or sterilization. Chemosphere 100:139–145
Michelini E, Magliulo M, Leskinen P, Virta M, Karp M, Roda A (2005) Recombinant cell-based bioluminescence assay for androgen bioactivity determination in clinical samples. Clin Chem 51:1995–1998
Miller CA (1999) A human aryl hydrocarbon receptor signaling pathway constructed in yeast displays additive responses to ligand mixtures. Toxicol Appl Pharmacol 160:297–303
Miller CA, Tan X, Wilson M, Bhattacharyya S, Ludwig S (2010) Single plasmids expressing human steroid receptors and a reporter gene for use in yeast signaling assays. Plasmid 63:73–78
Mulchandani A, Rogers KR (eds) (1998) Enzyme and microbial biosensors: techniques and protocols. Humana Press, Totowa
Pham HTM, Chamas A, Nieter A, Giersberg M, Rutten T, Gehrmann L, Hettwer K, Tuerk J, Uhlig S, Simon K, Baronian K, Kunze G (2016) Determination of glucocorticoids using photometric (A-YGS) and spectrofluorometric (A-YGFS) bioassays based on modified *Arxula adeninivorans* cells: applications in environmental analysis. Sensor Actuator B-Chem 223:540–549
Pham HTM, Giersberg M, Gehrmann L, Hettwer K, Tuerk J, Uhlig S, Hanke G, Weisswange P, Simon K, Baronian K, Kunze G (2015) The determination of pharmaceuticals using a recombinant *Arxula adeninivorans* whole-cell biosensor. Sensor Actuator B-Chem 211:439–448
Pham HTM, Giersberg M, Uhlig S, Hanke G, Simon K, Kunath K, Baronian K, Kunze G (2012) EstraMonitor – a monitor for amperometric detection of estrogenic activity with *Arxula adeninivorans* yeast cells as the biocomponent. Sens Actuator B-Chem 161:137–145
Pham HTM, Kunath K, Gehrmann L, Giersberg M, Tuerk J, Uhlig S, Hanke G, Simon K, Baronian K, Kunze G (2013) Application of modified *Arxula adeninivorans* yeast cells in an online biosensor for the detection of estrogenic compounds in wastewater samples. Sens Actuator B-Chem 185:628–637
Rajasärkkä J, Virta M (2013) Characterization of a bisphenol A specific yeast bioreporter utilizing the bisphenol A-targeted receptor. Anal Chem 85:10067–10074
Riedel K, Kunze G, Baronian KHR (eds) (2006) Biosensors and bioassays based on microorganisms. Research Signpost, Kerala

Riedel K, Kunze G, Konig A (2002) Microbial sensors on a respiratory basis for wastewater monitoring. In: Scheper T (ed) Advances in biochemical engineering/biotechnology. Springer, Berlin, pp 81–118

Routledge EJ, Sumpter JP (1996) Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen. Environ Toxicol Chem 15:241–248

Sanseverino J, Elridge ML, Layton AC, Easter JP, Yarbrough J, Schultz TW, Sayler GS (2008) Screening of potentially hormonally active chemicals using bioluminescent yeast bioreporters. Toxicol Sci 107:122–134

Sanseverino J, Gupta RK, Layton AC, Patterson SS, Ripp SA, Saidak L, Simpson ML, Schulz TW, Sayler GS (2005) Use of *Saccharomyces cerevisiae* BLYES expressing bacterial bioluminescence for rapid, sensitive detection of estrogenic compounds. Appl Environ Microbiol 71:4455–4460

Shantilatha P, Varma S, Mitra CK (2003) Designing a simple biosensor. In: Malhotra BD, Turner APF (eds) Advances in biosensors: perspectives in biosensors. JAI Press, Elsevier, Netherlands, pp 1–17

Shiizaki K, Asai S, Ebata S, Kawanishi M, Yagi T (2010) Establishment of yeast reporter assay systems to detect ligands of thyroid hormone receptors alpha and beta. Toxicol In Vitro 24:638–644

Shrivastava A, Gupta VB (2011) Methods for the determination of limit of detection and limit of quantification of the analytical methods. Chron Young Scientists 2:21–25

Tag K, Hahn T, Riedel K, Kunze G (2006) Assays and microbial biosensors for detection of endocrine disruptors. In: Riedel K, Kunze G, Baronian KHR (eds) Biosensors and bioassays based on microorganisms. Research Signpost, Kerala, pp 211–228

Turner APF, Karube I, Wilson GS (eds) (1992) Biosensors: fundamentals and applications. Mir Publishers, Moscow

Viswanath G, Halder S, Divya G, Majumder CB, Roy P (2008) Detection of potential (anti) progestagenic endocrine disruptors using a recombinant human progesterone receptor binding and transactivation assay. Cell Mol Endocrinol 295:1–9

Wang H, Lang Q, Liang B, Liu A (2015) Electrochemical glucose biosensor based on glucose oxidase displayed on yeast surface. Methods Mol Biol 1319:233–243

Yeast-Based Systems for Environmental Control

13

Galina Gayda, Mykhailo Gonchar, and Andriy Sibirny

Abstract

A wide range of dangerous pollutants, namely, heavy and transition metals, volatile toxicants, pesticides, plasticizers, pharmaceuticals, and natural and synthetic hormones, have to be tested in different environmental media to predict potential hazard for humans and nature as well to control the effectiveness of wastewater treatment. A lot of physicochemical analytical approaches have been developed, but they are not suitable for high-throughput control of chemicals in environmental samples. To support environmental safety, microbial analytical systems are considered to be very promising. In this review, the main achievements in the elaboration of analytical systems based on yeast cells for environmental monitoring of toxic pollutants are described, and perspectives of their implementation are discussed.

Contents

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13.1 Introduction

Extensive research over the past decade has found the widespread presence of wastewater, air, and soil contaminants around the globe. The increasing amount of pollutants in the environment is an alarming concern to the ecosystem (Fiore et al. 2015). These dangerous pollutants, namely, heavy and transition metals, volatile toxicants, pesticides, plasticizers, pharmaceuticals, and natural and synthetic hormones, being chemical spills, may be introduced into surface waters by runoff from land application of biosolids, through leaking sewer lines and septic systems, or by incomplete removal from wastewater treatment systems (Eldridge et al. 2011). A number of organic pollutants, such as polychlorinated biphenyls, polyaromatic hydrocarbons, and pesticides, are resistant to biodegradation and therefore represent toxicological threat to wildlife as well as human beings (Fiore et al. 2015). These chemicals affect on blood, cardiovascular, central nervous, respiratory, endocrine, reproductive, hormonal, and immune systems, causing even premature mortality (Rajasärkkä 2013; Thurston et al. 2016; Watanabe et al. 2017).

It is obvious that the special methods for environmental safety (monitoring dangerous chemicals and pollutant detoxification or removing) are necessary.

To improve environment quality, various physiological and biological methods have been employed globally. Several in vivo as well as in vitro assays were developed; however, as a rule, they are not suitable for high-throughput control of chemicals in environmental samples. Chemical analysis methods are sensitive, but they are also laborious and expensive and require specialized instruments. Consequently, microbiological methods have become valuable tools to test chemicals in environmental samples. While the European Union chemical legislation REACH has increased the need of chemical testing methods, one of its targets is also to decrease the use of animals in these tests. It has been proposed that inexpensive high-throughput in vitro assays could be used for initial screening of chemicals for further testing with other methods. To support environmental safety, microbiological methods based on utilization of yeast cells are considered as very promising (Chatterjee et al. 2008; Shimomura-Shimizu and Karube 2010; Sibirny et al. 2011; Eldridge et al. 2011; Rajasärkkä 2013; Dragone et al. 2014; Sigawi et al. 2014; Bui et al. 2016; Sun et al. 2015; He et al. 2016; Vigneshvar et al. 2016).

13.2 General Remarks

13.2.1 The Advantages of Yeast-Based Biosensors

Yeast-based biosensors (YBBs) have the ability to accurately detect a lot of compounds (see Table 13.1). YBBs appear to be quite promising for online wastewater control; their advantage is that organic compounds in samples can be directly measured without any pretreatment such as filtration and dialysis (Shimomura-Shimizu and Karube 2010; Dragone et al. 2014; Gutierrez et al. 2015; Jarque et al. 2016). High-specificity biosensing can be carried out in a micron-scale unit (the cell) without power requirements or the need for a highly trained operator. This can be contrasted with a Western blot or a gas chromatograph coupled with mass spectrometer, which is orders-of-magnitude larger and requires power and operators (Adeniran et al. 2015). Biosensors incorporating various yeasts have been proposed as the effective approach of synthetic biology to detect an incredibly large range of environmental pollutants including but not limited to metals, carcinogens, etc. (Hou et al. 2013; Bereza-Malcolm et al. 2015; Yudina et al. 2015). Although YBBs show promise for environmental monitoring, some disadvantages, as poor selectivity and low sensitivity, still remain. To overcome these limitations, YBBs have been integrated with innovative micro-/nanotechnologies and applied to a wide range of detection purposes. Micro-/nanofabrication showed remarkable potential for YBBs due to enhanced optical and electrochemical measurements, improved immobilization, and automated culture environments, as well as high portability and more practical in comparison with conventional analytical methods (Garcia-Alonso et al. 2011; Lim et al. 2015; Safarik et al. 2015).

Some yeast species, being facultative anaerobes, are ideal for engineering biosensors and suitable for online environmental monitoring for a number of reasons: (1) they can be made in "active dry" form cheaply and stored for long periods of time; (2) they can serve as a chassis for higher-eukaryotic sensing modalities (e.g., G-protein-coupled receptors, GPCRs); and (3) they can tolerate rather harsh environments, especially compared to bacteria. These advantages combine with the wealth of background information on *Saccharomyces cerevisiae* and its role as a model organism with simple genetic manipulations to make yeast an excellent choice for use in YBBs (Walmsley and Keenan 2000; Benton et al. 2007; Bovee et al. 2007, 2011; Adeniran et al. 2015; Chap. 12).

The most common reporting systems for YBBs are based on optical and electrical methods (Fig. 13.1) as well as cell growth monitoring (Nakamura et al. 2008; Vigneshvar et al. 2016). The advantages of optical methods are high sensitivity and simplicity; colorimetric variant is promising due to visible to naked eye results. Fluorescence and luminescence detection don't need substrate addition (Shetty et al. 2004; Leskinen et al. 2005; Garcia-Alonso et al. 2009; Eldridge et al. 2011). The main advantages of electric methods are high sensitivity and fastness of procedure (Schwartz-Mittelman et al. 2005; Ino et al. 2009; Chelikani et al. 2012; Adeniran et al. 2015).

Table 13.1 Yeast-based biosensors for detection of environmental pollutants

Group	Detected compound(s)	Mode of detection	Limit of detection, range (μM)	Yeast	References
Metals	Copper	Amperometry	0.11–500	*Saccharomyces cerevisiae*	Jarque et al. (2016)
		Colorimetry	1–100		
		Fluorescence	0.5		
		Luminescence	2.0		Vopalenska et al. (2015)
					Shetty et al. (2004)
					Roda et al. (2011)
		Pulse stripping voltammetry	0.1–100	*Rhodotorula mucilaginosa*	Turdean (2011)
	Cadmium	Fluorescence	0.2	*S. cerevisiae*	Matsuura et al. (2013)
			1–900	*Hansenula polymorpha*	Park et al. (2007) and Jarque et al. (2016)
	Methylmercury	Fluorescence	0.44 ng/L	*S. cerevisiae*	Tafurt-Cardona et al. (2015)
Endocrine disruptors	Thyroids	Luminescence	3.7×10^{-3}		Li et al. (2014) and Jarque et al. (2016)
		Colorimetry	0.5×10^{-3} to 0.075		Li et al. (2014, 2008) and Shizaki et al. (2010)
	Retinoids	Colorimetry	0.01		Shizaki et al. (2014) and Jarque et al. (2016)
		Luminescence	0.06		
					Kabiersch et al. 2013
	Tributyltin	Luminescence	0.03		Kabiersch et al. (2013)

	Androgens	Colorimetry	0.2×10^{-3}	*Arxula adeninivorans*	Gerlach et al. (2014)
		Luminescence	0.05×10^{-3}	*A. adeninivorans*, *S. cerevisiae*	
		Luminescence	0.05×10^{-3}		Michelini et al. (2008)
		Fluorescence	0.01×10^{-3}		Leskinen et al. (2005)
	Estrogens	Colorimetry	0.01×10^{-3}		Kaiser et al. (2010) and Jarque et al. (2016)
		Luminescence	$(0.05-2.8) \times 10^{-3}$		
		Luminescence	0.03×10^{-3}		
		Amperometry	0.01×10^{-3}		Sanseverino et al. (2005)
					Leskinen et al. (2005)
					Schwartz-Mittelman et al. (2005)
		Amperometry	5.3 ng/L	*A. adeninivorans*	Pham et al. (2012, 2013)
	Progestogens	Fluorescence	0.1×10^{-3} to 1	*S. cerevisiae*	Chatterjee et al. (2008)
		Colorimetry	1		
					Noguerol et al. (2006)
	Dioxin-like	Fluorescence	5×10^{-3}		Noguerol et al. (2006)
	Glucocorticoids	Fluorescence	0.5–10		Bovee et al. (2011)
	Bisphenol	Luminescence	0.11	*S. cerevisiae*	Rajasärkkä (2013)
	Serotonin	Fluorescence	10		Nakamura et al. (2015)
Genotoxins and cytotoxins	Chemicals	Fluorescence	0.1		Bui et al. (2016)
			10		
			50		

(continued)

Table 13.1 (continued)

Group	Detected compound(s)	Mode of detection	Limit of detection, range (μM)	Yeast	References
			10		Wei et al. (2013)
			3–45		Benton et al. (2007)
		Luminescence	NA		Leskinen et al. (2005)
	Pharmaceuticals	Amperometry	83.65 μg/L	*A. adeninivorans*	Pham et al. (2015)
	Methyl methanesulfonate	Microfluidic system		*Saccharomyces*, *Yarrowia*, *Kluyveromyces*, *Rhodotorula*	Safarik et al. (2015)
		Fluorescence	0.28 μM ~ 450	*S. cerevisiae*	Lim et al. (2015)
			0.1%		Garcia-Alonso et al. (2009)
	Formaldehyde	Amperometry	100	*H. polymorpha*	Sibirny et al. (2011)
	Inhibitors of respiratory system	Amperometry	0.8–1.2	*S. cerevisiae*	Goldblum et al. (1990)
	ROS inducers	Fluorescence	0.1		Yang et al. (2005)
Biodegradable organics	Chemicals which support the growth of microorganisms	Biochemical oxygen demand (BOD)	1.24 mg/L	*A. adeninivorans*	Chan et al. (2000)
			2.4–80 mg/L	Artificial mix: *Pichia angusta*, *A. adeninivorans*, *Debaryomyces hansenii*	Yudina et al. (2015)
		Fluorescence	2.1 $mg \cdot L^{-1}$, till 550 $mg \cdot L^{-1}$	*A. adeninivorans*	Renneberg et al. (2004)
		BOD	$(20–70) \cdot 10^3$		Nakamura et al. (2007)

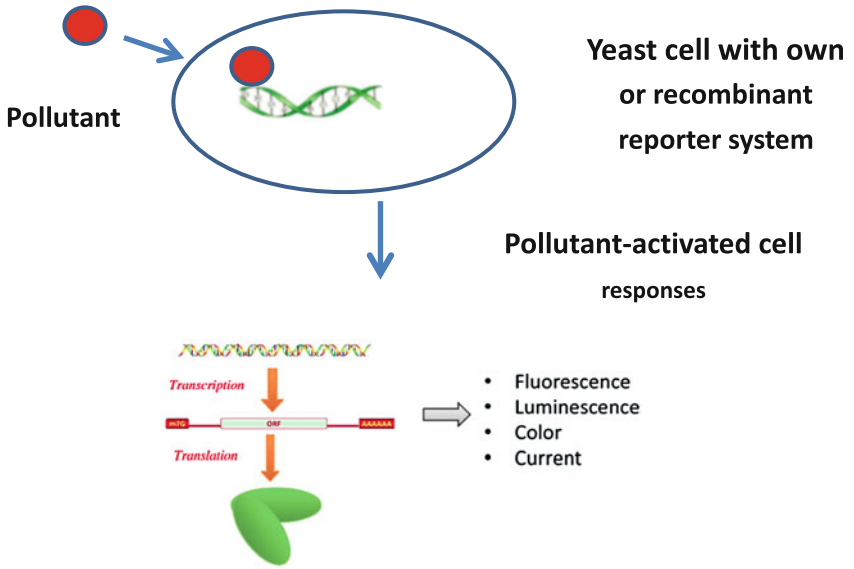

Fig. 13.1 Scheme of pollutant-activated yeast cell responses mediated via specific (own or recombinant) reporter system [modification of the scheme given by Adeniran et al. (2015)]

13.2.2 Types of YBBs

Two types of YBBs could be differentiated: dependent upon transcription ($YBBs^{DT}$) of a reported gene to detect a desired molecule and independent ($YBBs^{IDT}$) of this reporting mechanism (Adeniran et al. 2015).

$YBBs^{DT}$ frequently depend on heterologous expression of sensing elements from non-yeast organisms, a strategy that has greatly expanded the range of molecules available for detection by these biosensors. $YBBs^{DT}$ recognize the ligand of interest, typically via a native or heterologous receptor protein. When activated, the protein interacts with the cell's transcription machinery, either directly or via a signaling cascade, to induce (or cease) production of a reporter gene. $YBBs^{DT}$ could include receptors specific to organic compounds or heavy metals. In $YBBs^{DT}$ a fluorescence, luminescence, color, and current signal are usually detected (Eldridge et al. 2011; Adeniran et al. 2015).

$YBBs^{IDT}$ are promising for sensing difficult-to-detect analytes by modifying the yeast metabolism to generate easily detected molecules (redox potential, dissolved oxygen, etc.). $YBBs^{IDT}$ convert signals that are difficult to measure into ones that can easily be detected, using either by-products of metabolism or reporting systems triggered directly by analyte recognition. The changes in a fluorescence, luminescence, or current as well as of pH or oxygen signal were detected (Adeniran et al. 2015).

13.2.3 G-Protein-Coupled Receptors in Bioassays

G-protein-coupled receptors (GPCRs), being the members of the largest family of human membrane proteins and having similar basic structure, are able to selectively detect an incredibly diverse range of molecules including photons, ions, small organic molecules, and proteins. GPCRs undergo highly dynamic structural transitions during signal transduction, from binding of extracellular ligands to coupling with intracellular effector proteins (Jacobson 2015; Miao and McCammon 2016).

GPCRs are promising for development of YBBs, including bionose, due to (1) key similarities between the yeast mating pathway and the signaling mechanisms of higher organisms; (2) the fact that yeast has only two endogenous, non-interacting GPCR pathways; and (3) the ability of GPCRs to mediate intracellular changes in response to extracellular signals.

Many YBBs require expression of heterologous GPCRs that interface with the *S. cerevisiae* pheromone mating pathway. The incorporation of GPCRs from higher organisms with functionalities not naturally available to yeast greatly expands the capability of YBBs (Adeniran et al. 2015). To develop bionose, two strategies were proposed: (1) to replace or slightly modify the alpha subunit, which plays a key role in both activating and resetting the G-protein after a ligand-binding event (Fukutani et al. 2012); and (2) to replace the portions of the receptor itself, specifically the terminal domains and the first transmembrane regions (Hara et al. 2012).

13.3 Monitoring of Organic Pollutants Using Bioreporting Systems in *S. cerevisiae*

13.3.1 Recombinant Human Receptors in Assay of Endocrine-Disrupting Compounds

There is a growing need for suitable testing methods to screen compounds that may activate or inhibit human receptors and/or screen environmental samples for the presence of exogenous ligands or inhibitors, classified as hazardous endocrine disrupting compounds (EDC). The relative ease, portability, and rapidity of the YBBs' assay, in addition to chemical methods, make it a cost-effective choice for preliminary screening EDC potential of chemicals on humans and wildlife. For this purpose, several *S. cerevisiae* yeast-cell-based bioreporters (YBBRs) utilizing different nuclear receptors have been developed (Rajasärkkä 2013).

The most prolific class of YBBs is based on heterologous expression of human receptors: steroid estrogen and its analogs, as well as thyroid, androgen, glucocorticoid, mineralocorticoid and progesterone receptors, have all been recombinantly expressed, often multiple times with different reporting strategies (Kaiser et al. 2010). To assay estrogens, the sensitive analytical system BioLuminescent Yeast Estrogen Screen (BLYES) was proposed: the receptor is expressed continuously with reporter genes induced by the receptor-ligand dimer (Sanseverino et al. 2005).

YBBR assays have several advantages in environmental analytics. In addition to being inexpensive, they are particularly useful in determining the bioavailability of contaminants. Yeast is also very tolerant toward toxicity of different sample matrices. The use of the native human steroid receptors provides the most physiologically relevant information when screening an environmental sample (Rajasärkkä 2013).

13.3.2 The Main Strategies to Improve Biosensors for Organic Pollutants

The most of YBBR assays are usually not specific. To determine the total hormonal activity of wastewater samples, the number of YBBR assays was proposed. To improve assay specificity, directed evolution of intracellular receptors has been performed. The best of resulting mutant was shown to be fourfold more sensitive to bisphenol A and 166,000-fold less sensitive to the native ligand (Rajasärkkä 2013).

The yeast-based bacterial bioluminescent bioreporters were developed and used for the detection of EDCs (Eldridge et al. 2011; Rajasärkkä 2013). In addition to the major human endocrine receptors, there are organic compounds which influence other human nuclear receptors such as the aryl hydrocarbon receptor and retinoid X receptor. YBBs have been built around both of these receptors. However, two other strategies have also emerged for engineering: (1) engineering receptor fusions capable of detecting the desired ligand in yeast and (2) using a functional genomics approach to identify native yeast promoter activation that correlated with exposure to the desired ligand (Eldridge et al. 2011; Rajasärkkä 2013).

In certain situations, heterologous expression of the receptor system is not adequate to create an operational biosensor. With the use of a luciferase reporter, several organotin compounds (Caseri 2014) were detected at nanomolar levels (Kabiersch et al. 2013; Rajasärkkä 2013). This approach thus offers another method to construct YBBs when no native sensing elements are available. By fusing heterologous domains to well-characterized transactivators, the range of sensing domains available in yeast could be greatly expanded (Adeniran et al. 2015).

An alternative approach is to use functional genomics, where native yeast promoters are identified that have the desired properties. Organophosphates (pesticides and chemical weapons) are also a suitable target for YBBs. The applicability of functional genomics for the development of organophosphate-sensitive YBBs, when specific receptors are not known, was demonstrated. It is also possible, at least theoretically, to engineer promoters to get more desirable properties. It has been demonstrated that simple sequence changes in *S. cerevisiae* promoters can lead to a variety of phenotypic effects (Nevoigt et al. 2007). Such techniques will be promising for further improvement of biosensor performance.

A prototype semiautomated bioassay for genotoxic compounds, based on the expression of a green fluorescent protein (GFP) in genetically modified yeast cells in response to DNA damage, has been developed and successfully tested with the alkylating agent methyl methanesulfonate (0.01%). A flow-through detector has

been developed for GFP expressed in genetically modified yeast cells upon exposure to toxic chemicals. The detector forms the basis of a prototype semiautomated bioassay for genotoxic compounds, by biological fluorescence induction, and detection. The selection and optimization of the various optical components of the system are discussed. The complete system was successfully tested with a known DNA-damaging agent and was able to monitor the expression of GFP online, in situ, and in real time (Benton et al. 2007; García-Alonso et al. 2011).

Innovative microfluidic technologies showed many advantages by minimizing the sample and reagent volumes required, shortening analysis time with high resolution as well as repeatability, and demonstrating multiple assays on a chip in a high-throughput manner (Ino et al. 2009; Jarque et al. 2016). Microfluidic biosensor (MFB) containing eight multi-parallel channels has been developed to enable the screening of chemicals for toxicity. The recombinant yeast *S. cerevisiae* used was GreenScreen trademark which expresses GFPs when exposed to genotoxins. After exposure of the yeast to methyl methanesulfonate, the fluorescence emission was detected using an inverted microscope. Qualitative and quantitative comparisons of the fluorescent emission were performed. The device has the potential for use by industrial manufacturers to detect and reduce the production and discharge of toxic compounds, as well as to characterize already polluted environments (García-Alonso et al. 2009; Safarik et al. 2015).

To evaluate toxic chemicals that disrupt thyroid hormone (TH), several YBBR bioassays were developed (Shiizaki et al. 2010; Kabiersch et al. 2013; Li et al. 2014). Four tested compounds, tetrabromobisphenol A, tetramethyl bisphenol A, 2-isopropylphenol, and *o-t*-butylphenol, which are suspected to have TH-disrupting activity, were demonstrated to have agonistic activities in both assay yeasts. So, these YBBR bioassays will be powerful tools for assessing TH ligand activity of industrial chemicals and environmental pollutants (Kabiersch et al. 2013). For rapid and sensitive evaluation of TH disruption at the level of thyroid receptor (TR), two simple and fast modified bioassays, a colorimetric (CBA) and a chemiluminescent (ChBA) with the usage of β-galactosidase, were developed. The compounds tested included the known thyroid hormone 3,3′,5-triiodo-L-thyronine, the specific TR antagonist amiodarone hydrochloride (AH) and phthalate esters (PAEs), which potentially disrupt thyroid hormone signaling. None of the tested PAEs induced β-galactosidase expression, but diethylhexyl phthalate, benzyl butyl phthalate, and dibutyl phthalate demonstrated TR antagonism. Although TR agonism was not observed, antagonism was detected in all water samples and is expressed as AH equivalents. The toxicology equivalent quantity values obtained by the ChBA ranged from 21.2 ± 1.6 to 313.9 ± 28.8 μg/L AH, and similar values were obtained for the CBA (Li et al. 2014).

13.4 Other Yeasts in Environmental Control

The number of biochemical oxygen demand (BOD)-type biosensors for different chemicals was proposed (Chan et al. 2000; Ponomareva et al. 2011; Yudina et al. 2015). YBB with thick-film oxygen electrode as a transducer contains immobilized yeast *Arxula adeninivorans* as bioelement. Agonistic and antagonistic actions of phenols, phthalates, and organochlorine pesticides were tested by this YBB in the absence and presence of 5 μM 9-cis RA, at which maximal beta-galactosidase activity could be induced. The main analytical parameters of the developed YBB were determined, and domestic wastewater was analyzed (Chan et al. 2000). In the other variant of YBB, artificial microbial cocultures of the yeasts *Pichia angusta*, *A. adeninivorans*, *and Debaryomyces hansenii* were formed to develop the receptor element. This YBB, containing the cocultures immobilized in *N*-vinylpyrrolidone-modified poly(vinyl alcohol), possessed broad substrate specificities and enabled assays of water and fermentation products within a broad BOD range (2.4–80 mg/L) with a high correlation ($R = 0.9988$) to the standard method (Yudina et al. 2015).

Yeasts *Saccharomyces*, *Kluyveromyces*, *Rhodotorula*, and *Yarrowia* modified with magnetic micro- and nanoparticles (NPs) were shown to be employed as YBBs in toxicity microscreening devices as well as efficient adsorbents for removing different xenobiotics. Magnetically modified yeast cells can be used as part of cost-effective microfluidic biosensor (MFB) systems (see Table 13.1). One such screening method used viable yeast cells containing genetically modified GFP reporter. Cells were magnetically functionalized by biocompatible positively charged magnetic NPs, with diameters of 15 nm and held within an MFB. The GFP reporter yeast cells were used to detect genotoxicity by monitoring the exposure of the cells to methyl methanesulfonate; effective fluorescence emitted from the produced GFP was measured. The magnetically enhanced retention of the yeast cells, with their facile subsequent removal and reloading, allowed very convenient and rapid screening of genotoxic compounds (Safarik et al. 2015).

A YBB with cells of *A. adeninivorans* LS3 immobilized in the hydrogel poly (carbamoyl)sulfonate has been developed for measurement of biodegradable substances. The immobilized yeast membrane was placed in front of a BOD with −600 mV versus Ag/AgCl. *Arxula* is salt tolerant; it can give a stable signal up to 2.5 M NaCl in sample (120 mM in measuring cell). The YBB has a wide linear range (up to a corresponding BOD value of 550 mg/L) and very high stability (Renneberg et al. 2004).

The number of other effective YBBs was proposed with recombinant yeast *A. adeninivorans*: amperometric sensors for the determination of pharmaceuticals and chemicals such as omeprazole, lansoprazole, β-naphthoflavone, and methylcholanthrene, semi-*online* operation device, and the EstraMonitor for assay of estrogenic substances (Pham et al. 2012, 2013, 2015).

Formaldehyde (FA) is a highly reactive compound with a toxic effect on all organisms due to nonspecific interactions with proteins and nucleic acids. To develop effective methods for FA detection, the cells of the thermotolerant

methylotrophic yeast *Hansenula polymorpha* were employed (Sibirny et al. 2011). The cells of recombinant strain Tf-6 and mutant strain C-105 (*gcr1 catX*) were used as catalytic elements in amperometric YBBs. The chosen yeast cells were shown to be resistant to elevated concentrations of FA in the medium due to their ability to overproduce the corresponding yeast enzymes: formaldehyde dehydrogenase and alcohol oxidase. Experimental data confirm the possibility of exploiting the developed YBBs and bioreactors, based on methylotrophic yeast cells, for sensitive FA assay and its detoxification (Sigawi et al. 2011, 2014; Gayda et al. 2015). The reliability of the proposed analytical approaches was tested on model solutions and on real samples of FA-containing industrial wastes. A strong cross-correlation was found between FA content values obtained by the developed methods and routinely used chemical ones (Sibirny et al. 2011).

13.5 Monitoring of Heavy and Transitional Metals

Environmental sensing of heavy metals through whole cell microbial biosensors, including YBBs, was described in detail recently by Adeniran et al. (2015), Bereza et al. (2015), Gutiérrez et al. (2015), Vopálenská et al. (2015), Jarque et al. (2016), and others. The main types of described YBBs and their characteristics are presented in Table 13.1.

Contamination of water by heavy metals represents a potential risk for both aquatic and terrestrial organisms, including humans (Teo and Wong 2014). Heavy metals in water resources can come from various industrial activities, and drinking water can be ex post contaminated by heavy metals such as Cu^{2+} from house fittings (e.g., water reservoirs) and pipes. A new copper biosensor that based on cells of a specifically modified *S. cerevisiae* strain immobilized in alginate beads was developed. This optical biosensor was capable of detecting copper ions at concentrations of 1–100 μM. The YBB was successfully tested in the determination of copper concentrations in real samples of water contaminated with copper ions. In contrast to analytical methods or other biosensors based on fluorescent proteins, the newly designed YBB does not require specific equipment and allows the quick detection of copper in many parallel samples (Vopálenská et al. 2015).

YBBs are well suited to monitor heavy metals due to their availability to distinguish between bioavailability and concentration—an important distinction, as only certain forms of ions have biological effects. Bacterial heavy metal biosensors have seen significantly more developed than yeast-based systems, including biosensors for Cd, Pb, Hg, Cr, Ni, Co, Zn, Cu, and As (Adeniran et al. 2015). This disparity may be due to knowledge about the numerous metal-specific resistance operons in *Escherichia coli* which made it easy to identify specific metal responsive promoters. Only two metal-specific toxicity resistance genes (for As and Cd) are well characterized in *S. cerevisiae* (Wysocki and Tamás 2010). Nevertheless, several yeast-based metal biosensors have been developed using either optical or electrical reporting systems (Bereza-Malcolm et al. 2015; Matsuura et al. 2013).

Two such systems measure copper, which is an essential cofactor for all life forms at low concentrations, but is toxic and causes an environmental concern at high concentrations. Both systems take advantage of the Cu^{2+} inducible promoter which regulates expression of the *CUP1* copper-binding protein. The electrical method uses the promoter to drive expression of *LacZ* to enable lactose metabolism (Lehmann et al. 2000; Tag et al. 2007). The yeasts are incubated with lactose and the increase in metabolism, and therefore oxygen consumption, associated with promoter activation, is measured by an electrode, similar to the BOD systems. The optical approach uses the *CUP1* promoter to drive the firefly luciferase gene (Leskinen et al. 2003). The luciferase gene was modified from the wild type to remove peroxisome-targeting peptides, which improved cellular viability and signal strength. Both systems report a similar detection limit (1 μM). The electrical system requires cell immobilization and more specialized flow injection analysis equipment; however, the assay itself is faster as no incubation period is required. The electrical biosensor demonstrated agreement with chemical analyses on wastewater samples (Tag et al. 2007), while the optical biosensor was used to demonstrate copper bioavailability in a series of metal nanoparticle toxicity studies (Aruoja et al. 2009; Kasemets et al. 2009).

The advantages and disadvantages of using eukaryotic microorganisms to design biosensors for monitoring environmental heavy metal pollution in soil or aquatic habitats were discussed. A comparative analysis of the promoter genes used to design biosensors, based on yeast (*S. cerevisiae*), microalgae, and ciliated protozoa, is carried out, and the sensitivity and reproducibility of the main reporter genes used is calculated (Gutiérrez et al. 2015).

YBB optical sensing system for copper was developed based on recombinant *S. cerevisiae* cells harboring plasmid pYEX-GFPuv. The basis of this system was the ability of the transcriptional activator protein Ace1 present in *S. cerevisiae* to control the expression of the reporter protein, GFPuv. When copper ions are present in the sample, the Ace1 protein activates the *CUP1* promoter located upstream from the GFPuv gene in plasmid pYEX-GFPuv, thus inducing the production of GFPuv. The concentration of copper ions in the sample can then be related to the GFPuv expressed in the yeast. The amount of GFPuv produced in the system was determined by monitoring the fluorescence emitted at 507 nm after excitation at 397 nm. This system can detect Cu^{2+} at concentrations as low as 0.5 μM and is selective for Cu^{2+} over a variety of metal ions, with the exception of silver. The applicability of this sensing system to different analytical platforms and in real samples was demonstrated (Shetty et al. 2004).

For selective determination of methylmercury (MeHg), *S. cerevisiae* cells were used. Cells were immobilized in agarose gel as binding phase and polyacrylamide as diffusive layer in the diffusive gradient in thin films (DGT) technique. Deployment tests showed good linearity in mass uptake up to 48 h (3276 ng). When coupling the DGT technique with cold vapor atomic fluorescence spectrometry, the method has a limit of detection of 0.44 ng/L (preconcentration factor of 11 for 48 h deployment). Influence of ionic strength (from 0.5 mM to 0.1 M NaCl) and pH (from 3.5 to 8.5) on MeHg uptake were evaluated. For these ranges, recoveries of

84–105% and 84–98% were obtained for ionic strength and pH, respectively. Potential interference due to the presence of Cu, Fe, Mn, and Zn was also assessed showing good recoveries (70–87%). The selectivity of the proposed approach was tested by deployments in solutions containing MeHg and Hg(II). Results obtained showed recoveries of 102–115% for MeHg, while the uptake of Hg(II) was insignificant. The proposed approach was successfully employed for in situ measurements in the Negro River (Tafurt-Cardona et al. 2015).

13.6 Conclusion

Yeast cells, specially engineered by introducing specific receptors and highly sensitive reporter systems, seem to be very promising and prospective for rapid and sensitive monitoring of dangerous environmental pollutants. It can be predicted that after standardization, such systems will be candidates for commercialization and a broad use in different fields related to environmental control.

References

Adeniran A, Sherer M, Tyo KEJ (2015) Yeast-based biosensors: design and applications. FEMS Yeast Res 15:1–15

Aruoja V, Dubourguier H-C, Kasemets K, Kahru A (2009) Toxicity of nanoparticles of CuO, ZnO and TiO2 to microalgae *Pseudokirchneriella subcapitata*. Sci Total Environ 407:1461–1468

Benton MG, Glasser NR, Palecek SP (2007) The utilization of a *Saccharomyces cerevisiae* HUG1P-GFP promoter–reporter construct for the selective detection of DNA damage. Mutat Res 633:21–34

Bereza-Malcolm LT, Mann G, Franks AE (2015) Environmental sensing of heavy metals through whole cell microbial biosensors: a synthetic biology approach review. ACS Synth Biol 4 (5):535–546

Bovee TFH, Helsdingen RJR, Hamers ARM et al (2007) A new highly specific and robust yeast androgen bioassay for the detection of agonists and antagonists. Anal Bioanal Chem 389:1549–1558

Bovee TFH, Helsdingen RJR, Hamers ARM et al (2011) Recombinant cell bioassays for the detection of (gluco)corticosteroids and endocrine-disrupting potencies of several environmental PCB contaminants. Anal Bioanal Chem 401:873–882

Bui VN, Nguyen TTH, Mai CT et al (2016) Procarcinogens – determination and evaluation by yeast-based biosensor transformed with plasmids incorporating RAD54 reporter construct and cytochrome P450 genes. PLoS One 11(12):e0168721. doi:10.1371/journal.pone.0168721

Caseri W (2014) Initial organotin chemistry. J Organomet Chem 751:20–24

Chan C, Lehmann M, Chan K et al (2000) Designing an amperometric thick-film microbial BOD sensor. Biosens Bioelectron 15(7-8):343–353

Chatterjee S, Kumar V, Majumder CB, Roy P (2008) Screening of some anti-progestin endocrine disruptors using a recombinant yeast based *in vitro* bioassay. Toxicol In Vitro 22:788–798

Chelikani V, Downard AJ, Kunze G et al (2012) Investigating yeast cell responses to oestrogen by electrochemical detection. Electrochim Acta 73:136–140

Dragone R, Frazzoli C, Grasso G, Rossi G (2014) Sensor with intact or modified yeast cells as rapid device for toxicological test of chemicals. J Agric Chem Environ 3:35–40

Eldridge M, Sanseverino J, Umbuzeiro GA, Sayler GS (2011) Analysis of environmental samples with yeast-based bioluminescent bioreporters. In: Ekundayo E (ed) Environmental monitoring. InTech, Rijeka. doi:10.5772/27139

Fiore AM, Naik V, Leibensperger EM (2015) Air quality and climate connections. J Air Waste Manag Assoc 65(6):645–685

Fukutani Y, Nakamura T, Yorozu M et al (2012) The N-terminal replacement of an olfactory receptor for the development of a yeast-based biomimetic odor sensor. Biotechnol Bioeng 109:205–212

García-Alonso J, Greenway GM, Hardege JD, Haswell SJ (2009) A prototype microfluidic chip using fluorescent yeast for detection of toxic compounds. Biosens Bioelectron 24:1508–1511

García-Alonso J, Fakhrullin RF, Paunov VN et al (2011) Microscreening toxicity system based on living magnetic yeast and gradient chips. Anal Bioanal Chem 400:1009–1013

Gayda G, Demkiv O, Klepach H et al (2015) Formaldehyde: detection and biodegradation, Chapter 6. In: Patton A (ed) Formaldehyde: synthesis, applications and potential health effects. Nova Science, New York, pp 117–142. ISBN:978-1-63482-412-5

Gerlach T et al (2014) Development and assessment of a novel *Arxula adeninivorans* androgen screen (A-YAS) assay and its application in analysis of cattle urine. Sci Total Environ 490:1073–1081

Goldblum DK, Holodnick SE, Mancy KH, Briggs DE (1990) Oxygen transport in biofilm electrodes for screening of toxic chemicals. AIChE J 36:19–28

Gutiérrez JC, Amaro F, Martín-González A (2015) Heavy metal whole-cell biosensors using eukaryotic microorganisms: an updated critical review. Front Microbiol 6:48. doi:10.3389/fmicb.2015.00048

Hara K, Inada Y, Ono T et al (2012) Chimeric yeast G-protein α subunit harboring a 37-residue C-terminal gustducin-specific sequence is functional in *Saccharomyces cerevisiae*. Biosci Biotechnol Biochem 76:512–516

He W, Yuan S, Zhong WH et al (2016) Application of genetically engineered microbial whole-cell biosensors for combined chemosensing. Appl Microbiol Biotechnol 100(3):1109–1119

Hou QH, Ma AS, Zhuang XL, Zhuang GQ (2013) Advance in the bioavailability monitoring of heavy metal based on microbial whole-cell sensor. Huan Jing Ke Xue 34(1):347–356

Ino K, Kitagawa Y, Watanabe T et al (2009) Detection of hormone active chemicals using genetically engineered yeast cells and microfluidic devices with interdigitated array electrodes. Electrophoresis 30:3406–3412

Jacobson KA (2015) New paradigms in GPCR drug discovery. Biochem Pharmacol 98 (4):541–555

Jarque S, Bittner M, Blaha L, Hilscherova K (2016) Yeast biosensors for detection of environmental pollutants: current state and limitations review article. Trends Biotechnol 34 (5):408–419

Kabiersch G, Rajasärkkä J, Tuomela M et al (2013) Bioluminescent yeast assay for detection of organotin compounds. Anal Chem 85:5740–5745

Kaiser C, Uhlig S, Gerlach T et al (2010) Evaluation and validation of a novel *Arxula adeninivorans* estrogen screen (nAES) assay and its application in analysis of wastewater, seawater, brackish water and urine. Sci Total Environ 408:6017–6126

Kasemets K, Ivask A, Dubourguier HC, Kahru A (2009) Toxicity of nanoparticles of ZnO, CuO and TiO2 to yeast *Saccharomyces cerevisiae*. Toxicol In Vitro 23:1116–1122

Lehmann M, Riedel K, Adler K, Kunze G (2000) Amperometric measurement of copper ions with a deputy substrate using a novel *Saccharomyces cerevisiae* sensor. Biosens Bioelectron 15:211–219

Leskinen P, Virta M, Karp M (2003) One-step measurement of firefly luciferase activity in yeast. Yeast 20(13):1109–1113

Leskinen P, Michelini E, Picard D, Karp M, Virta M (2005) Bioluminescent yeast assays for detecting estrogenic and androgenic activity in different matrices. Chemosphere 61(2):259–266

Li J, Ma M, Wang Z (2008) A two-hybrid yeast assay to quantify the effects of xenobiotics on thyroid hormonemediated gene expression. Environ Toxicol Chem 27(1):159
Li J, Ren S, Han S, Li N (2014) A yeast bioassay for direct measurement of thyroid hormone disrupting effects in water without sample extraction, concentration, or sterilization. Chemosphere 100:139–145
Lim JW, Ha D, Lee J et al (2015) Review of micro/nanotechnologies for microbial biosensors. Front Bioeng Biotechnol 3:61. doi:10.3389/fbioe.2015.00061
Matsuura H, Yamamoto Y, Muraoka M et al (2013) Development of surface-engineered yeast cells displaying phytochelatin synthase and their application to cadmium biosensors by the combined use of pyrene-excimer fluorescence. Biotechnol Prog 29:1197–1202
Miao Y, McCammon JA (2016) G-protein coupled receptors: advances in simulation and drug discovery. Curr Opin Struct Biol 41:83–89
Michelini E, Cevenini L, Mezzanotte L et al (2008) A sensitive recombinant cell-based bioluminescent assay for detection of androgen-like compounds. Nat Protoc 3:1895–1902
Nakamura H, Suzuki K, Ishikuro H et al (2007) A new BOD estimation method employing a double-mediator system by ferricyanide and menadione using the eukaryote *Saccharomyces cerevisiae*. Talanta 72:210–216
Nakamura H, Shimomura-Shimizu M, Karube I (2008) Development of microbial sensors and their application. Adv Biochem Eng Biotechnol 109:351–394
Nakamura Y, Ishii J, Kondo A (2015) Applications of yeast-based signaling sensor for characterization of antagonist and analysis of site-directed mutants of the human serotonin 1A receptor. Biotechnol Bioeng 112(9):1906–1915
Nevoigt E, Fischer C, Mucha O et al (2007) Engineering promoter regulation. Biotechnol Bioeng 96:550–558
Noguerol T-N, Boronat S, Jarque S et al (2006) Detection of hormone receptor ligands in yeast by fluorogenic methods. Talanta 69:351–358
Park JN, Sohn MJ, Oh DB et al (2007) Identification of the cadmium-inducible *Hansenula polymorpha SEO1* gene promoter by transcriptome analysis and its application to whole-cell heavy-metal detection systems. Appl Environ Microbiol 73(19):5990–6000
Pham HTM, Giersberg M, Uhlig S et al (2012) EstraMonitor – a monitor for amperometric detection of estrogenic activity with *Arxula adeninivorans* yeast cells as the biocomponent. Sens Actuators B 161(1):137–145
Pham HTM, Kunath K, Gehrmann L et al (2013) Application of modified *Arxula adeninivorans* yeast cells in an online biosensor for the detection of estrogenic compounds in wastewater samples. Sens Actuators B 185:628–637
Pham HTM, Giersberg M, Gehrmann L et al (2015) The determination of pharmaceuticals in wastewater using a recombinant *Arxula adeninivorans* whole cell biosensor. Sens Actuators B 211:439–448
Ponamoreva ON, Arliapov VA, Alferov VA et al (2011) Microbial biosensors for detection of biological oxygen demand (a review). Prikl Biokhim Mikrobiol 47(1):5–15
Rajasärkkä J (2013) Development of high-throughput yeast-cell-based bioreporter assays for specific monitoring of bisphenol A and chemical testing of endocrine disrupting compounds. Academic Dissertation, Helsinki, 50 p
Renneberg T, Kwan RCH, Chan C et al (2004) A salt-tolerant yeast-based microbial sensor for 24 hour community wastewater monitoring in coastal regions. Microchim Acta 148:235–240
Roda A, Roda B, Cevenini L et al (2011) Analytical strategies for improving the robustness and reproducibility of bioluminescent microbial bioreporters. Anal Bioanal Chem 401:201–211
Safarik I, Maderova Z, Pospiskova K et al (2015) Magnetically responsive yeast cells: methods of preparation and applications. Yeast 32:227–237
Sanseverino JA, Gupta RKA, Layton ACA et al (2005) Use of *Saccharomyces cerevisiae* BLYES expressing bacterial bioluminescence for rapid, sensitive detection of estrogenic compounds. Appl Environ Microbiol 71:4455–4460

Schwartz-Mittelman A et al (2005) Electrochemical detection of xenoestrogenic and antiestrogenic compounds using a yeast twohybrid-17-b-estradiol system. Bioelectrochemistry 65:149–156

Shetty RS, Deo SK, Liu Y, Daunert S (2004) Fluorescence-based sensing system for copper using genetically engineered living yeast cells. Biotechnol Bioeng 88(5):664–670

Shiizaki K, Asai S, Ebata S et al (2010) Establishment of yeast reporter assay systems to detect ligands of thyroid hormone receptors alpha and beta. Toxicol In Vitro 24:638–644

Shiizaki K, Yoshikawa T, Takada E et al (2014) Development of yeast reporter assay form screening specific ligands of retinoic acid and retinoid X receptor subtypes. J Pharmacol Toxicol Methods 69:245–252

Shimomura-Shimizu M, Karube I (2010) Yeast based sensors. Adv Biochem Eng Biotechnol 117:1–19

Sibirny V, Demkiv O, Sigawi S et al (2011) Formaldehyde oxidizing enzymes and genetically modified yeast hansenula polymorpha cells in monitoring and removal of formaldehyde, Chapter 6. In: Einschlag FSG (ed) Waste water – evaluation and management. InTech Open Access Publisher, Rijeka, pp 115–154. ISBN: 978-953-307-233-3

Sigawi S, Smutok O, Demkiv O et al (2011) Immobilized formaldehyde-metabolizing enzymes from *Hansenula polymorpha* for removal and control of airborne formaldehyde. J Biotechnol 153:138–144

Sigawi S, Smutok O, Demkiv O et al (2014) Detection of waterborne and airborne formaldehyde: from amperometric chemosensing to a visual biosensor based on alcohol oxidase. Materials 7 (2):1055–1068

Sun JZ, Peter Kingori G, Si RW et al (2015) Microbial fuel cell-based biosensors for environmental monitoring: a review. Water Sci Technol 71(6):801–809

Tafurt-Cardona M, Eismann CE, Suárez CA et al (2015) In situ selective determination of methylmercury in river water by diffusive gradient in thin films technique (DGT) using baker's yeast (*Saccharomyces cerevisiae*) immobilized in agarose gel as binding phase. Anal Chim Acta 887:38–44

Tag K, Riedel K, Bauer H-J et al (2007) Amperometric detection of Cu^{2+} by yeast biosensors using flow injection analysis (FIA). Sens Actuators B 122:403–409

Teo SC, Wong LS (2014) Whole cell-based biosensors for environmental heavy metals detection. Annu Res Rev Biol 4(17):2663–2674

Thurston GD, Burnett RT, Turner MC et al (2016) Ischemic heart disease mortality and long-term exposure to source-related components of U.S. fine particle air pollution. Environ Health Perspect 124(6):785–794

Turdean GL (2011) Design and development of biosensors for the detection of heavy metal toxicity review article. SAGE-Hindawi Access to Research. Int J Electrochem, Article ID 343125, 15 pages. doi:10.4061/2011/343125

Vigneshvar S, Sudhakumari CC, Senthilkumaran B, Prakash H (2016) Recent advances in biosensor technology for potential applications - an overview. Front Bioeng Biotechnol 4:11. doi:10.3389/fbioe.2016.00011

Vopálenská I, Váchová L, Palková Z (2015) New biosensor for detection of copper ions in water based on immobilized genetically modified yeast cells. Biosens Bioelectron 72:160–167

Walmsley RM, Keenan P (2000) The eukaryote alternative: advantages of using yeasts in place of bacteria in microbial biosensor development. Biotechnol Bioprocess Eng 5:387–394

Watanabe M, Noma H, Kurai J et al (2017) A panel study of airborne particulate matter composition versus concentration: potential for inflammatory response and impaired pulmonary function in children. Allergol Int 66(1):52–58

Wei T, Zhang C, Xu X et al (2013) Construction and evaluation of two biosensors based on yeast transcriptional response to genotoxic chemicals. Biosens Bioelectron 44:138–145

Wysocki R, Tamas MJ (2010) How *Saccharomyces cerevisiae* copes with toxic metals and metalloids. FEMS Microbiol Rev 34:925–951

Yang H-C, Lim B, Lee Y (2005) In vitro assessment of biocompatibility of biomaterials by using fluorescent yeast biosensor. Curr Appl Phys 5:444–448

Yudina NY, Arlyapov VA, Chepurnova MA et al (2015) A yeast co-culture-based biosensor for determination of waste water contamination levels. Enzyme Microb Technol 78:46–53

Yeast-Based Biosensors for Clinical Diagnostics and Food Control

14

Mykhailo Gonchar, Oleh Smutok, Maria Karkovska, Nataliya Stasyuk, and Galina Gayda

Abstract

Science; medicine; clinical diagnostics; biotechnologies, including those in food and beverage industries; as well as environmental technologies need highly selective, sensitive, rapid, and reliable methods of identifying the key ingredients or metabolites which determine the quality of the product or serve as markers for diseases, the physiological state of human organism, or environmental safety. Biosensors are the most promising tool for these aims. Although the most of created biosensors are based on using enzymes as biocatalytic elements, cell sensors, especially microbial ones, have been actively developed only in recent years. A microbial biosensor consists of a transducer in conjunction with immobilized viable or nonviable microbial cells, an economical substitute for enzymes. The target analyte is usually either a substrate or an inhibitor of cell metabolism. In this review, the main achievements in the elaboration of microbial sensors, based on yeast cells, are described, and perspectives of their usage in clinical diagnostics and food control are discussed.

Contents

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14.1 Introduction

Modern science; medicine; clinical diagnostics; biotechnologies, including those in food and beverage industries; as well as environmental technologies need highly selective, sensitive, rapid, and reliable methods of identifying the key ingredients or metabolites which determine the quality of the product or serve as markers for diseases, the physiological state of human organism, or environmental safety (Turner et al. 1990).

Among different analytical approaches, a special role is attributed to *analytical biotechnology* which exploits the principles of biomolecular recognition, highly developed during the evolution. Biomolecular recognition is responsible for storing (on molecular level) and reading (on supramolecular level) of information and is the fundamental characteristic of the life. Such property of biomolecules and their complexes is used in analytical biotechnology. It can be defined as a branch of biotechnology related with the use of tissues, cells, organelles, biomolecules, their complexes, and even principles of their functioning for analytical purposes (Gonchar et al. 2002).

Biosensors are the most novel achievement of analytical biotechnology. Although up to now IUPAC has not accepted an official definition of the term biosensor, its electrochemical representative is defined as "a self-contained integrated device, which is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element (biochemical receptor) which is retained in direct spatial contact with an electrochemical transduction element" (Thevenot et al. 2001). Generally, a biosensor is a hybrid device containing two functional parts: a bioelement (*biorecognition unit*)—an immobilized biologically active material—and a physical transducer (*signal converting unit*). Figuratively speaking, biosensors may be called "chemical canaries" based on historical fact about miners using such birds to detect toxic methane.

Basically, biosensors can be regarded as information transducers in which the energy of biospecific interactions is transformed into the information about the nature and concentration of an analyte in the sample. Pieces of tissue, microbial cells, organelles, natural biomembranes or liposomes, receptors, enzymes, antibodies and antigens, nucleic acids and other biomolecules, and even biomimetics which imitate structural and functional features of the natural analogue can be used as biorecognition elements. The bioelement is a recognition unit providing selective binding or biochemical/metabolic conversion of the analyte that results in

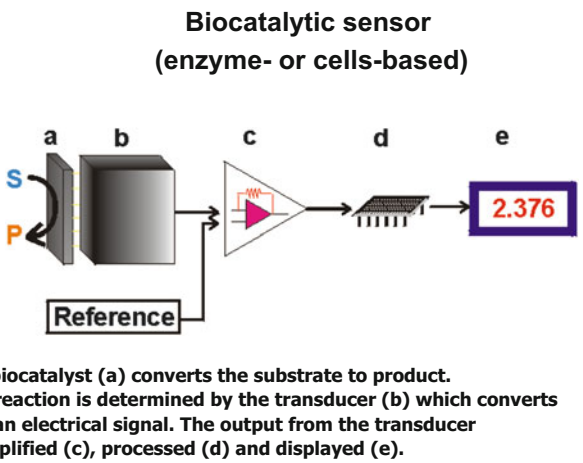

Fig. 14.1 Functional units of biosensor (Atta et al. 2011)

changes of physical or physicochemical characteristics of the transducer (Schmidt and Karube 1998). The output from the transducer is amplified, processed, and displayed (Fig. 14.1).

The biosensor bioelement is usually prepared in the immobilized form and often covered with an outer membrane (or placed between two membranes in a sandwich manner), which either prevents the penetration of interfering substances into a sensitive bioselective layer and transducer surface or creates a diffusion barrier for the analyte. Such membrane structures increase the stability of the biorecognizing element, enhance its selectivity, and provide the diffusion limitations for biochemical reactions. Electrochemical, optical, piezoelectric, thermoelectric, transistor, acoustic, and other elements are used as transducers in biosensor systems.

The most essential advantages of biosensors are their excellent chemical selectivity and high sensitivity, possibility of miniaturization, and compatibility with computers. Their drawbacks are limited stability and rather a complicated procedure of preparing the biologically active material.

The enzyme biosensors are the most widespread devices; some of them are produced commercially. The world market of biosensors was estimated at approx. $11.39 billion in 2013 and is expected to reach $22.68 billion by 2020 with an annual growth of 10.00% from 2014 to 2020 (http://www.marketsandmarkets.com/PressReleases/biosensors.asp).

The commercially produced biosensors are used mostly in clinical medicine (as points of care), in home diagnostics, in research labs, in biodefense, in environmental monitoring, for online control of biotechnological processes, in food industry, and for military or law enforcement (Fig. 14.2). The use of biosensors for fundamental research, e.g., in metabolomics, is also very promising (Kraly et al. 2009).

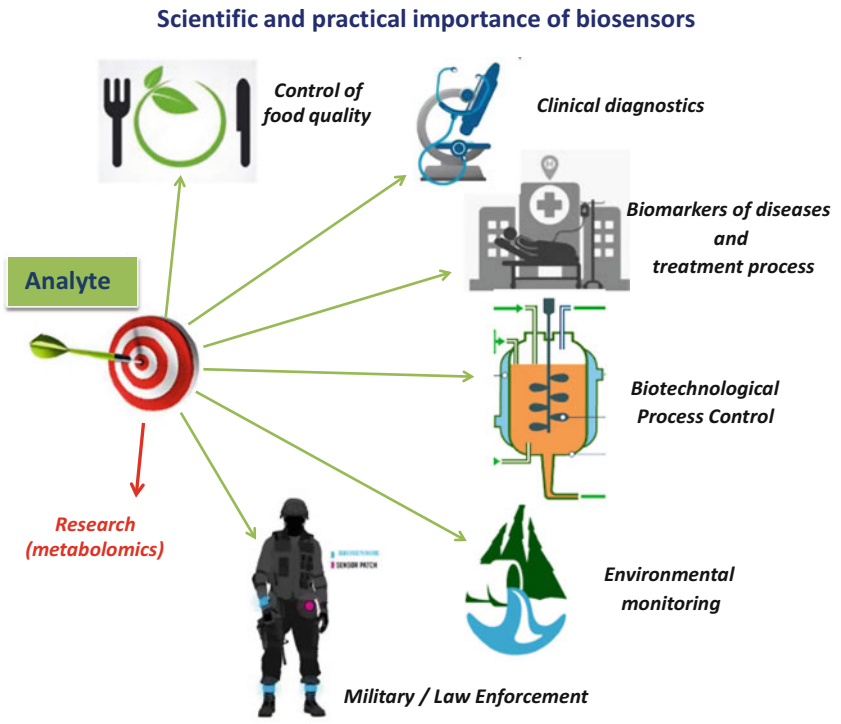

Fig. 14.2 Application of biosensors in practice and research

Biosensors are characterized by their high selectivity. They also provide fast output due to high activity and/or high local concentration of catalytic or binding element in a biosensitive layer. The drawbacks of biosensors are their insufficient stability and the high price of some purified biomolecules.

Cell sensors, especially microbial ones, have been actively developed only in recent years. A microbial biosensor consists of a transducer in conjunction with immobilized viable or nonviable microbial cells, an economical substitute for enzymes (D'Souza 2001; Lei et al. 2006). The target analyte is usually either a substrate or an inhibitor of cell metabolism.

Cell biosensors have a range of considerable advantages when compared to their enzyme analogues: availability of cells, low price and simple procedure of cell isolation, possibility to use long metabolic chains, avoiding purification of enzymes and coenzymes, advanced opportunity for genetic manipulations of metabolic pathways, integrity of the cell response (important in assaying total toxicity and mutagenic action of environmental pollutants), possibility to retain viability of sensing cells and even to provide their propagation, and, in some cases, higher stability of cell elements compared to enzyme ones (Gonchar et al. 2002; Nakamura et al. 2008; Shimomura-Shimizu and Karube 2010a, b). The main drawbacks of microbial biosensors are a long response time due to a lower concentration of

enzymes involved in cellular response, as well as low selectivity of cell output (e.g., in case of microbial O_2 electrode sensors due to the broad substrate specificity of cellular respiration).

These drawbacks are not absolute, taking into account recent progress in metabolic and genetic engineering and the possibility to overexpress the key analytical enzyme in the cell. The most known microbial sensors which exploit genetically constructed cells and are used for toxicity and bioavailability testing are bioluminescence-based biosensors created by fusing the *lux* gene with an inducible gene promoter(Nunes-Halldorson and Duran 2003; Yagi 2006; Narsaiah et al. 2012).

In recent years, nanotechnology approaches have been successfully used for improvement of functional properties of microbial sensors (Lim et al. 2015; Adeniran et al. 2015). The integration of micro- and nanotechnologies seems to be very promising in further development and production of such biosensors.

Many types of yeast-based biosensors (YBB) have been developed as analytical tools since the first microbial sensors (MS) were proposed by Karube et al. in 1977. The characteristics of MS are in absolute contrast to those of enzyme sensors or immunosensors, which are highly specific for the substrates of interest, although the specificity of MS has been improved by genetic modification of the microbe used as the sensing element. MS have the advantages of tolerance to measuring conditions, a long lifetime, and cost performance, but also the disadvantage of a long response time (Nakamura et al. 2008; Shimomura-Shimizu and Karube 2010a).

Integrated approaches, including the best advantages of nanotechnology and synthetic biology, provided a better perspective for developing specific and sensitive biosensors with high regenerative potentials for diverse usage (Bereza-Malcolm et al. 2015; Adeniran et al. 2015; He et al. 2016; Vigneshvar et al. 2016; Ponamoreva et al. 2011; Yudina et al. 2015; Jarque et al. 2016). MS appear to be quite promising for online wastewater control; their advantages lie in the fact that organic compounds in samples can be directly measured without any pretreatment such as filtration and dialysis (Dragone et al. 2014; Shimomura-Shimizu and Karube 2010a).

YBB, based on electrochemical devices, usually contain one of three types of electrodes: (1) oxygen electrodes (BOD) used for respiration testing (Chan et al. 1999; Shimomura-Shimizu and Karube 2010a); (2) fuel cell electrodes (MFC), self-powered portable devices of the next-generation biosensing innovative technology (Sun et al. 2015); and (3) membrane electrodes.

The YBB, based on genetically engineered cells, use a reporter gene under the control of an inducible promoter. The promoters are activated in the presence of a ligand of interest, either directly by the ligand/receptor dimer or via a signaling cascade. Both the receptor and the promoter may be native to yeast or adapted from other organisms. The most common reporting systems for YBB are based on optical methods for detection, namely, fluorescence (GFP, RFP, YFP), luminescence (Lux bacterial and Luc firefly), colorimetry (beta-galactosidase), as well as electrical methods (amperometry, potentiometry, conductometry, voltammetry) and growth activity (*HIS3*, *TRP1*, *LEU2*). The advantages of optical methods are their high

sensitivity and simplicity; the colorimetric variant is promising due to the visibility of its results to the naked eye. Fluorescence and luminescence detection does not need any substrate addition. The main advantages of electric methods are their high sensitivity and fastness of procedure (Eldridge et al. 2011; Adeniran et al. 2015).

Many YBB are dependent upon the signaling capabilities of G-protein-coupled receptors (GPCRs)—the largest family of human membrane proteins that mediate cellular signaling and represent primary targets of about one third of currently marketed drugs. Molecular dynamic simulations have been utilized to investigate GPCR signaling mechanisms, to design novel small-molecule drug candidates and to develop the methods of its analysis. The incorporation of GPCRs from higher organisms with functionalities, not naturally available to yeast, greatly expands the capability of YBB (Jacobson 2015; Miao and McCammon 2016).

In this review, the main achievements in the elaboration of microbial sensors, based on yeast cells, are described, and perspectives of their usage in clinical diagnostics and food control are discussed.

14.2 Microbial Sensors for Clinical Diagnostics

Widely used in vitro diagnostic approaches have a number of shortcomings; e.g., chemical methods suffer from low selectivity and sensitivity; ELISA is labor-intensive and challenging to implement multiplex detection; PCR requires a complicated sample preparation and has potential for false positives. When compared to the described above approaches in clinical diagnostics, biosensors have a number of advantages which include small sample volume, required for analysis, short assay time, low energy consumption, low prices, high portability, high throughput, and multiplexing ability.

Most of the described yeast-/fungi-based biosensors for clinical diagnostics are of amperometric mode. The measurements are carried out at a fixed potential regarding a reference electrode and involve the detection of the current generated by the oxidation or reduction of the substrate, mediators, or products of enzymatic reaction at the surface of the electrode. Glucose, lactate, and ethanol are the most frequently detected metabolic substrates in clinical diagnostics.

14.2.1 Glucose

The most well-known example in use today is the glucose sensor, which has a great effect on the management of diabetes. About 3% of the population worldwide suffers from diabetes, a leading cause of death, and its incidence is growing fast. Diabetic individuals are at the greater risk of heart disease, stroke, high blood pressure, blindness, kidney failure, neurological disorders, and other health-related complications without diligent monitoring of blood glucose concentrations. Although glucose oxidase-based sensors are widely used for glucose assay, specially treated *Saccharomyces cerevisiae cells were* used in combination with an

oxygen electrode, to be applied in aerobic conditions, or with a carbon dioxide sensor, to be used in anaerobic conditions (Mascini and Memoli 1986). To reduce the interference due to the changes in oxygen and CO_2 pressure, a potentiometric sensor was proposed based on the use of *Hansenula anomala for monitoring of* pH change correlating with glucose concentration (Racek 1991). To reduce the interference due to the changes in oxygen and CO_2 pressure, a potentiometric sensor was proposed based on the use of *Hansenula anomala for monitoring of* pH change correlating with glucose concentration (Wang et al. 2013). Microbial sensors for glucose determination based on co-immobilized *Gluconobacter oxydans* and *Saccharomyces cerevisiae* and co-immobilized *G. oxydans* and *Kluyveromyces marxianus* cells were also developed (Svitel et al. 1998).

To address the challenges of biosensors with low selectivity and inability to distinguish between substrates, *Ogataea methanolica* was combined with *Gluconobacter oxydans* to make a biosensor able to determine ethanol and glucose concentrations in various ethanol–glucose mixtures. The use of an artificial neural network to analyze the data provided accurate estimation of ethanol and glucose concentrations from 1 to 10 mM (Lobanov et al. 2001).

14.2.2 Lactate

Lactate measurement is helpful in respiratory insufficiencies, diabetes, shocks, heart failure, metabolic disorders, intoxification, and monitoring the physical condition of athletes.

The earliest analysis of L-lactate in blood plasma and whole blood was carried out by the sensor based on *H. anomala* cells. The sensor was based on yeast cells, suspended in a semipermeable membrane attached to an amperometric electrode (Racek and Musil 1987a). *H. anomala* cells have also been used in conjunction with a carbon paste electrode for lactate determination in serum (Kulys et al. 1992).

The analysis of lactate in blood plasma and whole blood was carried out by another sensor based on *H. anomala* cells. The sensor has high reproducibility, stability, and rate of assay. The analysis results were in agreement with the data obtained using conventional spectrophotometric technique and lactate dehydrogenase-based enzyme sensor (Racek and Musil 1987b). There are a few described microbial amperometric biosensors for L-lactate analysis based on baker's yeast *S. cerevisiae* (Garjonyte et al. 2006, 2008). Drying temperature was found to have an effect on lactic acid sensor performance. The K2 killer-type strain of *S. cerevisiae* was dried at temperatures as high as 90 °C for the use in a sensor that showed higher sensitivity, lower noise, and quicker response time than previous *S. cerevisiae* lactate sensors (Garjonyte et al. 2009).

In recent years, a number of L-lactate-selective amperometric biosensors based on genetically modified *H.* (*Ogataea) polymorpha* (*gcr1 catX*/*prAOX_CYB2*) overproducing L-lactate: cytochrome *c*-oxidoreductase (EC 1.1.2.3, flavocytochrome b_2, FC b_2) have been reported (Smutok et al. 2007; Shkil et al. 2009). The usage of permeabilized cells of *H. polymorpha* containing FC b_2 at a

high level in combination with free-defuse electron transfer mediator demonstrated better sensor characteristics: fast response, prolonged linearity, and operation at low working potential (Smutok et al. 2007). Another amperometric microbial sensor is based on the phenomenon of bioelectrochemical monitoring of L-lactate respiration. It was shown that in the case of *H. polymorpha* cells, overproducing FC b_2, the O_2 reduction current is decreased upon the addition of L-lactate to the electrolyte solution in contrast to the cells from the parental strain (Shkil et al. 2009). Recently, a new approach based on the additional enrichment of the recombinant yeast cell *H. polymorpha* by the FC b_2 bound with nanoparticles to improve the analytical parameters of microbial amperometric sensor has been reported (Karkovska et al.2015). Moreover, D-lactate-selective amperometric biosensor based on the cell debris of recombinant yeast *H. polymorpha* overproducing D-lactate: cytochrome *c*-oxidoreductase (EC 1.1.2.4, DLDH) in combination with cytochrome *c* and OS-contained dye was constructed. The sensor was characterized by high selectivity to D-lactate due to the deletion of *CYB2* gene responsible for the synthesis of L-lactate: cytochrome *c*-oxidoreductase (Smutok et al. 2014).

14.2.3 Hormones

It is known that the decreased levels of neurotensin and estrogen are associated with the occurrence of Parkinson's disease, as well as with many other disorders. On the other hand, a deficiency in serotonin causes the serotonin syndrome.

The modulation in optical properties such as fluorescence caused by the interaction of the biocatalyst with the target analyte is the basis for optical microbial biosensors. There are a number of described fluorescent microbial sensors for the analysis of human hormones based on recombinant yeast cells with incorporated fluorescent reporters.

Neurotensin receptor type-1 (NTSR1) is a member of the G-protein-coupled receptor (GPCR) family. The natural ligand of NTSR1 is neurotensin (NT), a neuromodulator of the central nervous system. Because NT is also involved in many oncogenic events, the signaling mediator NTSR1 is a significant molecular target in medicinal and therapeutic fields. It was used to construct a fluorescence-based microbial *S. cerevisiae*-based biosensor that can monitor the activation of human NTSR1 signaling responding to its agonist (Ishii et al. 2014).

The monoamine neurotransmitter serotonin (5-HT) regulates a wide spectrum of human physiology through the 5-HT receptor family. Yeast-based fluorescent reporter systems have proven to be especially useful for GPCR assays, since the detection using a fluorescent reporter considerably simplifies measurement procedures. A refined yeast-based GPCR biosensor employing *S. cerevisiae* cells with both incorporated Gα-engineered receptor and a fluorescent reporter (*ZsGreen*) was elaborated (Nakamura et al. 2015).

In addition to fluorescence, there are reports on the construction of amperometric microbial sensor for estrogen analysis. A phytase under the control of the estrogen-induced promoter in the yeast *Arxula adeninivorans* was used as a biorecognition

element. When the appropriate substrate was added, the induced phytase catalyzed the formation of *p*-aminophenol, which can be oxidized at an electrode and thus monitored amperometrically (Pham et al. 2012).

14.2.4 Vitamin B_1

Vitamin B_1 (thiamine) is one of the most important members of the B group of vitamins. Vitamin B_1 deficiency can be caused by a number of factors and diseases such as alcoholism, HIV, vomiting, and some gastrointestinal illnesses.

An amperometric whole-cell biosensor based on *S. cerevisiae* cells immobilized in gelatin was developed for selective determination of vitamin B_1. The biosensor was constructed using gelatin and cross-linking agent glutaraldehyde to immobilize *S. cerevisiae* cells on the Teflon membrane of dissolved oxygen (DO) probe used as the basic electrode system combined with a digital oxygen meter. The cells were induced by vitamin B_1 in the culture medium and used as a carbon source in the absence of glucose. So, when the vitamin B_1 solution was injected into the whole-cell biosensor system, an increase in the respiration activity of the cells resulting from the metabolic activity and causing a decrease in the DO concentration related to vitamin B_1 content was observed. Certainly, such a biosensor is unselective and can be used only for the assay of the target analyte in vitamin B_1 pills (Akyilmaz et al. 2006).

14.2.5 L-Lysine

A naturally occurring amino acid, L-lysine is one of the only 20 amino acids that serve as building blocks for proteins. The analysis of L-lysine is helpful in the diagnostics of a variety of health conditions in medicine. As a drug, Lys can be used to control cholesterol levels, prevent osteoporosis and other age-related bone density conditions, support growth, and reduce anxiety and stress. The nutrient is also effective in preventing herpes, treating shingles, relieving pain due to inflammation, and maintaining heart health.

An amperometric microbial biosensor for sensitive determination of L-lysine based on the cells of *S. cerevisiae* NRRL-12632, producing L-lysine oxidase (EC 1.4.3.14), was developed. The cells were immobilized on a pretreated oxygen-sensitive Teflon membrane using gelatin and glutaraldehyde. The assay procedure for the microbial biosensor was based on the determination of the differences in the respiration activity of the cells using oxygen meter in the absence and presence of L-lysine (Akyilmaz et al. 2007).

14.2.6 Urate

Uric acid is a final purine intermediate of purine nucleotide catabolism in mammals. Normally, uric acid is not accumulated in human fluids, and its availability in amounts exceeding the threshold levels in body fluids (e.g., serum and urine) is a clinically valuable diagnostic indicator. The presence of elevated uric acid levels is a sign of gout, hyperuricemia, or Lesch–Nyhan syndrome. An elevated uric acid concentration is related also to increased alcohol and cholesterol consumption, obesity, diabetes, and kidney and heart diseases. A urate-selective microbial biosensor was developed using cellular debris of recombinant methylotrophic yeast *H. polymorpha* overproducing urate-oxygen oxidoreductase (urate oxidase, uricase, 1.7.3.3) as a bioselective element. The cellular debris coupled with peroxidase were either immobilized onto the graphite electrode by physical entrapment of the suspension by means of a dialysis membrane or by integration in the electrochemically generated layer using a cathodic electrodeposition paint as well as an Os-complex modified cathodic electrodeposition paint or their combinations. The uric acid content in urine samples was determined by this sensor, and the values obtained exhibited a good correlation with the reference method (Dmytruk et al. 2011).

14.2.7 Intracellular Metabolites

Mitochondria play a pivotal role in energy metabolism, programmed cell death, and oxidative stress. Mutated mitochondrial DNA in diseased cells compromises the structure of key enzyme complexes and, therefore, mitochondrial function, which leads to a myriad of health-related conditions such as cancer, neurodegenerative diseases, diabetes, and aging. Early detection of mitochondrial and metabolic anomalies is an essential step toward effective diagnosis and therapeutic intervention. Reduced nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD) play important roles in a wide range of cellular oxidation–reduction reactions (Heikal 2010).

Cellular energy and redox carriers can also be directly monitored by yeast-based sensors. FRET-based sensors have been employed to monitor cytosolic ATP, using a subunit from bacterial ATP synthase as the recognition element (Bermejo et al. 2010). The protein can also be targeted specifically to the mitochondria by adding a mitochondrial targeting sequence from cytochrome *c* to the N-terminal, allowing in vivo monitoring of mitochondrial function (Vevea et al. 2013). Bulk NADH and NADPH redox states can be monitored electrically with the use of mediator compounds that shuttle electrons from the cell to an electrode (Baronian et al. 2002; Heiskanen et al. 2004). This technique also allows for nearly real-time monitoring—for example, monitoring NAD(P)H levels upon substrate addition to the yeast with an overexpressed reductase enzyme (Kostesha et al. 2009). The system has even been implemented onto a microfluidic platform (Heiskanen et al. 2013). NAD(P)H can also be assayed by autofluorescence, although this method

has been criticized for a low sensitivity and specificity (Hung et al. 2011). Recently, genetically encoded fluorescent NADH sensors with subcellular resolution have been developed using mammalian cells (Hung et al. 2011; Bilan et al. 2014); however, to our knowledge they have not, as yet, been implemented in yeast. Finally, yeast cells have been engineered to report their own pH. This is commonly achieved through pH-sensitive GFP variants (Miesenböck et al. 1998; Ullah et al. 2012), although a new bioluminescence resonance energy transfer (BRET)-based system can report pH without excitation from an external light source (Zhang et al. 2012).

14.2.8 Toxic Compounds

14.2.8.1 Alcohols

Ethanol determination is of great relevance in clinical toxicological tests. Although alcohol is used very often in human nutrition, alcoholism is considered an ailment. Ethanol ingestion could affect nervous, circulatory, or digestive system. On the other hand, methanol intoxication is an uncommon but serious poisoning. The consumption of methanol could cause coma or even death.

Besides chemosensors and enzyme-based biosensors, the microbial sensors have been proposed for alcohol determination. The methylotrophic yeasts of genera *Hansenula*, *Pichia*, and *Candida* are generally characterized by a high intracellular content of alcohol oxidase (alcohol:O_2 oxidoreductase, AOX, EC 1.1.3.13) which has rather broad specificity to primary alcohols. The improvement of AOX selectivity by biochemical or genetic manipulations is very important (Voronova et al. 2008; Akyilmaz and Dinçkaya 2005; Korpan et al. 1993). In the papers (Gonchar et al. 1998, 2002), two microbial alcohol sensors based on recombinant *H. polymorpha* (*gcr1 catX*) cells have been described. The first sensor utilized the cells with highly active alcohol oxidase immobilized on an oxygen electrode, while the second one was based on catalase-deficient cells and involved a peroxide electrode as a transducer. A conductometric microbial sensor for ethanol based on alginate-immobilized yeast cells attached to gold planar electrodes was also described (Korpan et al. 1994).

The co-immobilization *of O. methanolica* and *Gluconobacter oxydans* on chromatographic paper and fixation on the Clark-type oxygen electrode was used for the construction of another alcohol biosensor. Among the tested substrates (ethanol, methanol, isopropanol, glucose, xylose, xylitol, arabinose, arabitol, glycerol, pyruvate, citrate, acetate), the *O. methanolica*-based sensor demonstrated higher selectivity and was sensitive only to ethanol and methanol and insignificantly sensitive to isopropanol (Reshetilov et al. 2001).

An amperometric biosensor based on *C. tropicalis* cells which contain alcohol oxidase, immobilized in gelatin by glutaraldehyde, was described for sensitive determination of ethanol. This microorganism detects alcohol oxidase which is specific to several short-chain primary alcohols; therefore, the sensor was characterized by low selectivity to ethanol (Akyilmaz and Dinçkaya 2005).

A microbial biosensor for ethanol determination was prepared by the immobilization of yeast *S. ellipsoideus* cells on the surface of the oxygen electrode with the electrolyte in nonaqueous medium. The analytical determination was based on the respiratory activity of the microorganism in the presence of the analyte (Rotariu and Bala 2003).

14.2.8.2 Hormone-Active Chemicals (Xenoestrogens)

Endocrine disruptors that act like hormones in the endocrine system might have toxic effects. The yeast cells were used as biosensors since they were genetically engineered to respond to the presence of hormone-active chemicals by synthesizing beta-galactosidase as a reporter. To achieve higher sensitivity, the interdigitated array (IDA) electrodes were incorporated into the analytical chamber of the microfluidic device. The yeast cells, precultured with a hormone-active chemical, 17-beta-estradiol (E2), were trapped from the main channel of the device to the analytical chamber by electrophoresis. After trapping in the analytical chamber, electrochemical detection of beta-galactosidase induced in the yeast cells with the IDA electrodes was performed. Actually, electrochemical detection was also performed on *p*-aminophenol, formed from *p*-aminophenyl-beta-D-galactopyranoside, with beta-galactosidase. The electrochemical signals from the yeast cells precultured with 17-beta-estradiol were successfully determined with the device. Furthermore, the inhibitory effects of antagonists such as tamoxifen were also detected electrochemically using the device (Ino et al. 2009).

14.2.8.3 Poisons

The microbial sensor for cyanide detection, based on *S. cerevisiae* cells and the oxygen electrode, was described. The principle of detection is based on the inhibition of cell respiration and hence the decrease of oxygen consumption rate in the presence of cyanide, which is the approach typical for most toxicity sensors (Nakanishi et al. 1996; Ikebukuro et al. 1996).

14.2.8.4 Toxins

Toxins are classified according to their mode of action as follows: (1) membrane pore-forming toxins (hemolysin), diarrheagenic toxins (activating secondary messenger pathways—cholera toxin), superantigens (activating immune responses—staphylococcal enterotoxin B), neurotoxins (botulinum toxin), and protein synthesis inhibitory toxins (*Shiga* toxin). Both prokaryotic (bacteria) and eukaryotic (yeast, invertebrate, and vertebrate) cells have been used in developing of biosensors. The biotoxins are usually detected using higher eukaryotic cells (such as insect, fish, mammalian, or other vertebrate origins) or bacterial cells (Woutersen et al. 2011). But in scientific literature, there is also a description of the biosensor based on yeast cells. Such biosensor was developed for detection of mycotoxins. Mycotoxins are compounds produced by mold fungi in moist conditions. Approximately 25% of the world's crops are contaminated with mold or fungal growth, and mycotoxins may be produced both before and after harvest (Bryden 2007). In both humans and animals ingestion of food or feed contaminated by mycotoxins can lead to

mycotoxicoses, the possible symptoms of which are acute intoxication, losses in productivity, reduced weight gain, immunosuppression, and increased risk of cancer (Bryden 2007; Fink-Gremmels 2008). The mycotoxin zearalenone produced by *Fusarium* species as well as its metabolites zearalanone, α-zearalanol, and ß-zearalanol are harmful for health mainly due to their estrogenic activity. The estrogenic response was detected by a whole-cell biosensor based on a genetically modified *Saccharomyces cerevisiae* strain that in the presence of an estrogenic compound produces firefly luciferase enzyme and further light emission within a system provided with D-luciferin substrate. The results show that the yeast sensor reacts to mycotoxins with typical sigmoidal response at nanomolar concentrations. The response differs in different milk products with regards to the fat content of milk (Valima et al. 2010).

14.3 Microbial Sensors Based on Yeast Cells for Food Analysis

The development of biosensors for food safety applications has been an area of tremendous activity in recent years. Food safety is of great concern for many countries; e.g., the European Commission has identified it as a priority area of attention. The Commission has formulated a new food policy to ensure human health and protect consumer health. Testing and identifying foods that are unsafe is the first step for ensuring food safety (Sankarankutty 2014; Mello and Kubota 2002).

The food quality depends essentially on its biochemical composition (Mello and Kubota 2002). Quick and specific analytical tools are needed for monitoring of nutritional parameters and food contaminants (D'Souza 2001). Microbial biosensors are a rapid and affordable instrument to ensure the quality of products. There are a lot of described biosensors for the assay of various components in food samples and during fermentation processes in winemaking and other biotechnological processes (Karube and Suzuki 1983; Karube and Tamiya 1987; Karube and Sode 1989; Lim et al. 2015; Dai and Choi 2013).

14.3.1 Ethanol Assay

Ethanol assays are necessary in many industries and biotechnologies (Otles and Yalcin 2012; Shimomura-Shimizu and Karube 2010a). The content of ethanol should be monitored in its production in fermentation processes as well as when it is utilized as a substrate in different biotechnologies, for instance, in production of liquors, alcoholic beverages, and some foodstuffs (Rotariu and Bala 2003). The development of simple and sensitive biosensors for estimating the content of ethanol is an urgent problem. There are a lot of known biosensors for ethanol detection, and almost all of them are based on the usage of enzymes in the bioselective layer, but the construction of biosensors based on yeast cells is also described (Tkac et al. 2003; Rotariu et al. 2003; Rotariu and Bala 2003; Rotariu

et al. 2004; Korpan et al. 1994). Most of these biosensors exhibit good stability and a sensitive response to the target analyte. The main analytical characteristics of the proposed sensors for ethanol and other analytes are presented in Table 14.1.

One of the described biosensors is based on the cells of the yeast *Saccharomyces ellipsoideus* immobilized on the surface of an oxygen electrode with electrolyte in nonaqueous medium. The analytical signal is based on the respiratory activity of the microorganism in the presence of ethanol. The response time of approximately 5 min for steady-state method and 2 min for kinetic method was registered. The detection limit of 6 μM of the proposed biosensor was achieved (Rotariu and Bala 2003) (see Table 14.1). The similar mechanism of biosensor functioning was proposed by Rotariu and coauthors (Rotariu et al. 2004). The biosensor was used for selective determination of ethanol in the presence of glucose using a Teflon membrane. The biosensor is practically specific to ethanol, while the interference of glucose in determination of ethanol for a biosensor with dialysis membrane is about 30%. This biosensor was used to determine ethanol concentration in alcoholic beverages. Good correlation of the results obtained using the biosensor and by the spectrometric method with alcohol dehydrogenase was observed (Table 14.1).

Amperometric biosensors for simultaneous determination of glucose, sucrose, and lactose based on the cells of *S. cerevisiae* and *Kluyveromyces marxianus* immobilized on the surface of an oxygen electrode were proposed too (Svitel et al. 1998). Selective potentiometric microbial sensors using *Pichia methanolica* (Reshetilov et al. 1998) and *S. cerevisiae* (Rotariu et al. 2002, 2004) cells were described for the analysis of sucrose in soft drinks (Lobanov et al. 2001) as well as glucose and ethanol in alcoholic beverages.

To analyze the ethanol level in some alcoholic beverages, a conductometric biosensor based on immobilized *S. cerevisiae* cells was constructed (Korpan et al. 1994). To develop the conductometric biosensor, a membrane with yeast cells, immobilized in 2% Ca-alginate gel, was attached to gold planar electrodes. The changes in conductivity due to the specific consumption of ethanol by the yeast cells were registered by the computer-controlled sensor system. The response time of the constructed microbial sensor was less than 5 min; the linearity was observed in the range of 5–100 mM alcohol concentration (see Table 14.1). The minimal detectable level of ethanol was 1 mM, and the relative standard deviation was found to be 10–12% for 15 repeated assays. When the system was operated and stored at 20–25 °C, the biosensor response was stable for only 3 days. Good correlation between the results obtained by a conductometric cell biosensor and gas chromatography was observed.

Table 14.1 Comparison of different types of microbial biosensors based on yeast cells for food analysis

Sample of food	Analyte	Microorganism	Mode of signal registration	Detection limit, μM	Linearity range, mM	Reference
Alcoholic beverages	Ethanol	*S. ellipsoideus*	Amperometric	6	–	Rotariu and Bala (2003)
		S. ellipsoideus	Potentiometric	–	0.02–50	Rotariu et al. (2004)
		S. cerevisiae	Conductometric	1000	5–100	Korpan et al. (1994)
	L-Lysine	*S. cerevisiae*	Amperometric	–	1×10^{-3}–0.01	Akyilmaz et al. (2007)
Wines and juices	L-Arginine	*H. polymorpha*	Amperometric	85	Till 0.6	Stasyuk et al. (2014)
Milk, sour milk, and yogurts	L-Lactic acid	*S. cerevisiae*	Amperometric	–	Up to 1 mM	Garjonyte et al. (2006)
Fruit brandies	Cyanide	*S. cerevisiae*	Amperometric	8	–	Filipović-Kovačević et al. (2002)
Milk	Estrogenic mycotoxin	*S. cerevisiae*	Luminescence	–	1×10^{-6}–2.6×10^{-4}	Valima et al. (2010)

14.3.2 Amino Acid Assay

Amino acids serve as the nitrogen source for yeast during the fermentation of wine. Hernández-Orte et al. (2002) reported that amino acids are also a mark of the quality of wine because they contribute to wine taste, aroma, and color. Amino acids serve as the nitrogen source for yeast during the fermentation of wine. The industry of fermented foods and beverages, especially winemaking, is an important area of L-arginine (Arg) assay. Urea, a final product of L-Arg metabolism in the urea cycle during juice fermentation and wine maturation, is partly assimilated by the cells of microorganisms. In case of elevated Arg level in grape juice, urea remains in the fermentation medium and spontaneously reacts with ethanol to produce ethyl carbamate (EC) or urethane (Huang and Ough 1989). Citrulline, formed from Arg metabolism *via* the arginine deiminase pathway by wine malolactic bacteria, is the second significant precursor of EC in wine (Uthurry et al. 2006; Spayd et al. 1994).

Both urea and citrulline accumulation in the fermentation medium mainly depend on the Arg level and the type of microorganism. If L-Arg concentration in juice is higher than 1000 mg L^{-1} (5.0 mM), the potential EC concentration in wine will be above 15 μg L^{-1}, the current voluntary limit in the United States. To ensure high quality of the final product and avoid potential health hazard of urethane, the determination of Arg content in fruit juices and wine is necessary. The biosensor approach for Arg determination seems to be perspective. The construction of Arg biosensor with *Hansenula (Ogataea) polymorpha* cells in biorecognition layer has already been reported (Stasyuk et al. 2014). Both urea and citrulline accumulation in the fermentation medium mainly depend on the Arg level and the type of microorganism. If L-Arg concentration in juice is higher than 1000 mg L^{-1} (5.0 mM), the potential EC concentration in wine will be above 15 μg L^{-1}, the current voluntary limit in the United States. To ensure high quality of the final product and avoid potential health hazard of urethane, the determination of Arg content in fruit juices and wine is necessary. The biosensor approach for Arg determination seems to be perspective. The construction of Arg biosensor with *Hansenula (Ogataea) polymorpha* cells in biorecognition layer has already been reported.

L-Lysine (Lys) is the most easily damaged essential amino acid, and therefore, Lys is a vital marker for the nutritional value of food. It is abundant in animal proteins, unlike plant proteins, which are often low in lysine content, making it the limiting amino acid in most cereal proteins (Kelly et al. 2000). However, lysine is an indicator of the effect on foods by various industrial processes or during cooking treatment (Karalemas et al. 2000).

In addition, Lys is an essential constituent of the diet and of great interest in the nutrition and pharmaceutical fields. Lys is often added as a dietary supplement to food and drugs in order to correct possible deficiencies of this amino acid in humans (Saurina et al. 1999). To provide high quality of a food product, it is necessary to determine Lys content. Recently, a new amperometric microbial biosensor based on *S. cerevisiae* cells for Lys determination in alcoholic beverages has been developed

(Akyilmaz et al. 2007). The proposed amperometric sensor is suitable for the determination of Lys content in food products in the absence and presence of other amino acids (see Table 14.1).

14.3.3 L-Lactate Assay

Reliable determination of L-lactate is important in food technology, fermentation, and wine industries (Garjonyte et al. 2006, 2008). The use of cell-based amperometric biosensors seems to be very promising due to the favorable coupling of the selectivity of the biological recognition element and the sensitivity of electrochemical transducer (Smutok et al. 2007; Shkil et al. 2009). Most of the proposed amperometric biosensors for the detection of L-lactate are based on baker's yeast *S. cerevisiae* and *H. polymorpha* cells (sources of flavocytochrome b_2) and different types of mediators (potassium ferricyanide, phenazine methosulphate, 1,2-naphthoquinone-4-sulfonic acid sodium salt, and *p*-benzoquinone) (Akyilmaz et al. 2007; Shkil et al. 2009). The suitability of the developed bioelectrodes for the determination of L-lactic acid in milk and dairy products such as sour milk and yogurt was also suggested (Garjonyte et al. 2009).

Recently, the microbial L-lactate-selective sensor based on the recombinant *H. polymorpha* cells overproducing flavocytochrome b_2 (FC b_2), as well as additionally fortified by the immobilized FC b_2 with gold nanoparticles (nAu), has been proposed (Karkovska et al. 2015). The FC b_2-nAu-enriched living and permeabilized yeast cells were used for construction of a bioselective membrane of microbial L-lactate-selective amperometric biosensor. The main analytical characteristics of the proposed sensor are presented in Table 14.1. The obtained results confirm that the additional enrichment of the recombinant yeast cell by the enzyme bound with nanoparticles improves the analytical parameters of the microbial sensor.

14.3.4 Assay of Contaminants

Contaminants should be carefully detected in order to ensure the quality of the food products. Mycotoxins of zearalenone family are common contaminants in milk which can cause mycotoxicoses (Bryden 2007). In order to ensure the quality of milk, genetically modified *S. cerevisiae* cells were used as the bioelement of the microbial biosensor for the detection of zearalenone mycotoxins in milk (Valima et al. 2010).

S. cerevisiae cells, immobilized on an oxygen electrode, were used for cyanide detection in fruit brandies (Filipović-Kovačević et al. 2002). For quality control of meat freshness, a yeast-based sensor using *Trichosporon cutaneum* was developed. The detection of polyamines and amino acids from the meat in washwater was estimated by this sensor system (Yano et al. 2001).

References

Adeniran A, Sherer M, Tyo K (2015) Yeast-based biosensors: design and applications. FEMS Yeast Res 15(1):1–15

Akyilmaz E, Dinçkaya E (2005) An amperometric microbial biosensor development based on *Candida tropicalis* yeast cells for sensitive determination of ethanol. Biosens Bioelectron 20:1263–1269

Akyilmaz E, Yaşa I, Dinçkaya E (2006) Whole cell immobilized amperometric biosensor based on *Saccharomyces cerevisiae* for selective determination of vitamin B1 (thiamine). Anal Biochem 354(1):78–84

Akyilmaz E, Erdogan A, Ozturk R et al (2007) Sensitive determination of L-lysine with a new amperometric microbial biosensor based on *Saccharomyces cerevisiae* yeast cells. Biosens Bioelectron 22(6):1055–1060

Atta NF, Galal A, Ali S (2011) Nanobiosensors for health care, Chapter 4. In: Serra PA (ed) Biosensors for health, environment and biosecurity. doi:10.5772/17996

Baronian KH, Downard AJ, Lowen RK et al (2002) Detection of two distinct substrate-dependent catabolic responses in yeast cells using a mediated electrochemical method. Appl Microbiol Biotechnol 60(1–2):108–113

Bereza-Malcolm LT, Mann G, Franks AE (2015) Environmental sensing of heavy metals through whole cell microbial biosensors: a synthetic biology approach review. ACS Synth Biol 4 (5):535–546

Bermejo C, García R, Straede A et al (2010) Characterization of sensor-specific stress response by transcriptional profiling of *wsc1* and *mid2* deletion strains and chimeric sensors in *Saccharomyces cerevisiae*. OMICS 14(6):679–688

Bilan DS, Matlashov ME, Gorokhovatsky AY et al (2014) Genetically encoded fluorescent indicator for imaging NAD_+/NADH ratio changes in different cellular compartments. Biochim Biophys Acta 1840:951–957

Bryden WL (2007) Mycotoxins in the food chain: human health implications. Asia Pac J Clin Nutr 16(1):95–101

Chan C, Lehmann M, Tag K, Lung M, Kunze G, Riedel K, Gruendig B, Renneberg R (1999) Measurement of biodegradable substances using the salt-tolerant yeast *Arxula adeninivorans* for a microbial sensor immobilized with poly(carbamoyl) sulfonate (PCS) part I: construction and characterization of the microbial sensor. Biosens Bioelectron 14(2):131–138

D'Souza SF (2001) Microbial biosensors. Biosens Bioelectron 16(6):337–353

Dai C, Choi S (2013) Technology and applications of microbial biosensor. OJAB 2:83–93

Dmytruk K, Smutok O, Dmytruk O et al (2011) Construction of uricase-overproducing strain of *Hansenula polymorpha* and its application as biological recognition element in microbial urate biosensor. BMC Biotechnol 11:58–66

Dragone R, Frazzoli C, Grasso G, Rossi G (2014) Sensor with intact or modified yeast cells as rapid device for toxicological test of chemicals. J Agric Chem Environ 3:35–40

Eldridge M, Sanseverino J, Umbuzeiro GA, Sayler GS (2011) Analysis of environmental samples with yeast-based bioluminescent bioreporters, environmental monitoring. In: Ekundayo E (ed) InTech. doi:10.5772/27139. http://www.intechopen.com/books/environmental-monitoring/analysis-of-environmental-samples-with-yeast-based-bioluminescent-bioreporters

Filipović-Kovačević Ž, Mikšaj M, Šalamon D (2002) Cyanide determination in fruit brandies by an amperometric biosensor with immobilised *Saccharomyces cerevisiae*. Eur Food Res Technol 215(4):347–352

Fink-Gremmels J (2008) Mycotoxins in cattle feeds and carry-over to dairy milk: a review. Food Addit Contam 25:172–180

Garjonyte R, Melvydas V, Malinauskas A (2006) Mediated amperometric biosensors for lactic acid based on carbon paste electrodes modified with baker's yeast *Saccharomyces cerevisiae*. Bioelectrochemisty 68(2):191–196

Garjonyte R, Melvydas V, Malinauskas A (2008) Effect of yeast pretreatment on the characteristics of yeast-modified electrodes as mediated amperometric biosensors for lactic acid. Bioelectrochemistry 74:188–194
Garjonyte R, Melvydas V, Malinauskas A (2009) Amperometric biosensors for lactic acid based on baker's and wine yeast. Microchim Acta 164:177–183
Gonchar MV, Maidan MM, Moroz OM et al (1998) Microbial O_2- and H_2O_2- electrode sensors for alcohol assays based on the use of permeabilized mutant yeast cells as the sensitive bioelements. Biosens Bioelectron 139:945–952
Gonchar M, Maidan M, Korpan Y et al (2002) Metabolically engineered methylotrophic yeast cells and enzymes as sensor biorecognition elements. FEMS Yeast Res 2:307–314
He W, Yuan S, Zhong WH et al (2016) Application of genetically engineered microbial whole-cell biosensors for combined chemosensing. Appl Microbiol Biotechnol 100(3):1109–1119
Heikal AA (2010) Intracellular coenzymes as natural biomarkers for metabolic activities and mitochondrial anomalies. Biomark Med 4(2):241–263
Heiskanen A, Yakovleva J, Spegel C et al (2004) Amperometric monitoring of redox activity in living yeast cells: comparison of menadione and menadione sodium bisulfite as electron transfer mediators. Electrochem Commun 6:219–224
Heiskanen J, Saksa T, Luoranen J (2013) Soil preparation method affects outplanting success of Norway spruce container seedlings on till soils susceptible to frost heave. Silva Fennica 47(1). http://www.silvafennica.fi/article/893
Hernández-Orte P, Cacho JF, Ferreira V (2002) Relationship between varietal amino acid profile of grapes and wine aromatic composition. Experiments with model solutions and chemometric study. J Agric Food Chem 50(10):2891–2899
Huang Z, Ough CS (1989) Effect of vineyard locations, varieties and rootstocks on the juice amino acid composition of several cultivars. Am J Enol Vitic 40:135–139
Hung KW, Suen MF, Chen YF et al (2011) Detection of water toxicity using cytochrome P450 transgenic zebrafish as live biosensor: for polychlorinated biphenyls toxicity. Biosens Bioelectron 31(1):548–553
Ikebukuro K, Honda M, Nakanishi K et al (1996) Flow-type cyanide sensor using an immobilized microorganism. Electroanalysis 8(10):876–879
Ino K, Kitagawa Y, Watanabe T et al (2009) Detection of hormone active chemicals using genetically engineered yeast cells and microfluidic devices with interdigitated array electrodes. Electrophoresis 19:3406–3412
Ishii J, Oda A, Togawa S et al (2014) Microbial fluorescence sensing for human neurotensin receptor type 1 using Gα-engineered yeast cells. Anal Biochem 1(446):37–43
Jacobson KA (2015) New paradigms in GPCR drug discovery. Biochem Pharmacol 98 (4):541–555
Jarque S, Bittner M, Blaha L, Hilscherova K (2016) Yeast biosensors for detection of environmental pollutants: current state and limitations review article. Trends Biotechnol 34 (5):408–419
Karalemas ID, Georgiou CA, Papastathopoulos DS (2000) Construction of a L-lysine biosensor by immobilizing lysine oxidase on a gold-poly(o-phenylenediamine) electrode. Talanta 53:391–402
Karkovska M, Smutok O, Stasyuk N et al (2015) L-lactate-selective microbial sensor based on flavocytochrome b_2-enriched yeast cells using recombinant and nanotechnology approaches. Talanta 144:1195–1200
Karube I, Sode K (1989) Biosensors for fermentation process control. In: Ghose TK (ed) Bioprocess engineering: the first generation. Chapman Hall, London
Karube I, Suzuki S (1983) Application of biosensor in fermentation processes, Chapter 8. In: Tsao GT (ed) Annual reports on fermentation processes, vol 6. Academic, New York
Karube I, Tamiya E (1987) Biosensors for the food industry. Food Biotechnol 1:147–165
Kelly SC, O'Connell PJ, O'Sullivan CK et al (2000) Development of an interferent free amperometric biosensor for determination of L-lysine in food. Anal Chim Acta 412:111–119

Korpan YI, Gonchar MV, Starodub NF et al (1993) A Cell biosensor specific for formaldehyde based on pH-sensitive transistors coupled to methylotrophic yeast cells with genetically adjusted metabolism. Anal Biochem 215(2):216–222

Korpan YI, Dzyadevich SV, Zharova VP et al (1994) Conductometric biosensor for ethanol detection based on whole yeast cells. Ukr Biokhim Zh 66(1):78–82

Kostesha NV, Almeida JR, Heiskanen AR et al (2009) Electrochemical probing of *in vivo* 5-hydroxymethyl furfural reduction in *Saccharomyces cerevisiae*. Anal Chem 81 (24):9896–8901

Kraly JR, Holcomb RE, Guan Q et al (2009) Review: microfluidic applications in metabolomics and metabolic profiling. Anal Chim Acta 653(1):23–35

Kulys J, Wang L, Razumas V (1992) Sensitive yeast bioelectrode to L-lactate. *Electroanalysis* 4:527–532

Lei Y, Chen W, Mulchandani A (2006) Microbial biosensors. Anal Chim Acta 568:200–210

Lim JW, Ha D, Lee J et al (2015) Review of micro/nanotechnologies for microbial biosensors. Front Bioeng Biotechnol 3(61):1–13

Lobanov AV, Borisov IA, Gordon SH et al (2001) Analysis of ethanol-glucose mixtures by two microbial sensors: application of chemometrics and artificial neural networks for data processing. Biosens Bioelectron 16:1001–1007

Mascini M, Memoli A (1986) Comparison of microbial sensors based on amperometric and potentiometric electrodes. Anal Chim Acta 182:113–122

Mello LD, Kubota LT (2002) Review of the use of bio-sensors as analytical tools in the food and drink industries. Food Chem 77(2):237–256

Miao Y, McCammon JA (2016) G-protein coupled receptors: advances in simulation and drug discovery. Curr Opin Struct Biol 41:83–89

Miesenböck G, De Angelis DA, Rothman JE (1998) Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins. Nature 394(6689):192–195

Nakamura H, Shimomura-Shimizu M, Karube I (2008) Development of microbial sensors and their application. Adv Biochem Eng Biotechnol 109:351–394

Nakamura Y, Ishii J, Kondo A (2015) Applications of yeast-based signaling sensor for characterization of antagonist and analysis of site-directed mutants of the human serotonin 1A receptor. Biotechnol Bioeng 112(9):1906–1915

Nakanishi K, Ikebukuro K, Karube I (1996) Determination of cyanide using a microbial sensor. Appl Biochem Biotechnol 60(2):97–106

Narsaiah K, Jha SN, Bhardwaj R et al (2012) Optical biosensors for food quality and safety assurance – a review. J Food Sci Technol 49(4):383–406

Nunes-Halldorson VS, Duran NL (2003) Bioluminescent bacteria: *lux* genes as environmental biosensors. Braz J Microbiol 34(2):91–96

Otles S, Yalcin B (2012) Review on the application of nanobiosensors in food analysis. Acta Sci Pol Technol Aliment 11(1):7–18

Pham H, Giersberga M, Uhligb S et al (2012) Estra monitor – a monitor for amperometric detection of estrogenic activity with *Arxula adeninivorans* yeast cells as the biocomponent. Sens Actuators Chem 161:137–145

Ponamoreva ON, Arliapov VA, Alferov VA, Reshetilov AN (2011) Microbial biosensors for detection of biological oxygen demand (a review). Prikl Biokhim Mikrobiol 47(1):5–15

Racek J (1991) A yeast biosensor for glucose determination. Appl Microbiol Biotechnol 34:473–477

Racek J, Musil J (1987a) Biosensor for lactate determination in biological fluids. I. Construction and properties of the biosensor. Clin Chim Acta 162:129–139

Racek J, Musil J (1987b) Biosensor for lactate determination in biological fluids. 2. Interference studies. Clin Chim Acta 167:59–65

Reshetilov AN, Lobanov AV, Morozova NO et al (1998) Detection of ethanol in a two-component glucose/ethanol mixture using a nonselective microbial sensor and a glucose enzyme electrode. Biosens Bioelectron 13:787–793

Reshetilov AN, Trotsenko JA, Morozova NO et al (2001) Characteristics of *Gluconobacter oxydans* B-1280 and *Pichia methanolica* MN4 cell based biosensors for detection of ethanol. Process Chem 36(10):1015–1020

Rotariu L, Bala C (2003) New type of ethanol microbial biosensor based on a highly sensitive amperometric oxygen electrode and yeast cells. Anal Lett 36(11):2459–2471

Rotariu L, Bala C, Magearu V (2002) Yeast cells sucrose biosensor based on a potentiometric oxygen electrode. Anal Chim Acta 458(1):215–222

Rotariu L, Bala C, Magearu V (2003) Microbial biosensors for ethanol determination in alcoholic beverages. Ann Univ Buchar XII(I–II):69–76

Rotariu L, Bala C, Magearu V (2004) New potentiometric microbial biosensor for ethanol determination in alcoholic beverages. Anal Chim Acta 513:119–123

Sankarankutty KM (2014) Biosensors and their applications for ensuring food safety. Global J Pathol Microbiol 2:15–21

Saurina J, Hernández-Cassou S, Alegret S, Fàbregas E (1999) Amperometric determination of lysine using a lysine oxidase biosensor based on rigid-conducting composites. Biosens Bioelectron 14:211–220

Schmidt RD, Karube I (1998) Biotechnology. In: Rehm HJ, Reed G (eds) Biosensors and bioelectronics, vol 6b. VCH Verlagsgesellschaft, Weinheim, pp 317–365

Shimomura-Shimizu M, Karube I (2010a) Applications of microbial cell sensors. Biochem Biotechnol 118:1–30

Shimomura-Shimizu M, Karube I (2010b) Yeast based sensors. Adv Biochem Eng Biotechnol 117:1–19

Shkil H, Stoica L, Dmytruk K et al (2009) Bioelectrochemical detection of L-lactate respiration using genetically modified *Hansenula polymorpha* yeast cells overexpressing flavocytochrome b_2. Bioelectrochemistry 76:175–179

Smutok O, Dmytruk K, Gonchar M et al (2007) Permeabilized cells of flavocytochrome b_2 over-producing recombinant yeast *Hansenula polymorpha* as biological recognition element in amperometric lactate biosensors. Biosens Bioelectron 23:599–605

Smutok O, Dmytruk K, Karkovska M et al (2014) D-lactate-selective amperometric biosensor based on the cell debris of the recombinant yeast *Hansenula polymorpha*. Talanta 125:227–232

Spayd SE, Wample RL, Evans RG et al (1994) Nitrogen fertilization of white Riesling grapes in Washington. Must and wine composition. Am J Enol Vitic 45:34–42

Stasyuk NY, Gayda GZ, Gonchar MV (2014) L-arginine-selective microbial amperometric sensor based on recombinant yeast cells over-producing human liver arginase I. Sens Actuat B Chem 204:515–521

Sun JZ, Peter Kingori G, Si RW et al (2015) Microbial fuel cell-based biosensors for environmental monitoring: a review. Water Sci Technol 71(6):801–809

Svitel J, Curilla O, Tkac J (1998) Microbial cell-based biosensor for sensing glucose, sucrose or lactose. Biotechnol Appl Biochem 27(2):153–158

Thevenot D, Toth K, Durst R et al (2001) Electrochemical biosensors: recommended definitions and classification. Biosens Bioelectron 16:121–131

Tkac J, Vostiar I, Gorton L et al (2003) Improved selectivity of microbial biosensor using membrane coating. Application to the analysis of ethanol during fermentation. Biosens Bioelectron 18:1125–1134

Turner AP, Karube I, Wilson GS (eds) (1990) Biosensors: fundamentals and applications. Oxford University Press, London

Ullah A, Orij R, Brul S et al (2012) Quantitative analysis of the modes of growth inhibition by weak organic acids in Saccharomyces cerevisiae. Appl Environ Microbiol 78(23):8377–8387

Uthurry CA, Lepe JA, Lombardero J et al (2006) Ethyl carbamate production by selected yeasts and lactic acid bacteria in red wine. Food Chem 94:262–270

Valima A, Kivisto AT, Leskinen PI et al (2010) A novel biosensor for the detection of zearalenone family mycotoxins in milk. J Microb Met 80(1):44–48

Vevea JD, Wolken DM, Swayne TC et al (2013) Ratiometric biosensors that measure mitochondrial redox state and ATP in living yeast cells. J Vis Exp 77:e50633

Vigneshvar S, Sudhakumari CC, Senthilkumaran B, Prakash H (2016) Recent advances in biosensor technology for potential applications an overview. Front Bioeng Biotechnol 4 (11):1–9

Voronova E, Iliasov P, Reshetilov A (2008) Development, investigation of parameters and estimation of possibility of adaptation of *Pichia angusta* based microbial sensor for ethanol detection. Anal Lett 41(3):377–391

Wang S, Horn PJ, Liou LC et al (2013) A peroxisome biogenesis deficiency prevents the binding of alpha-synuclein to lipid droplets in lipid-loaded yeast. Biochem Biophys Res Commun 438 (2):452–456

Woutersen M, Belkin S, Brouwer B et al (2011) Are luminescent bacteria suitable for online detection and monitoring of toxic compounds in drinking water and its sources? Anal Bioanal Chem 400:915–929

Yagi K (2006) Applications of whole-cell bacterial sensors in biotechnology and environmental science. Appl Microbiol Biotechnol 3(6):1251–1258

Yano Y, Numata M, Hachiya H et al (2001) Application of a microbial sensor to the quality control of meat freshness. Talanta 54:255–262

Yudina NY, Arlyapov VA, Chepurnova MA, Alferov SV, Reshetilov AN (2015) A yeast co-culture-based biosensor for determination of waste water contamination levels. Enzyme Microb Technol 78:46–53

Zhang J, Liu M, Su Y et al (2012) A targeted in vivo RNAi screen reveals deubiquitinases as new regulators of notch signaling. G3 (Bethesda) 2(12):1563–1575