Chapter 9
Molecular Mechanisms in Yeast Carbon Metabolism: Bioethanol and Other Biofuels

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Abstract Biofuels, such as ethanol, biodiesel and biogas, have the potential to replace a large proportion of transportation fuels that presently are mainly produced from fossil raw materials. Bioethanol, which is the product of the fermentative energy metabolism of yeasts, is currently the major biofuel on the global market. It is to a large extent generated from first-generation substrates, i.e. food grade raw materials. There are huge research efforts to develop ethanol processes based on non-food lignocellulosic materials. Using—omics technologies, metabolic and evolutionary engineering, strains of, predominantly, *Saccharomyces cerevisiae* have been isolated that display enhanced inhibitor and general stress tolerance, lowered glycerol production and a broadened substrate spectrum (including the fermentation of pentose sugars released from hemicellulose). Expression of these features in industrial isolates may within a relatively short time generate strains robust enough for commercial ethanol production from lignocellulose. *S. cerevisiae* has also been modified to produce the advanced biofuel butanol. Although yields and production rates are still below the threshold for industrial applications, tools for further developments are now available. Biodiesel production by either oleaginous yeast species that can naturally accumulate high amounts of lipids or by genetically engineered *S. cerevisiae* are further examples of how yeasts can be used for biofuel production. Sustainable production of biofuels requires the integration of all steps of handling biomass, including preservation, pretreatment, fermentation and conversion of side products into high value compounds. In all these steps, yeasts have great technological potential.

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9.1 Introduction

Biofuels are reduced organic compounds, whose oxidation energy is used for heating, producing electricity and running combustion engines, mainly for transportation. The metabolic activity of photosynthetic organisms is the basis for both the origin of fossil fuels and biofuel generation, but, in contrast to fossil fuels, biofuels are generated from renewable biomass. Currently, bioethanol, biogas and biodiesel are those biofuels that are commercially used in transportation. However, the present global society primarily runs on fossil fuels; biofuels represent less than 4 % of the total global transportation energy. To move away from this large-scale consumption of fossil resources requires both, measures to reduce the global energy consumption and a considerable development of sustainable energy technologies (Cheng and Timilsina 2011; Vanholme et al. 2013). Yeasts are able to produce a variety of reduced organic molecules and have good potential to play a central role in these developments (Nielsen et al. 2013).

Bioethanol is the major biofuel on the global market (Cheng and Timilsina 2011; Caspeta et al. 2013; Amorim et al. 2011). It is mainly produced by the yeast *Saccharomyces cerevisiae* as the final product of fermentative sugar metabolism. The production of ethanol is one of the mankind's oldest biotechnologies. Ethanol has had a major impact on the development of human civilisation as a beverage component, conservation agent and drug (Vallee 1998; McGovern et al. 2004). It also has a history as a transportation fuel. The combustion engine developed by Nikolaus Otto in 1860 ran on ethanol, and, similarly, cars developed by Henry Ford at the end of the nineteenth and the beginning of the twentieth centuries could be driven with ethanol. However, at the beginning of the twentieth century, ethanol was no longer competitive with the relatively cheap gasoline made from mineral oil. In the following years, the interest in ethanol as a fuel declined, except on certain occasions when the supply with mineral oil was perturbed. During the oil crisis of the 1970s, the interest in ethanol as a fuel increased again, but with the exception of Brazil, this interest decreased with decreasing oil prices. Only towards the end of the twentieth and the beginning of the twenty-first centuries did ethanol became increasingly regarded as an alternative motor fuel, at first because it can replace lead-containing compounds as an octane booster, but nowadays, more and more because of growing concerns about the environmental impact of using fossil fuels and issues of supply and access (Solomon et al. 2007; Gnansounou 2010).

Considering the long history of ethanol production, it might be surprising that there is still a huge demand for research in this field. However, biofuel production has been debated during recent years, mainly because of low efficiency of production, low or occasionally negative impact on greenhouse gas and fossil fuel balance, and potential conflicts between food and energy production (Caspeta et al. 2013). New substrates derived from second-generation, lignocellulosic materials must be introduced into the production process to address these shortcomings. Lignocellulose provides the most abundant biomass resource on earth, and the

amount of lignocellulose produced by land plants has been estimated to be about 10–200 $\times$ 10^9 t per year (Vanholme et al. 2013). However, due to its recalcitrance and its heterogenic composition, conversion of lignocellulose into biofuels requires new methods of pretreatment and modified yeast strains. Newly developed methods of metabolic analyses and metabolic engineering are providing a variety of opportunities for optimising ethanol production (Nielsen et al. 2013; Van Vleet and Jeffries 2009). Even ethanol production from first-generation substrates could still be greatly improved by identifying optimal strains, metabolic engineering and optimising fermentation conditions (Amorim et al. 2011; Nielsen et al. 2013).

Apart from ethanol production, research efforts have also focused on producing biofuels such as biodiesel or biobutanol with the help of yeasts. Microbial biodiesel could overcome the low energy yield per hectare obtained from oil plants, and if produced from lignocellulose a food versus fuel conflict might be avoided (Caspeta et al. 2013). Biodiesel production can be achieved either by oleaginous yeasts, which can accumulate lipids to more than 30 % of their biomass, or by genetically engineered *S. cerevisiae*. Investigation and manipulation of lipid metabolism in yeasts will also provide new insights into the carbon metabolism of yeasts, as pathways towards lipid accumulation require oxygen, in contrast to the well-investigated alcoholic fermentation (Buijs et al. 2013; Ratledge and Wynn 2002).

Butanol, compared to ethanol, has a higher energy density, can be better blended with gasoline and is less hygroscopic. In 2008, 2.8 million t were produced, corresponding to a market value of about 5 billion US dollars. Most butanol is currently produced by chemical synthesis from mineral oil compounds. A fermentative process based on solventogenic clostridia was commercialised already in 1912, but is currently not competitive with chemical synthesis (Green 2011). Several approaches for producing biobutanol with genetically engineered yeasts have been developed (Buijs et al. 2013).

Sustainable conversion of biomass into biofuels and chemicals requires the integration of production, storage, pretreatment, processing of the feedstock in a biorefinery, treatment and generating value from the remnant feedstock (Vanholme et al. 2013). Yeasts can play important roles in such processes. This chapter aims to provide a survey on the efforts to understand and manipulate the yeast carbon metabolism to develop ethanol, butanol and biodiesel production for a biofuel refinery.

9.2 Ethics of Biofuel Production: Food Versus Fuel?

Biofuels are seen as a step towards a more sustainable society that is less dependent on fossil raw materials and that produces less or no surplus greenhouse gases (Cheng and Timilsina 2011; Vanholme et al. 2013; Solomon et al. 2007). However, production of biofuels, as well as human food, is based on plant

biomass. Recent years have seen an increased demand for biofuels and, in parallel, a dramatic increase in worldwide food prices. Moreover, there are examples where the effect of biofuel production on saving fossil resources and reducing greenhouse gas emission was negligible (Hill et al. 2006). In some cases, biofuel production has had negative impacts on the environment, for instance, when vast areas of rainforest in Borneo were deforested to grow oil palms (Graham-Rowe 2011). Given that marginalised members of the population, particularly in developing countries, struggle to meet their basic nutritional requirements, this raises the question of whether it is ethically acceptable to use food grade raw materials for producing biofuels (Thompson 2012). There is, however, no simple answer. Prices of food are influenced by additional factors beyond the competitive use of agricultural products for biofuel generation. Current agriculture is to a large extent dependent on an input of fossil fuels and increasing oil prices have a major influence on food prices. Moreover, there is also a global increase in average meat intake per capita, and animal production requires 2.5–10-fold more energy input per generated nutritive calorie compared to plant-based food, which is also driving prices up (Pelletier et al. 2011). Increasing prices should not necessarily be viewed as a negative phenomenon *per se*, they may support food producers in developing countries. According to the United Nations, about 50 % of people in extreme poverty (i.e. with an income of less than 1 Euro per day) are food producers, 30 % are to some extent involved in agricultural production, and thus, a majority of extremely poor people might benefit from price increases. Moreover, farmers might be encouraged by higher prices to produce more food, which might in the long term even help marginalised non-producers. On the other hand, there are a number of examples where poor farmers have been evicted due to the increasing value of land. Such cases may not specifically be linked issues of biofuel production, but rather may stem from political circumstances (Thompson 2012). Arising from this background, principles have been formulated to evaluation situations in which production of biofuels could be considered ethically acceptable. These criteria include: keeping essential rights of people (including food, water, health rights or land entitlements); sustainability of biofuels; greenhouse gas savings; and fair trade. If all of these criteria are met even a duty to develop these biofuels is postulated (Buyx and Tait 2011).

There is a certain consensus that the development of lignocellulose as raw material for biofuel production will solve the issue of competing food and fuel production. Exploitation of underutilised feedstocks may indeed provide a broader basis for biofuel production (Tilman et al. 2009). However, if second-generation energy plants turn out to be profitable, it is likely that they will also be produced in areas that can be used for food production (Graham-Rowe 2011). In this case, second-generation biofuels would also compete with food production. Moreover, if technologies are developed to gain access to the nutrients stored in the lignocellulose structure, it might be possible that these nutrients can also be used for food production (Thompson 2012). This has, in principle, been a long-established alternative in the feed industry by producing fodder yeast from lignocellulose residues (Johnson 2013).

Current biofuel production cannot be seen as the major cause of hunger in developing countries. The refinement of second- and higher generation biofuels will indeed provide essential technologies for a sustainable global economy, but technologies cannot in themselves change political circumstances. However, the scientific community developing these technologies can and should emphasise well-formulated ethical criteria regarding biofuels when communicating with society at large. Moreover, by sharing knowledge with colleagues from developing countries and especially supporting projects that enable local farmers to introduce low-tech innovations for food preservation or processing into their food value chains, scientists can actively support a development that empowers people and preserves their fundamental rights. Yeast-based technology can also play a role in such low-tech applications (e.g. Hellström et al. 2010; Leong et al. 2012).

9.3 First- and Second-Generation Ethanol Production

9.3.1 Towards Optimisation of First-Generation Biofuel Production

As ethanol production from first-generation substrates is still of great economic importance, significant research efforts are directed towards improving these well-established processes, especially in Brazil. Most Brazilian ethanol factories rely on a fermentation method in which yeast cells are recycled and inoculated to the next round of fermentation. This decreases the amount of sugar spent on biomass formation, increases the ethanol yield and shortens the fermentation time. On the other hand, the yeast cells are exposed to considerable stresses, e.g. high ethanol concentrations, high temperatures, osmotic stress, low pH, sulphite and contamination by bacteria. Thus, there are specific demands placed on strains used in these environments (Amorim et al. 2011; Della-Bianca et al. 2013). During a time frame of 12 years highly competitive, non-foaming and non-flocculating strains have been isolated and further tested for fermentation performance and competitiveness in commercial distilleries. However, most of the isolates had a poor implantation capability, highlighting that many factors influencing population dynamics in industrial ethanol fermentation are still unknown (Basso et al. 2008). In fermentations running with cell recirculation contamination with *Dekkera bruxellensis* has frequently been observed (de Souza Liberal et al. 2007; Passoth et al. 2007). *D. bruxellensis* is a slow-growing yeast with a highly efficient energy metabolism and, most probably, a high affinity to the limiting sugar substrate (Blomqvist et al. 2010, 2012; Tiukova et al. 2013), indicating that these characteristics play a major role in the ecosystem of industrial ethanol fermentation. Several genome analyses of *S. cerevisiae* isolates obtained from industrial ethanol fermentations have been performed. Almost all isolates were diploid strains with a high degree of heterozygosity, i.e. with a high number of polymorphisms between different alleles.

The number of transposable elements was comparably low, which may contribute to genome stability (Della-Bianca et al. 2013; Zheng et al. 2012). On the other hand, chromosomal rearrangements have been observed, but these were mostly restricted to the chromosome ends. Suppression of flocculation-related genes may contribute to competitiveness of Brazilian fermentation strains (Babrzadeh et al. 2012; Della-Bianca et al. 2013). A variety of genes may be responsible for efficiency in industrial ethanol production, including those involved in vitamin and glycerol metabolism (Babrzadeh et al. 2012; Della-Bianca et al. 2013; Zheng et al. 2012).

9.3.2 Strains for Second-Generation Ethanol Production

The situation is more complicated for second-generation substrates. A variety of inhibitors are formed during lignocelluloses pretreatment (Fig. 9.1), including furfural, hydroxymethylfurfural (HMF), acetate and other organic acids, and aromatic compounds (Palmqvist and Hahn-Hägerdal 2000; Klinke et al. 2004). Apart from this, lignocellulose hydrolysate contains a number of sugars, mainly released from hemicellulose, which cannot be fermented by wild-type *S. cerevisiae*. The most prominent of these sugars is the pentose xylose, which is, after glucose, the second most abundant sugar in nature. Several studies have demonstrated that the ability to convert this sugar into a valuable product is a key factor for the economic feasibility of ethanol production from lignocellulose (Kuhad et al. 2011). Attempts have been taken to manipulate strains to convert xylose and other lignocellulose sugars into ethanol (see below).

Occasionally, strains have been isolated from spent sulphite liquor (SSL) plants (Margeot et al. 2009). One isolated strain exhibited enhanced ethanol productivity and yield in SSL. This strain had high furaldehyde reductase activity and flocculated heavily, which may contribute to resistance against inhibitors in the hydrolysate (Sanchez et al. 2012). In terms of flocculation, the optimal characteristics for a production strain in second-generation substrates may thus differ from those for first-generation substrates (see above). Metabolic manipulation of industrial isolates has been recently performed regarding xylose fermentation and may result in strains that can be used in commercial plants (Garcia Sanchez et al. 2010b).

9.4 Second-Generation Ethanol Production on Commercial and Pilot Scale

Commercially available bioethanol is currently mainly produced from first-generation substrates such as cereal grain or sugar cane. Although it is desirable to develop second-generation processes, for both environmental and global food security reasons, their costs are still too high to replace first-generation ethanol

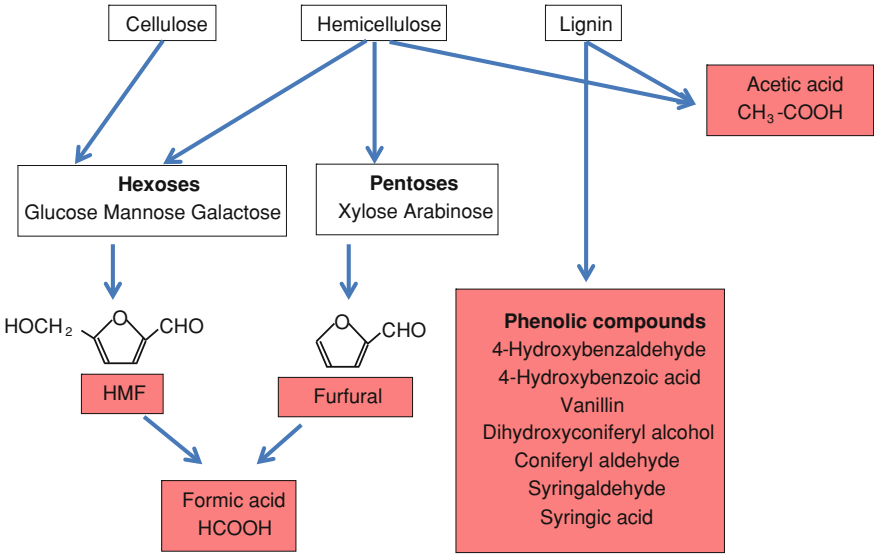


Fig. 9.1 Inhibitors (*red boxes*) released during pretreatment of lignocellulosic biomass. Acetic acid is released from acetylated hemicellulose and lignin. The major degradation products of lignin are phenolic compounds (the most prominent examples are given); sugars are primarily degraded to furans, especially furfural and hydroxymethylfurfural (HMF). Furans can be further degraded to weak acids, mainly formic acid

(Stephen et al. 2012). However, there are examples of commercial second-generation ethanol production and an increasing number of near-commercial ethanol production on a pilot scale. Borregaard (http://www.borregaard.com/Business-Areas/Borregaard-ChemCell) is a Norwegian company, mainly producing cellulose products from spruce. Sugars released from the cellulose process are used for producing second-generation ethanol, with an annual capacity of 20 million litres (Rødsrud et al. 2012). There is also a plant in Russia (Kirov Biochemical Plant) that converts wood biomass into pellets, furfural, fodder yeast, bioethanol and biogas. The ethanol process is based on thermophilic bacteria, and production capacity is not described on the company website (http://biochim.org/). In October 2013, the largest second-generation bioethanol plant to date was started in Crescentino (Italy), with a capacity of 75 million litres (60,000 metric tons) per year. The plant uses mixed feedstocks: wheat or rice straw, or the energy plant, giant cane (*Arundo donax*). Apart from this, energy is provided from burning of lignin. In the future, production of butanol and other chemicals are also planned. The plant's construction was driven by the BIOLYFE project, which was co-financed by the seventh framework program for Energy Research (FP7) of the European Union (http://www.biolyfe.eu/). The Danish company Inbicon AS has developed a process called Integrated Biomass Utilization System (IBUS). In this process, the cellulose of wheat straw is converted into ethanol, the lignin to a solid fuel, delivering process energy, and the hemicellulose fraction is planned to be converted into animal feed

(Larsen et al. 2008). With support of the Danish energy authority and FP7, a pilot plant has now been built and is able to fully operate 7 days per week. The production potential is 576 kg (730 l) per hour, which is still not at profitable levels. However, the produced ethanol is sold and distributed at more than 100 filling stations in Denmark (Larsen et al. 2012). In an ongoing European project (KACELLE), the fermentation of C5 sugars with genetically engineered strains (see below) will also be investigated (http://www.inbicon.com/projects/kacelle/pages/kacelle_project.aspx). A variety of international projects are currently supported by FP7, with the aim to develop new enzymes, new pretreatment strategies and new yeast strains for commercial second-generation ethanol production (http://www.biofuelstp.eu/cell_ethanol.html#ce2). Similar efforts are underway all around the world. In 2007, the US Department of Energy (DOE) announced an investment of up to $385 million for six biorefinery projects (http://www.biofuelstp.eu/cell_ethanol.html#ce8). In July 2013, INEOS Bio announced the start of a commercial ethanol plant in Vero Beach, Florida, using vegetative and yard waste, citrus, oak, pine and pallet wood waste. Its annual output is projected to be 8 million gallons (24,000 t) (http://www.ineos.com/en/businesses/INEOS-Bio/News/INEOS-Bio-Produces-Cellulosic-Ethanol/). There are also projects for demonstration and commercial plans in Canada and Brazil (http://www.biofuelstp.eu/cell_ethanol.html) and also China is encouraging efforts to obtain lignocellulosic ethanol production by offering specific subsidies (Qiu et al. 2012). The above-mentioned examples demonstrate that, although costs are still quite high compared to both first-generation ethanol and mineral oil, lignocellulosic ethanol has reached the threshold of commercial reality. Ongoing research efforts towards more efficient enzymes and pretreatment methods, novel strains fermenting all sugars present in hydrolysates, and learning effects when running large-scale lignocellulose-based ethanol production will most probably rapidly result in technologies with increased economical robustness and higher acceptance within society.

9.5 Manipulation of Yeast Carbon Metabolism to Obtain Higher Ethanol Production

9.5.1 Enabling Fermentation of Pentoses and Other Sugars

Lignocellulose mainly consists of the polysaccharides, cellulose and hemicellulose, and the polyaromatic compound, lignin. Hemicellulose, in contrast to cellulose that consists of polyglucose, contains a variety of sugars. Among them, xylose comprises the highest proportion. In softwood xylose comprises 5–10 % of the total lignocellulose biomass, however, in straw and hardwood its percentage can be higher than 20 and 25 %, respectively. Other sugars include the pentose arabinose (prominent in some softwoods and grasses, up to 10 % of the total

biomass) and the hexoses mannose (up to 15 % of the total biomass in softwoods) and galactose (about 4 % of the total biomass in softwoods and some agricultural materials) (Girio et al. 2010). Whereas the hexoses can be converted into ethanol by *S. cerevisiae*, this yeast is unable to assimilate pentoses (Kurtzman et al. 2011). The predominant pathway for xylose assimilation in fungi consists of two steps: NAD(P)H-dependent reduction by xylose reductase (XR) to xylitol and subsequent NAD^+-dependent re-oxidation by xylitol dehydrogenase (XDH) to xylulose. In most known xylose-assimilating fungi, the XR utilises only NADPH as co-factor, whereas for ethanol production from this sugar NADH utilisation by XR is essential (Bruinenberg et al. 1984). Interestingly, *S. cerevisiae* has enzymes that can reduce xylose to xylitol and oxidise xylitol to xylulose. The NADPH-dependent aldose reductase encoded by *GRE3* is the main XR in *S. cerevisiae*, and it is obviously involved in xylitol production by recombinant strains. Its disruption significantly decreases the xylitol production of those strains (Träff et al. 2001, 2002), and thus in many constructs that have been engineered during the recent years, this gene is knocked out (see below). There is also a gene encoding an XR in the *S. cerevisiae* genome (*YLR070c* or *XYL2*). This gene seems only to be expressed in the presence of a non-repressing carbon source and xylose. Thus, *S. cerevisiae* has the xylose assimilation pathway in its genome, but it is expressed at too low levels to enable the yeast to grow on this sugar (Richard et al. 1999). These results are supported by earlier findings that *S. cerevisiae*, although not able to grow on xylose as sole carbon source, can metabolise this sugar (van Zyl et al. 1989). A few years ago, *S. cerevisiae* strains were isolated that could grow slowly on xylose. When applying a series of mass matings and selection experiments on xylose medium, strains were obtained that could grow on xylose with doubling times down to 6 h, forming some ethanol (Attfield and Bell 2006). Several xylose-fermenting yeast species are known, but these have drawbacks such as low tolerance of ethanol and of inhibitors released during pretreatment of lignocellulose, or a requirement for a very controlled regime of oxygenation (Hahn-Hägerdal et al. 2007). Therefore, substantial efforts have been taken to engineer *S. cerevisiae* to ferment xylose. Bacteria directly convert xylose into xylulose using a co-factor-independent xylose isomerase (XI). However, early attempts to express bacterial XIs in *S. cerevisiae* did not result in active enzymes (Amore et al. 1989; Sarthy et al. 1987), and thus, a two-step, redox factor-dependent fungal xylose assimilation pathway was introduced. Xylose fermentation in *S. cerevisiae* could be achieved by expressing the genes of XR and XDH, encoded by *XYL1* (active both with NADPH and NADH) and *XYL2* (active with NAD^+) of the xylose-fermenting yeast *Scheffersomyces* (*Pichia*) *stipitis* (Kötter and Ciriacy 1993). The first recombinant strains produced substantial amounts of the side product xylitol, indicating a redox imbalance in the cell. Since then, several targets have been approached to improve *S. cerevisiae*'s ability to ferment xylose, including xylose transport, the xylose assimilation pathway, reduction of xylitol formation, redox factor regeneration or the general performance of the sugar metabolism (summarised in Hahn-Hägerdal et al. 2007). One example to approach the redox factor imbalance was the expression of *GDP1* of *Kluyveromyces lactis* in *S. cerevisiae*.

GDP1 encodes an $NADP^+$-dependent glycerol-aldehyde-phosphate dehydrogenase, which results in a lower production of NADH from glycolysis. Deletion of the gene encoding the naturally NADPH-generating glucose-6-phosphate dehydrogenase (*ZWF1*) resulted in an additional increase of ethanol production and lowered CO_2 production (Verho et al. 2003). The first functional XI expressed in *S. cerevisiae* was the xylA gene of the thermophilic bacterium *Thermus thermophilus;* however, as this enzyme operated far away from its temperature optimum, its activity was rather low (Walfridsson et al. 1996). Higher XI activity was obtained by expressing the XylA gene from the cellulolytic fungus *Pyromyces* sp. E2 (Kuyper et al. 2003). When this gene was expressed in a strain overexpressing all enzymes involved in converting xylulose into ethanol, i.e. xylulokinase, ribulose-5-phosphate isomerase, ribulose-5-phosphate epimerase, transketolase and transaldolase, and with a deleted *GRE3* gene encoding aldose reductase, xylose was relatively rapidly converted into ethanol with comparatively low xylitol production and an ethanol yield of 0.43 g per g consumed xylose (Kuyper et al. 2005). Karhumaa et al. compared strains either expressing XR/XDH or XI. The genetic background was the same as in the strain of Kuyper et al. The ethanol yield was quite high in the XI strain, and accordingly, the xylitol yield rather low. On the other hand, xylose uptake was lower than in the XR/XDH-strain and also lower as described by Kuyper et al. 2005. Table 9.1 summarises important fermentation characteristics of selected manipulated strains. Obviously, some unknown factors are influencing the performance during xylose fermentation. Apart from the laboratory strains, an industrial isolate was also engineered to ferment xylose, using XR/XDH. This isolate essentially behaved like the XR/XDH strain on xylose as sole carbon source. However, in contrast to the two strains, which were derived from the laboratory strain CEN.PK, the industrial strain could ferment un-detoxified lignocellulose hydrolysate. Interestingly, in contrast to the cultivation on pure xylose, no xylitol was formed from the hydrolysate, and a substantially higher ethanol yield was obtained (Karhumaa et al. 2007). Expression of an XI from *Orpinomyces* in an *S. cerevisiae* strain overexpressing the homologous xylulokinase and the *S. stipitis* sugar transporter *SUT1* resulted in a xylose-fermenting strain that obtained an ethanol yield of 0.39 g per g consumed xylose (Table 9.1). However, not all xylose was consumed and substantial amounts of xylitol were produced (Madhavan et al. 2009). XI activities were still quite low, resulting in a carbon-starvation response of an engineered strain (Bergdahl et al. 2012). High XI activity was reached by expressing a codon-optimised *XylA* gene from *Clostridium phytofermentans*. An industrial strain transformed with this gene was able to grow on xylose after four serial transfers in xylose medium, without additional manipulations. In contrast to the eukaryotic XI, this enzyme was not inhibited by xylitol, which is always formed in *S. cerevisiae* due to the action of unspecific aldose reductases (Brat et al. 2009).

To identify genes critical for xylose fermentation, genomes and transcriptomes of yeasts naturally fermenting or assimilating xylose have been compared to other yeasts. Forty-three genes were specific for xylose-assimilating yeasts, including a putative xylose transporter and a variety of endoglucanases. Global gene

Table 9.1 Ethanol production parameters of selected genetically engineered *Saccharomyces cerevisiae* strains

Strain	Expressed genes[a]	Initial sugar ($g\ l^{-1}$)[b]	Consumed sugar ($g\ l^{-1}$)[b]	Specific sugar consumption ($g\ g^{-1}\ h^{-1}$)[b]	Specific ethanol production ($g\ g^{-1}\ h^{-1}$)	Yields[c]	Final concentration (g/l)[c]	Reference
H2684	*XYL1*, *XYL2*, *XKS1*, *GDP1*, Δ*zwf1*	X 50	X 14.7	NP[e]	0.018	E 0.41, X-OH 0.35	E 4.6, X-OH 5.2	Verho et al. 2003
RWB 217	*XylA*, *XKS1*, araA, araB, araD, *RPE1*, *RKI1*, *TKL1*, *TAL1*, Δ*gre3*	X 20	X 20	X 1.06	NP[e]	E 0.43, X-OH 0.003	E 8.7, X-OH 0.058	Kuyper et al. 2005
		Mixed: G 20, X 20	G 20, X 20	NP[e]	NP[e]	E 0.43, X-OH 0.006[f]	E 17.1, X-OH 0.12	
TMB 3057	*XYL1*, *XYL2*, *XKS1*, *RPE1*, *RKI1*, *TKL1*, *TAL1*, Δ*gre3*	X 50	X 39.6	X 0.13	0.04	E 0.33, X-OH 0.22, Glyc 0.11	E 13.3, X-OH 8.7[d], Glyc 4.4[d]	Karhumaa et al. 2007
TMB 3066	*XylA*, *XKS1*, *RPE1*, *RKI1*, *TKL1*, *TAL1*, Δ*gre3*	X 50	X 16.8	X 0.05	0.02	E 0.43, X-OH 0.04, Glyc 0.07	E 7.3, X-OH 0.7[d], Glyc 1.2[d]	Karhumaa et al. 2007
TMB 3400	*XYL1*, *XYL2*, *XKS1*, *RPE1*, *RKI1*, *TKL1*, *TAL1*, Δ*gre3*	X 50	X 36.5	X 0.06	0.02	E 0.34, X-OH 0.29, Glyc 0.04	E 12.1, X-OH 10.6[d], Glyc 1.5[d]	Karhumaa et al. 2007
		LCH: G 16, M 10, Gal 4, X 7, A 3	G 16, X 7, M 8, Gal 0, A NP[e]	G 0.021, M 0.013, X 0.005	0.02	E 0.41, Glyc 0.035	E 16, X-OH 0, Glyc 1.4	

(continued)

Table 9.1 (continued)

Strain	Expressed genes[a]	Initial sugar ($g\ l^{-1}$)[b]	Consumed sugar ($g\ l^{-1}$)[b]	Specific sugar consumption ($g\ g^{-1}\ h^{-1}$)[b]	Specific ethanol production ($g\ g^{-1}\ h^{-1}$)	Yields[c]	Final concentration (g/l)[c]	Reference
INVSc1 (engineered)	*XKS1*, *XylA*, *SUT1*	X 50	X 15.55	NP[e]	NP[e]	E 0.39, X-OH 0.08	E 6.05, X-OH 1.28, Glyc 0.66	Madhavan et al. 2009
IMS0010	*XylA*, *XKS1*, araA, araB, araD, *RPE1*, *RKI1*, *TKL1*, *TAL1*, Δ*gre3*	Mixed: G 30, X 15, A 15	G 30, X 15, A 15	X 0.35 A 0.53 [g]	NP[e]	E 0.43	E 25.8, X-OH 0, A-OH 0	Wisselink et al. 2009
BWY10Xyl	xylA	X 25	X 18	X 0.06	0.03	E 0.42	E 7.9, X-OH 3	Brat et al. 2009
TMB 3130	*XYL1*, *XYL2*, *XKS1*, araA, araB, araD	Mixed: G 20, X 20, A 20	G 20, X 17.89, A 18.39 [g]	X 0.09, A 0.1 [g]	NP[e]	E 0.29, X-OH 0.31[f], A-OH 0.95, Glyc 0.04		Garcia-Sanchez et al. 2010a, b
GS1.11-26	*XylA*, *XKS1*, *RPE1*, *RKI1*, *TKL1*, *TAL1*, *TKL2*, *TAL2*, *HXT7* araA, araB, araD, *ARAT*	Mixed: G 36, X 37	G 36, X 37	G 2.71 X 1.1	1.38	E 0.46, X-OH 0.04[f], Glyc 0.06	E 33.6, X-OH 1.5, Glyc 4.3	Demeke et al. 2013b
		LCH (*Arundo donax*): G 59.3, X 22.18, M 6.19	G 59.3, X 22.18, M 6.19			E 0.47, X-OH 0.07[f], Glyc 0.04	E 41.2, X-OH 1.5, Glyc 3.5	

(continued)

Table 9.1 (continued)

Strain	Expressed genes[a]	Initial sugar ($g\ l^{-1}$)[b]	Consumed sugar ($g\ l^{-1}$)[b]	Specific sugar consumption ($g\ g^{-1}\ h^{-1}$)[b]	Specific ethanol production ($g\ g^{-1}\ h^{-1}$)	Yields[c]	Final concentration (g/l)[c]	Reference
GSF767	*XylA*, *XKS1*, *RPE1*, *RKI1*, *TKL1*, *TAL1*, *TKL2*, *TAL2*, *HXT7* araA, araB, araD, *ARAT*	Mixed: G 36, X 37	G 36, X 37	G NP, X 0.65	NP[e]	E 0.46	E 33, X-OH 4, Glyc 4.5	Demeke et al. 2013a
		LCH (amended with sugars) G 62, X 18, M 15	G 62, X 16, M 15	NP[e]	NP[e]	E 0.42[d], X-OH 0, Glyc 0.05	E 39.8, X-OH 0, Glyc 4.65[d]	

[a] Overexpressed genes have been derived from *S. stipitis* (*XYL1*, *XYL2*, *SUT1*, *ARAT*), *S. cerevisiae* (*XKS1*, *RPE1*, *RKI1*, *TKL1*, *TAL1*, *HXT7*) and *K. lactis* (*GPD1*). Genes of the arabinose pathway (araA, araB, araD) were from *L. plantarum* (strains IMS0010 and GS1.11-26), or *B. subtilis* (araA) and *E. coli* (araB, araD) (strain TMB 3130). Xylose isomerase genes were from *Pyromyces* (*XylA*, strains RWB217, TMB 3066 and IMS0010), *Orpinomyces* (*XylA*, strain INVSc1), *C. phytofermans* (xylA, strains BWY10Xyl, GS1.11-26 and GSF767)

[b] G—glucose, X—xylose, M—mannose, Gal—galactose, LCH—lignocellulose hydrolysate

[c] E—ethanol, X-OH—xylitol, A-OH—arabitol, Glyc—glycerol

[d] Calculated from values provided in the original publications

[e] Information not provided by the original article

[f] Calculated on consumed xylose

[g] Xylose and arabinose were consumed after glucose was exhausted

expression analysis of cells grown in glucose or xylose revealed additional genes critical for xylose assimilation and fermentation. Besides the known genes of the xylose assimilation pathway, genes involved in plant biomass degradation like glucosidases and cellulases were strongly induced. Moreover, genes involved in redox metabolism and the pentose phosphate pathway were activated. Several of the identified genes were expressed in an *S. cerevisiae* strain engineered to ferment xylose. Two genes, a *Candida tenuis* aldo/keto reductase and a *Spathaspora passalidarum* gene, with homology to uncharacterised fungal-specific proteins had the greatest effect on xylose assimilation (Wohlbach et al. 2011).

L-Arabinose is the second most abundant pentose in plant biomass, and several attempts have been made to generate yeast strains able to ferment this sugar. In L-arabinose-assimilating fungi, the sugar is first reduced to L-arabinitol (NADPH dependent), which is then re-oxidised to L-xylulose (NAD^+ dependent). L-xylulose is reduced to xylitol (NADPH dependent), which is subsequently converted into D-xylulose by the NAD^+-dependent XDH. The redox factor imbalance generated by this pathway makes it almost impossible to ferment L-arabinose in fungi (Richard et al. 2002). In contrast, the bacterial pathway of L-arabinose assimilation is independent of redox factors and consists of L-arabinose isomerase, ribulokinase and L-ribulose-phosphate 4-epimerase. Genes encoding this pathway, derived from several species, have been expressed in *S. cerevisiae* and L-arabinose-fermenting strains have been obtained (Weber et al. 2010). Expressing AraA, AraB and AraD from *Lactobacillus plantarum* in an *S. cerevisiae* strain did not immediately result in arabinose assimilation. At first, cells were precultivated in galactose, as it has been shown that cells grown on galactose can transport arabinose into the cell. Subsequently, cells were cultivated in several passages in medium containing arabinose as sole carbon source. Finally, arabinose-assimilating cells were transferred to oxygen limited conditions, which resulted in arabinose-fermenting cells. The strain used for this selection had also been engineered to ferment xylose. However, this ability was lost during the selection procedure (Wisselink et al. 2007). Therefore, a strain containing genes for xylose and arabinose assimilation was selected in consecutive selection cycles on medium containing (i) glucose, xylose and arabinose, (ii) xylose and arabinose and (iii) only arabinose as carbon sources. The resulting strain (Table 9.1) was able to ferment all provided sugars (30 g/l glucose, 15 g/l xylose and 15 g/l arabinose) within 35 h, reaching an ethanol yield of 0.44 g per g total sugar (Wisselink et al. 2009). Another potential step towards efficient arabinose-fermenting *S. cerevisiae* strains may be the cloning and expression of L-arabinose transporters from naturally L-arabinose-fermenting yeasts (Verho et al. 2011).

The majority of the above-mentioned manipulations were performed in laboratory strains, which are very efficient tools in research but most probably not competitive under harsh industrial conditions. Recently, the xylose and arabinose fermentation pathways have been introduced in industrial strains. *S. stipitis XYL1* and *XYL2*, the *S. cerevisiae XKS1* and bacterial genes of the arabinose assimilation pathway were overexpressed in the diploid wine strain USM21 (Garcia Sanchez et al. 2010b; Westhuizen and Pretorius 1992). The resulting strain was then further

improved by evolutionary engineering using continuous cultivation for about 65 generations with xylose and arabinose as carbon sources, and with gradually increasing dilution rates as soon as a steady state was reached. In test fermentations with mixed sugars, the evolved strain completely consumed xylose and arabinose. A significant amount of ethanol was produced from xylose, however, also a substantial amount of xylitol. Arabinose was almost completely converted into arabitol in the mixed sugar fermentation (Garcia Sanchez et al. 2010b). In another attempt (Demeke et al. 2013b), the industrial Ethanol Red strain was transformed with a cassette containing a modified *HXT7* (transporting both glucose and xylose into the cell), the codon-optimised *C. phytofermentans XylA* gene, and genes coding enzymes of the pentose phosphate pathway. In addition, bacterial genes of the arabinose assimilation pathway and a codon-optimised arabinose transporter from *S. stipitis* (Subtil and Boles 2011) were introduced. However, these manipulations still did not result in efficient xylose fermentation. Resulting strains were chemically mutagenised and xylose-assimilating strains were further manipulated by genome shuffling (mass mating of isolated spores) with each other and the parental strain. The isolated strain fermented both glucose and xylose at the same time with comparable high rates and ethanol yields. Xylitol production was substantially diminished (0.04 g per g consumed xylose) compared to other studies. On the other hand, the evolved strain showed a decreased glucose uptake rate compared to the strain before random mutagenesis (2.71 and 3.83 g g^{-1} cell dry weight h^{-1}, respectively) and also a reduced ethanol productivity (1.38 and 1.79 g g^{-1} cell dry weight h^{-1}). Moreover, it was less ethanol tolerant and had a reduced respiratory capacity compared to the parental strain. Fermentation of arabinose has not been tested yet, although the arabinose assimilation pathway had also been expressed in the strain (Demeke et al. 2013b). To improve the fermentation capacity, several crossing/segregation experiments of this strain with isolates showing exceptional inhibitor tolerance have been performed. Three superior strains have been isolated. Although showing a lower xylose consumption rate compared to the original strain in complete medium, they exhibited significantly improved tolerance to spruce hydrolysate, higher glucose consumption rates, higher aerobic growth rates and higher maximum ethanol accumulation in high gravity ethanol production (Demeke et al. 2013a). These results illustrate that although there are still unknown factors when it comes to the manipulation of industrial isolates, the application of engineered pentose-fermenting strains in industrial fermentations may soon become a reality.

Apart from xylose and arabinose fermentation, some efforts have been directed towards a more efficient fermentation of other sugars. Overexpressing the *PGM2* gene encoding a phosphoglucomutase improved galactose fermentation, but at the same time also xylose fermentation. This indicates that interactive effects may occur when engineering multiple metabolic pathways in one strain (Garcia Sanchez et al. 2010a). Sucrose is the major substrate of sugar cane- and sugar beet-based ethanol production, and thus, its improvement would have a substantial effect on sustainability of global ethanol production. *S. cerevisiae* hydrolyses most of the sucrose extracellularly by secreting invertase. There is also some capacity to

directly transport sucrose into the cell via a proton symport system. This system consumes energy, hence, less biomass would be formed and thus more ethanol. Basso et al. constructed a strain where the signal sequence of the invertase was removed. This strain hydrolysed most of the sucrose intracellularly; however, growth rate was diminished and the residual sucrose concentration in the medium was high. Selection in anaerobic, sucrose-limited continuous fermentation finally resulted in a strain with higher affinity and higher growth and ethanol production rates and increased ethanol yield compared to the wild type (Basso et al. 2011). This indicates that even on a conventional substrate such as sucrose there is potential for process improvement.

Surface engineering of *S. cerevisiae* may provide an approach to obtain consolidated bioprocessing of biomass. Surface-engineered strains express extracellular, polymer-degrading enzymes such as cellulases, hemicellulases or amylases fused to a glycosylphosphatidylinositol anchoring system, resulting in the display of these enzymes on the surface of the engineered cells. Using those strains, direct conversion of lignocellulosic biomass into ethanol and other compounds has been demonstrated (Hasunuma and Kondo 2012).

9.6 Metabolic Engineering of Non-conventional Yeasts for Ethanol Production from Lignocellulose and Other Substrates

Apart from *S. cerevisiae*, other yeasts have been manipulated to ferment xylose and other sugars. Engineering of thermotolerant yeasts may be of special interest, as fermentation at higher temperatures reduces cooling costs, enables simultaneous saccharification and fermentation closer to the optimum temperature of polysaccharide-degrading enzymes and decreases the risk of contamination. The yeast, *Ogataea polymorpha* (widely known by its previous name *Hansenula polymorpha*), had originally not been described to ferment xylose to ethanol. However, carefully performed growth tests demonstrated that this yeast can convert xylose into ethanol at higher fermentation temperatures (Ryabova et al. 2003). Interestingly, expression of a bacterial XI gene (*xylA* of *Escherichia coli*) resulted in an active protein in this yeast. Expressing this gene in a strain with deleted natural XR/XDH-dependent xylose assimilation pathway resulted in higher ethanol production compared to the wild type (Dmytruk et al. 2008). Further improvements could be obtained for instance by overexpressing the *PDC1* gene, or by overexpressing heat shock proteins and at the same time deleting *ATH1*, encoding for an acid trehalase. The latter resulted in an even more thermotolerant strain with increased ethanol formation (Ishchuk et al. 2008, 2009). Expression of genes encoding for α-amylase, glucoamylase, xylanase and β-xylosidase resulted in a starch- and xylane-fermenting strain (Voronovsky et al. 2009). However, ethanol productivities and yields of all engineered *O. polymorpha* strains are still far below

levels obtained by other xylose fermenting yeasts or engineered *S. cerevisiae*; thus, further efforts are required to introduce this yeast into industrial bioethanol production.

A thermotolerant strain of *Kluyveromyces marxianus* has been manipulated by surface engineering to display endoglucanase and β-glucosidase at its cell surface. The resulting strain converted glucan into ethanol with a yield close to the theoretical maximum (Yanase et al. 2010).

9.7 Inhibitor Tolerance

Yeasts detoxify furfural and HMF (Fig. 9.1) by converting them into the less toxic alcohols, furfuryl alcohol (mainly NADH dependent) and 2, 5-bis-hydroxymethylfuran (mainly NADPH dependent), respectively (Liu 2006). Inhibitor-tolerant strains isolated from pilot-scale lignocellulose fermentations showed increased NADPH- and NADH-dependent furfural and HMF reduction abilities (Sanchez et al. 2012). In accordance with this, several successful attempts of manipulation included overexpression of enzymes with NADPH- or NADH-dependent aldehyde reduction activity. Other approaches increased the flux through the pentose phosphate cycle, which, apart from improving pentose fermentation, may also have provided higher amounts of NADPH, which can be utilised as co-factor in the reduction of furfural and HMF (Hasunuma and Kondo 2012; Liu et al. 2009b), and to detoxify reactive oxygen species (ROS). It has been shown that ROS are released in yeast cells upon exposure to furfural. Interestingly, in contrast to ROS generated by acetic acid (see below), this does not seem to induce programmed cell death (Allen et al. 2010). Overexpression of *ALD6*, encoding an $NADP^+$-dependent cytosolic aldehyde dehydrogenase also increased the tolerance against lignocellulose hydrolysate, probably by generating NADPH. However, a strain overexpressing this gene also generated more acetate (Zheng et al. 2012), which is itself a potent inhibitor present in lignocellulose hydrolysate (Fig. 9.1).

Acetic acid is a powerful antimicrobial agent, which is used for food preservation. At a pH below the pK_a value 4.76, acetic acid is protonated and can diffuse into the cell either directly through the hydrophobic cell membrane or via the facilitator Fps1. Inside the cell the molecule dissociates, which affects the intracellular pH, and by this a variety of metabolic reactions. Yeast cells exposed to acetic acid stress have been described to undergo programmed cell death (Ludovico et al. 2001). This is probably tightly connected to the development of ROS, which are formed as response to acetic acid stress. A variety of other disturbances are caused by acetic acid, including inhibition of amino acid uptake and inhibition of glycolysis (Sousa et al. 2012). As a response to acetic acid stress, the Hog1 MAP kinase and the transcription factor Haa1 are activated. These factors in turn deactivate Fps1 and activate a corresponding stress response, including energy demanding proton transport out of the cell (Piper 2011). Acetic acid-tolerant strains were isolated by long-time cultivation in increasing acetate

concentrations at pH below the pK_a of acetic acid: resulting strains could tolerate up to 6 g/l acetate. Remarkably, during the selection procedure, a very high specific xylose uptake rate was monitored, probably due to the increased energy demand of the cell to run the energy-dependent defence mechanisms (Wright et al. 2011). However, after storing the cells under non-selective conditions, the resistance phenotype disappeared and could only be re-established by precultivating in sub-lethal acetate concentration, indicating an inducible resistance mechanism. It has been shown that catalase is activated upon pre-incubation at sub-lethal concentrations of acetic acid, pointing towards a mechanism detoxifying ROS by the inducible resistance (Martani et al. 2013). A loss of resistance after cultivation under non-selective conditions has also been observed in other systems (e.g. Tiukova et al. 2014), indicating a demand for further investigation of the mechanism of adaptation. In another attempt, an acetate-tolerant strain was isolated by screening about 500 isolates from different origins. The strain showed higher expression of HAA1p- and HOG1p-regulated genes, although transcription of *HOG1* was less enhanced compared to a more sensitive strain. Remarkably, additional genes regulated by another transcription factor, Aft1p, were upregulated. These genes are mainly involved in iron transport. It is to date unclear which role they play in acetate resistance (Haitani et al. 2012). Genetic engineering of *S. cerevisiae* towards production of vitamin C (L-ascorbic acid) resulted in lowered production of ROS and a higher viability of cells exposed to acetic acid stress (Martani et al. 2013). Overexpression of *HAA1* resulted in constitutive increased acetate tolerance in *S. cerevisiae* (Tanaka et al. 2012). Guadalupe-Medina et al. expressed an NADH-dependent aldehyde dehydrogenase in *S. cerevisiae*. The resulting strain was able to use acetate as electron acceptor and converted it into ethanol, which is an example of combining detoxification with product generation (Guadalupe-Medina et al. 2013). Deletion of the *PHO13* gene, encoding *p*-nitrophenyl phosphatase, improved xylose fermentation in genetically engineered *S. cerevisiae* (Van Vleet et al. 2008). Moreover, increased ethanol production by the deletion strain was also observed in the presence of common inhibitors such as acetate, formic acid, furfural and HMF, and in lignocellulose (rice straw) hydrolysate. The physiological basis for this improvement is not clear, but increased expression of genes involved in the pentose phosphate cycle, glycolysis and alcoholic fermentation has been observed (Fujitomi et al. 2012).

A formic acid-tolerant strain has been constructed by overexpression of the *FDH1* gene, encoding formate dehydrogenase. The resulting strain produced ethanol in the presence of 10 mM formic acid, almost as efficient as the original strain in a control fermentation (Hasunuma et al. 2011). Several attempts have been made to construct strains resistant to inhibitors released from lignin, e.g. by expressing the *lcc2* gene from *Trametes versicolor*, encoding a laccase, in *S. cerevisiae* (Larsson et al. 2001).

9.8 Lowering Glycerol Formation

Glycerol is formed during alcoholic fermentation to re-oxidise NADH formed by processes other than glycolysis. It is also a compatible solute, which is produced in response to extracellular stress (Ansell et al. 1997). Glycerol production removes carbon from ethanol formation, decreasing the yield of ethanol production. Disrupting one or both genes encoding cytosolic glycerol-3-P-dehydrogenases in *S. cerevisiae*, *GPD1* and *GPD2*, resulted in decreased glycerol and increased ethanol yield. On the other hand, manipulating the glycerol production genes resulted in slow growth and low specific ethanol production rates. The double disruptant lacked the ability to grow anaerobically (Ansell et al. 1997; Valadi et al. 1998). This is similar to the situation in *D. bruxellensis*, which naturally produces low amounts of glycerol and has a high ethanol yield, but shows low growth and ethanol production rates and requires addition of amino acids for anaerobic growth (Blomqvist et al. 2010, 2012). Apart from directly manipulating the genes involved in glycerol production, attempts have been made to influence the redox balance in the cell. For instance, the NADPH-dependent pathway of ammonium assimilation has been replaced by an NADH- and ATP-dependent pathway in *S. cerevisiae* by disrupting the gene of the NADPH-dependent glutamate dehydrogenase, *GDH1*, and overexpressing *GLN1* and *GLT1*, encoding glutamine synthetase and glutamate synthase (Nissen et al. 2000). Replacement of the natural glyceraldehyde-3-P-dehydrogenase by a non-phosphorylating $NADP^+$-dependent bacterial equivalent also significantly decreased glycerol production. When an NADH-dependent aldehyde dehydrogenase was expressed in a Δ*gpd1*, Δ*gpd2* strain, anaerobic growth was restored in the presence of acetate, which served as alternative electron acceptor and was converted into ethanol (Guadalupe-Medina et al. 2010, 2013). This is a promising approach, as acetate is one of the inhibitors of fermentation released during pretreatment of lignocellulose (see above). The lowered tolerance towards osmotic stress of the Gpd^- strain could be compensated by overexpressing the genes of the trehalose pathway *TPS1* and *TPS2* (Guo et al. 2011). Apart from this, both approaches reduced the amount of ATP produced per mol sugar, and due to this, the flux through the fermentation pathway was increased, resulting in higher ethanol productivity. Deleting *FPS1* encoding an aquaglyceroporin involved in glycerol efflux and acetate uptake (see above) resulted in lower glycerol production but also slower ethanol formation (Wang et al. 2012; Zheng et al. 2012). However, strains with low capabilities of glycerol production are often sensitive to osmotic and other stresses, and thus, they are not suited to the stressful environment of industrial ethanol production. Apart from overexpressing stress-related genes like those of the trehalose synthesis pathway, strategies of genetic manipulation have been combined with genome shuffling by multi-parental protoplast fusion of strains with desired phenotypes, which resulted in the generation of stress-tolerant, low glycerol producing strains (Guo et al. 2011; Tao et al. 2012; Wang et al. 2012).

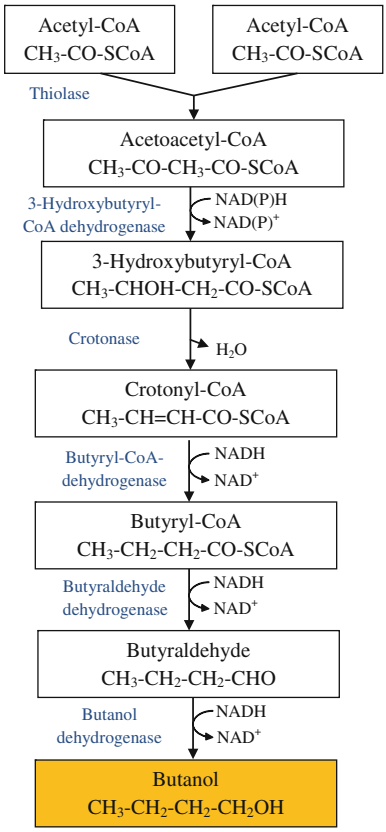


Fig. 9.2 Biosynthesis of n-butanol in engineered *S. cerevisiae*. Two acetyl-CoA are combined and subsequently reduced to n-butanol in a multi-enzyme cascade. Most of the enzymes required for n-butanol formation have been introduced by expressing the corresponding *Clostridium beijerinckii* genes in *S. cerevisiae*. Only thiolase (acetyl-coenzyme A acetyltransferase, encoded by *ERG10*) is present in *S. cerevisiae*. The most productive strain overexpressed thiolase from *S. cerevisiae* or *E. coli*. Expression of NADH-dependent *C. beijerinckii* 3-hydroxybutyryl-CoA dehydrogenase resulted in better n-butanol production than expression of NADPH-dependent enzymes from other bacteria (Steen et al. 2008)

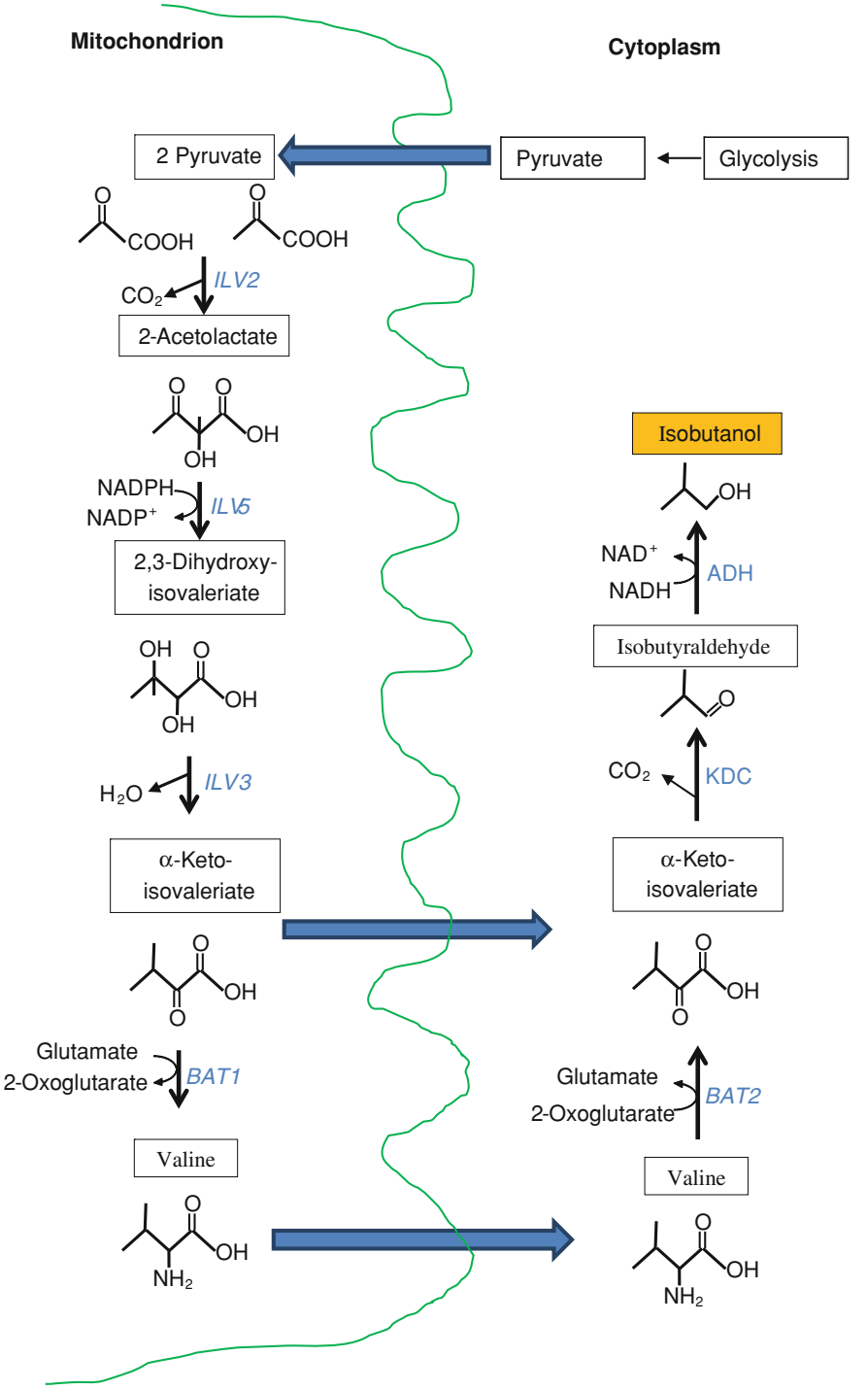
9.9 Manipulating Yeasts for Butanol Production

Yeasts may have several advantages for producing butanol compared to the established clostridia, as yeasts produce fewer side products, are less sensitive to oxygen and are probably more robust. An n-butanol production pathway has been introduced into *S. cerevisiae*. In this pathway two acetyl-CoA first form aceto-acetyl-CoA, which is then reduced by 3-hydroxybutyryl-CoA dehydrogenase to 3-hydroxybutyryl-CoA. This reduction step can be NADH or NADPH dependent in different organisms. From this, crotonyl-CoA is formed by the crotonase reaction. Crotonyl-CoA is then in several NADH-dependent steps finally reduced to butanol (Fig. 9.2). Since *S. cerevisiae* lacks most of the enzymes required for these steps, several genes from *Clostridium beijerinckii*, *E. coli*, *Ralstonia eutropha*, *Streptomyces collinus* and *S. cerevisiae* were overexpressed. Higher butanol production was obtained when an NADH-instead of an NADPH-dependent dehydrogenase was introduced for reduction of acetoacetyl-CoA. However, the highest concentration reached (2.5 mg/l) was below that of an engineered

E. coli strain and of the *Clostridium*-based system. Accumulation of butyryl-CoA indicated a bottleneck at the butyraldehyde dehydrogenase reaction (Steen et al. 2008).

More intense efforts have been made to produce isobutanol, which has similar characteristics as a biofuel compared to n-butanol. Yeasts naturally produce isobutanol during the catabolism of valine through the Ehrlich pathway (Hazelwood et al. 2008). Valine is first deaminated to α-ketoisovalerate, which is then decarboxylated and reduced to isobutanol. Valine biosynthesis starts from pyruvate, and α-ketoisovalerate is formed as an intermediate also in the synthesis pathway (Fig. 9.3). Thus, it is possible to establish an isobutanol production pathway by combining the valine synthesis and degradation pathways. However, the valine synthesis pathway is localised in the mitochondrial matrix, whereas valine degradation takes place in the cytoplasm. Thus, strategies of metabolic engineering aimed, in many cases, to express the corresponding enzymes in the cytoplasm (Buijs et al. 2013; Matsuda et al. 2012; Brat et al. 2012). Further optimisations included enhancement of the pyruvate levels by disrupting *PDC* genes, expressing an optimal α-ketoisovalerate decarboxylase (*Lactococcus lactis* KivD) and alcohol dehydrogenase (*ScADH6*) (Kondo et al. 2012; Matsuda et al. 2012). Brat et al. transferred the valine synthesis pathway into the cytosol by overexpressing codon-optimised *ILV2*, *ILV5* and *ILV3* with truncated mitochondrial import signal sequences. The *S. cerevisiae ARO10* (encoding an α-ketoacid decarboxylase, KDC) and *ADH2* were found to encode the optimal genes for the final two steps of isobutanol production. When these genes were expressed in a Pdc^- strain (Δ*pdc1*, Δ*pdc5*, Δ*pdc6*) with blocked mitochondrial valine synthesis pathway, up to 0.63 g/l, with a yield of 15 mg/g glucose could be obtained (Brat et al. 2012). When additionally XI, transaldolase and xylulokinase genes were overexpressed, the strain was able to ferment xylose to isobutanol, with a final concentration of 1.36 mg/g xylose and a yield of 0.16 mg isobutanol/g xylose (Brat and Boles 2013). A different strategy was employed by Avalos et al., who expressed the whole isobutanol production pathway inside the mitochondria. Overexpressing *S. cerevisiae ARO10* and the *Lactococcus lactis* AdhA (encoding *L. lactis* ADH7) with mitochondrial targeting sequences, together with overexpression of *ILV2*, *ILV3* and *ILV5*, resulted in a maximal isobutanol titer of 630 mg/l in complete medium. No further gene deletions were required to attain this level (Avalos et al. 2013). Although these values are still much lower than in bacterial production hosts, they can be regarded as starting points for further optimisation. In the patent literature, final concentrations up to 18.6 g/l and yields up to 0.33 g/g (i.e. about 80 % of the theoretical maximum) have been reported (Buijs et al. 2013).

Using amino acids as substrate may be another way of producing butanol and isobutanol with *S. cerevisiae*. Branduardi et al. (2013) obtained isobutanol and butanol production from glycine, and introducing a heterologous glycine oxidase gene from *Bacillus subtilis* (*goxB*) increased butanol/isobutanol formation. Maximum concentrations of 92 and 58 mg/l of butanol and isobutanol, respectively, were reached.


Mitochondrion
Cytoplasm
2 Pyruvate
Pyruvate
Glycolysis
COOH
COOH
CO2
ILV2
2-Acetolactate
OH
OH
NADPH
NADP+
ILV5
2,3-Dihydroxy-isovaleriate
OH
OH
H2O
ILV3
α-Keto-isovaleriate
OH
O
Glutamate
2-Oxoglutarate
BAT1
Valine
OH
NH2
Isobutanol
OH
NAD+
NADH
ADH
Isobutyraldehyde
O
CO2
KDC
α-Keto-isovaleriate
OH
O
Glutamate
2-Oxoglutarate
BAT2
Valine
OH
NH2

◀ **Fig. 9.3** Biosynthesis of isobutanol in *S. cerevisiae*. Isobutanol is produced as a result of the Ehrlich pathway of valine degradation. α-Ketoisovalerate is a common intermediate of both the mitochondrially localised valine synthesis and the cytoplasmatic degradation pathways, and by combining both pathways, isobutanol can be generated from pyruvate. α-Ketoacid decarboxylase (KDC) and alcohol dehydrogenase (ADH) activities are encoded by several genes, including all *PDC*-genes and *ARO10* (KDC), and a variety of *ADH* genes, respectively (Hazelwood et al. 2008). Increased isobutanol production was obtained by either expressing KDC and ADH proteins with a mitochondrial signal sequence, thus expressing the whole pathway in the mitochondria, or by expressing cytoplasmic *ILV2* (encoding acetolactate synthase), *ILV5* (acetohydroxyacid reductoisomerase), and *ILV3* (dihydroxyacid dehydratase) (Avalos et al. 2013; Brat et al. 2012). Transamination is performed by branched chain amino acid transaminase (*BAT1* and *BAT2*)

9.10 Yeasts for Biodiesel Production

Biodiesel is currently the only liquid biofuel that is produced on a commercial scale, apart from ethanol. It is generated from oil plants such as soy, oil palms or oilseed rape, which can accumulate triglycerides. The triglycerides are extracted from the plant material. Subsequently, the triglycerides are converted by the transesterification reaction, in which the glycerol is replaced by the short chain alcohols methanol or ethanol, forming fatty acid methyl or ethyl esters (FAME or FAEE), respectively (Fig. 9.4). However, the methanol that is preferably used is generated from mineral oil. Thus, biodiesel cannot completely be considered a renewable fuel. The oil plants are cultivated on arable land, and thus, biofuel production may compete with food production. The energy yield per hectare of oil plants is relatively low compared to sugar plants. Moreover, in contrast to sugar plants that are the basis of ethanol production, oil plants can be cultivated on rainforest areas; and examples of deforestation in such areas for oil plant production have been documented (Graham-Rowe 2011; Azócar et al. 2010).

To overcome the obvious disadvantages of plant-based biodiesel, microbial lipids may represent an alternative. Lipid-accumulating microalgae obtained considerable attention. Indeed, the concept of producing biofuels just from sunlight and assimilated CO_2 is appealing. However, algae have several disadvantages, including slow growth, low lipid accumulation rates, high costs and a high risk for contamination (Cheng and Timilsina 2011). Certain oleaginous yeasts can form triglycerides with high specific rates, and to a proportion of their biomass exceeding 50 %, which is higher than in all other known lipid-accumulating organisms. Thus, yeasts may have a great potential also for biodiesel production. There are about 30 known oleaginous yeast species. They belong to different phylogenetic groups, including ascomycetous species such as *Lipomyces starkeyi* and *Yarrowia lipolytica* or basidiomycetes such as *Rhodotorula glutinis* and *Rhodosporidium toruloides* (Ratledge and Wynn 2002).

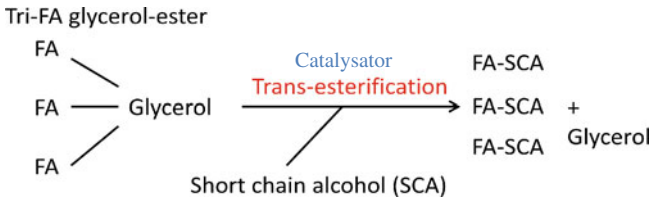


Fig. 9.4 Survey of the transesterification process. Glycerol is replaced by a short chain alcohol, either methanol or ethanol, generating fatty acid methyl or ethyl esters (FAME or FAEE). Alkaline (NaOH), acid (H_2SO_4) or lipases can be used as catalysator

9.11 Physiology of Lipid Accumulation in Yeasts

Lipid accumulation (Fig. 9.5) follows a common pattern in all known oleaginous yeasts (Ratledge and Wynn 2002), in spite of their phylogenetic distance: upon nitrogen limitation, AMP concentration decreases to less than 5 % of its value under C-limitation. The AMP is deaminated to inosine monophosphate by AMP deaminase. In oleaginous yeasts, isocitrate dehydrogenase (IDH) is strictly dependent on AMP. Thus, the activity of the tricarboxylic acid cycle (TCC) decreases, isocitrate accumulates and is equilibrated with citrate. Citrate is transported out of the mitochondria and in the cytoplasm it is converted into acetyl-CoA and oxaloacetate by the ATP citrate lyase (ACL). Oxaloacetate is channelled back to the mitochondria, while acetyl-CoA is the substrate of the acetyl-CoA carboxylase (ACC) which forms malonyl-CoA, the substrate of the fatty acid synthase (FAS) to elongate the acyl-CoA chain (Tehlivets et al. 2007). Nitrogen concentration should, however, not be below a certain threshold, as under those circumstances, citrate is secreted from the cells, decreasing the lipid yield (Morin et al. 2011; Ratledge and Wynn 2002). Lipid accumulation also occurs upon P and S and other non-carbon limitations, but in these cases, the cellular processes are less well documented. FAS requires NADPH as co-factor, and although there are several enzymes in the cell producing NADPH, malic enzyme was the sole enzyme supposedly involved in fatty acid synthesis. In the transhydrogenase cycle, pyruvate is carboxylated to oxaloacetate, which is converted into malate (NADH dependent). Malic enzyme converts malate into pyruvate, generating CO_2 and NADPH. A physical interaction between malic enzyme and other lipid synthesis enzymes has been suggested (Ratledge and Wynn 2002). However, in many yeasts, no cytoplasmic malic enzyme has been found. The oleaginous yeast *L. starkeyi* possesses a cytoplasmic malic enzyme, but this has a preference for NADH over NADPH (Tang et al. 2010). *Y. lipolytica* contains only a mitochondrial malic enzyme, and its overexpression did not affect lipid accumulation (Beopoulos et al. 2011). Malic enzyme has not been identified among the highly expressed enzymes in proteome studies of *L. starkeyi* and *R. toruloides*; instead, upregulation of the NADPH-generating 6-P-gluconat dehydrogenase has been reported (Liu et al. 2009a, 2011). However, an increased level of malic enzyme

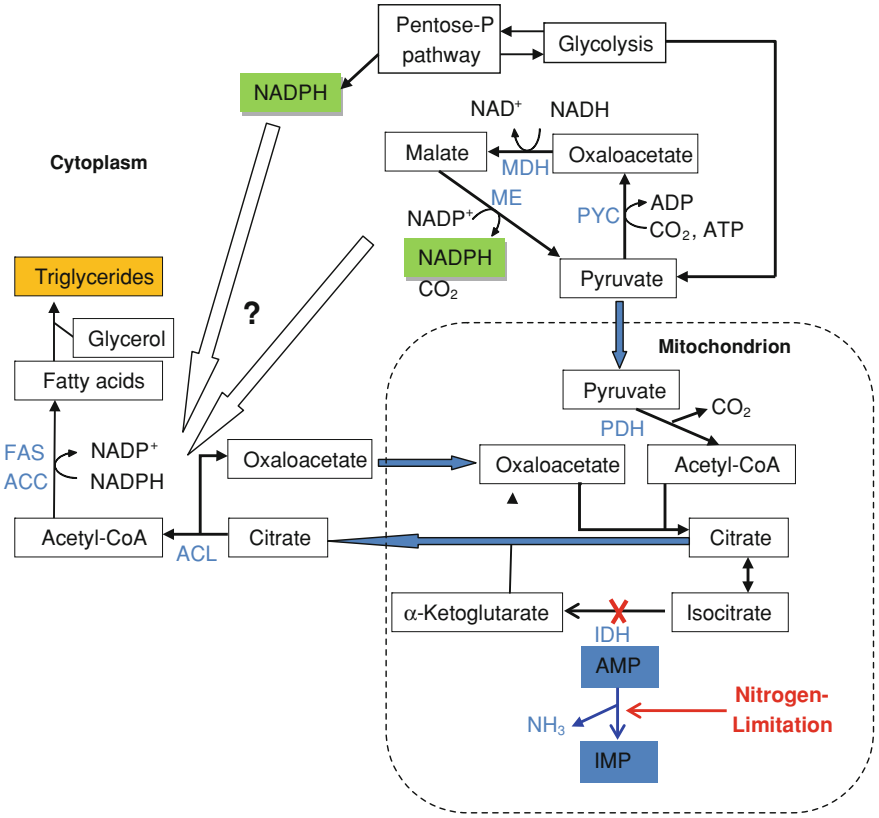


Fig. 9.5 Lipid production in oleaginous yeasts. Sugars are metabolised via glycolysis and the pentose phosphate pathway to pyruvate, which is transported into the mitochondria, converted into acetyl-CoA by PDH and further metabolised via the TCC. In oleaginous yeasts, isocitrate dehydrogenase (IDH) depends on AMP. At nitrogen limitation, AMP is deaminated to IMP, halting the IDH reaction. Isocitrate accumulates and is equilibrated with citrate. Citrate is transported out of the mitochondria and degraded to acetyl-CoA and oxaloacetate by the ACL. Oxaloacetate can be transported back to the mitochondria and feed the TCC. Acetyl-CoA is substrate of the fatty acid synthesising enzymes ACC and FAS. ACC forms malonyl CoA from two acetyl-CoA. Malonyl-CoA reacts in the FAS reaction with acyl-CoA, prolonging the chain by two carbons. Finally, the fatty acids react with glycerol and form, in several steps, triglycerides. The source of the NADPH that is required for the FAS reaction is unclear. It is supposedly generated in the transhydrogenase cycle of pyruvate carboxylase (PYC), malate dehydrogenase (MDH) and malate enzyme (ME), but some recent studies rather indicated that the pentose-P pathway might be the source of NADPH (see text)

upon lipid production conditions has been recently observed in *R. toruloides* (Zhu et al. 2012). Transcriptome analysis of *Y. lipolytica* in nitrogen-limited fed-batch culture showed significant transcriptional regulation of 569 genes. Interestingly, genes encoding assumed key enzymes for fatty acid synthesis like ACL, ACC, FAS or malic enzyme were not transcriptionally regulated, similar to genes

encoding the TCC enzyme IDH (Morin et al. 2011). In the *L. starkeyi* and *R. toruloides* proteomes, ACC was more abundant in lipid accumulation conditions (Liu et al. 2009a, 2011), indicating either a physiological difference to *Y. lipolytica* or regulation at the post-transcriptional level. However, fatty acid accumulation seems to be mainly dependent on regulation of enzyme activities, with inactivation of IDH due to AMP degradation as a key event. In general, nitrogen assimilation enzymes were upregulated at onset of lipid accumulation, which is most likely a physiological response to nitrogen limitation. On the other hand, enzymes involved in glycolysis and the pentose phosphate pathway were downregulated, probably as a response to prevent carbon overflow of the metabolism (Liu et al. 2009a, 2011; Morin et al. 2011). Similar results have been reported in a recently performed multi-omic study of *R. toruloides*, especially in terms of both transcription and protein concentrations of enzymes involved in nitrogen metabolism. However, enhanced transcription of genes encoding FAS has been noted; transcription of most of the other genes involved in fatty acid synthesis was not significantly altered. On a protein level, increased levels of several enzymes involved in fatty acid synthesis have been observed, including, apart from ACC, also ACL, FAS and ME. The behaviour of ME was, to some extent, unusual, as its transcription was decreased under these conditions. This once again shows that not all cellular processes influencing lipid production are yet well understood (Zhu et al. 2012). For lipid production from oleaginous yeasts, obviously cultivation conditions have to be carefully optimised. The optimal pH value differs from species to species, different C:N ratios from 25 to 100 have also been stated to be optimal (Ageitos et al. 2011; Shen et al. 2013; Ykema et al. 1986). Growth rate and lipid production have been found to be inversely correlated (although at very low growth rates, lipid content may decrease), and thus, a compromise between yield and volume-time productivity must be made (Shen et al. 2013; Ykema et al. 1986). Thus, fed-batch cultivation where the growth rate can be regulated probably represents the most efficient fermentation technique for lipid production (Beopoulos et al. 2011).

9.12 Metabolic Engineering to Improve Biodiesel Production by Yeasts

Analysing and manipulating the metabolism of oleaginous yeasts is severely hampered due to the paucity of molecular tools for genetic engineering of these yeasts. Recently, experiments to improve lipid formation in oleaginous yeasts have been performed by a kind of evolutionary engineering. After random mutagenesis, cells of the yeasts *R. glutinis* and *L. starkeyi* were plated onto medium containing cerulenin, an inhibitor of lipid synthesis. Isolates that were able to form bigger colonies accumulated higher amounts of intracellular lipids (Tapia et al. 2012; Wang et al. 2009). Identifying the altered genes in those mutants may identify

targets for improvement of lipid accumulation by oleaginous yeasts. *Y. lipolytica*, which can convert glucose, acids, glycerol and hydrophobic substances such as alkanes into fatty acids, is the only oleaginous yeast for which molecular genetic tools have been developed (Beopoulos et al. 2009). However, in some oleaginous yeasts, initial efforts have been taken to perform molecular manipulation (e.g. Tully and Gilbert 1985), and, with increasing interest in these yeasts, these tools may rapidly be developed. In *Y. lipolytica*, disruption of *GUT2*, encoding a glycerol-3-P dehydrogenase that converts glycerol-3-P into dihydroxyacetone, together with the disruption of genes encoding acyl-CoA oxidases (*POX1-6*) involved in beta oxidation of fatty acids, resulted in a substantial increase in lipid production (Beopoulos et al. 2008).

Several efforts have been undertaken to engineer *S. cerevisiae* to produce biodiesel. *S. cerevisiae* is not oleaginous; in fact, storage lipid synthesis is not essential for this yeast (Sandager et al. 2002). However, it is an established cell factory with well-developed tools for molecular manipulation and a well-investigated metabolism. In yeasts, acetyl-CoA, the precursor of fatty acid synthesis, is formed from pyruvate: in the cytosol, by the action of pyruvate decarboxylase, aldehyde dehydrogenase and acetyl-CoA synthase; and in the mitochondria by pyruvate dehydrogenase (PDH) (Holzer and Goedde 1957). In contrast to oleaginous yeasts, the pathway for synthesising cytosolic acetyl-CoA from surplus citrate from the mitochondria is absent in *S. cerevisiae* (Beopoulos et al. 2011). Thus, increasing the intracellular acetyl-CoA level and redirecting the flux from ethanol production towards producing precursors of lipids is one of the challenges when producing biodiesel or related products from *S. cerevisiae*. An increased acetyl-CoA level has been achieved by overexpressing the aldehyde dehydrogenase gene *ALD6* and a mutated acetyl-CoA synthase gene from *Salmonella enterica* in *S. cerevisiae* (Shiba et al. 2007). This system was further improved by (Chen et al. 2013), who additionally overexpressed *ADH2*, encoding the assimilatory alcohol dehydrogenase. Moreover, *ERG10*, encoding an acetyl-CoA acetyltransferase, was overexpressed. By this strategy, substantial amounts of acetyl-CoA were redirected from ethanol and biomass production towards desired compounds, in this case the production of α-santalene. This platform can be the basis for producing a variety of compounds, including biodiesel or n-butanol. Overexpression of the genes of the isoprenoid synthesis pathway and repressing ergosterol synthesis, or overexpressing a phosphatase dephosphorylating farnesol pyrophosphate resulted in strains overproducing farnesol, which can be used as biodiesel or jet fuel (Hong and Nielsen 2012; Zhang et al. 2011). Expression of heterologous wax synthases in *S. cerevisiae* for in vivo production of FAEE, which can be directly used as biodiesel, has also been reported (Kalscheuer et al. 2004; Shi et al. 2012). Overexpression of ACC additionally increased FAEE production by 30 %, resulting in a biodiesel production of 8.2 mg/l (Shi et al. 2012).

Glycerol is a side product from transesterification and its conversion into biofuels can contribute to sustainability of biodiesel production. By overexpression of genes of the glycerol assimilation pathway (glycerol kinase, *GUT1*) and the triaacylglycerol formation pathway (diacylglycerol acyltransferase, *DGA1* and

LRO1), lipid concentrations of 23 mg/l could be obtained from glycerol as sole carbon source (Yu et al. 2013). Overexpressing the glycerol assimilation pathway (including a glycerol transport protein) and disruption of the genes of dihydroxyacetone phosphate degradation and glycerol export resulted in ethanol production from glycerol in *S. cerevisiae*. When a wax ester synthase was also overexpressed, the engineered strain was able to condense the formed ethanol with externally added oleic acid to ethyl oleate, thus representing FAEE production from glycerol (Yu et al. 2012).

9.13 Biodiesel Production from Lignocellulose

Lignocellulose hydrolysate seems to have a good potential for lipid production, as it usually has a high C/N ratio (Hyvönen et al. 2000; Reinertsen et al. 1984). Moreover, most oleaginous yeasts (unfortunately, except the well-investigated yeast *Y. lipolytica*) have the potential to assimilate xylose and other sugars present in lignocellulose hydrolysate (Kurtzman et al. 2011). On the other hand, the inhibitors released during pretreatment (Fig. 9.1) also influence oleaginous yeasts. A variety of oleaginous yeast species have been tested to convert residue materials such as wheat and rice straw, corn stover hydrolysate or sewage sludge into lipids (Angerbauer et al. 2008; Galafassi et al. 2012; Huang et al. 2009; Yu et al. 2011). The final lipid concentrations in these experiments rarely reached more than 10 g/l, which was relatively low compared to, say, a high cell density cultivation of an *R. toruloides* strain on glucose, where a lipid concentration of 151.5 g/l was obtained (Li et al. 2007). However, most of these tests were performed in batch cultivation, so there is still a great potential to optimise the fermentations. Remarkably, the diversion between growth and lipid accumulation as observed in artificial growth medium was not seen in these experiments. Lipid accumulation mainly followed biomass formation. In later stages of fermentation, the lipid content remained constant or slightly decreased, while the biomass was still increasing. Thus, the lipid proportion on the total biomass was relatively low towards the end of the fermentation (Huang et al. 2009; Yu et al. 2011). Inhibitors had differing effects on growth and lipid accumulation. Reports on the inhibitory action of acetate are conflicting to some extent. In a screening experiment, 5 g/l acetate completely prevented growth of strains of *L. starkeyi*, *R. glutinis* and *R. toruloides*. *Trichosporon cutaneum* could grow at this concentration, but was strongly inhibited (Chen et al. 2009). Similarly, *Rhodotorula graminis* was already inhibited at acetate concentrations above 2 g/l (Galafassi et al. 2012). However, only a slight inhibitory effect of acetate on the growth and even some stimulation of lipid accumulation was observed for *R. toruloides* (Hu et al. 2009). Obviously, species and strains differ substantially. In some studies, acetate seemed to be rather a substrate than an inhibitor (Lian et al. 2012; Yu et al. 2011). In mixed substrate, it was consumed faster than the sugars in the medium, and it contributed to lipid accumulation

(Yu et al. 2011). HMF, which is a strong inhibitor during ethanol production, does not seem to have a similar deleterious effect on lipid production, whereas furfural and vanillin were toxic for the yeasts (Chen et al. 2009; Hu et al. 2009).

9.14 Lipid Extraction from Yeasts

In contrast to ethanol, lipids are not secreted into the medium and thus have to be extracted from the cells. The relatively robust cell walls represent a serious barrier for extraction. Moreover, several other lipophilic compounds are present in the lipid bodies, which have to be removed before using the fatty acids to produce biodiesel. Thus, lipid extraction from the cells represents a major challenge in establishing yeast-based biodiesel production. Several extraction methods have been tested at pilot scale, mainly by using ethanol–hexane mixtures, but further optimisation is required to obtain an optimal process running under commercial conditions (Ageitos et al. 2011; Jacob 1992). The final step in biodiesel production, transesterification, is currently mainly done with the help of alkaline catalysts, which can result in undesirable saponification reactions with biolipids, due to the high availability of free fatty acids. Thus, the development of an economically viable enzyme-based transesterification is critical for developing a commercial biodiesel process based on microbes (Azócar et al. 2010; Robles-Medina et al. 2009).

Forcing secretion of the formed lipids out of the cell might be an alternative to extraction. Recently, experiments were performed in bacteria to express transporters exporting hydrophobic molecules out of the cell (Dunlop et al. 2011). In particular, the approach of expressing specific ABC transporters may be also relevant for yeasts, as ABC transporters are ubiquitous among all kingdoms of life. When the transporters are expressed under an inducible promoter, the cells can, after a lipid-accumulating cultivation, be transferred into a biphasic system with an aquatic and an organic phase. Secreted lipids will then accumulate in the organic phase, while the cells stay in the liquid phase. After secreting the lipids, the cells can be re-used in further fermentations (Doshi et al. 2013). Another approach makes use of the native capacity of yeasts to excrete esterified fatty acids. Overexpression of a gene encoding Acyl-coenzyme A: ethanol O-transferase can result in the production of fatty acid ethyl esters. Moreover, it has been shown that *Candida tropicalis* excretes free fatty acids to the medium when transferred to oxygen limited conditions (Phadnavis and Jensen 2013).

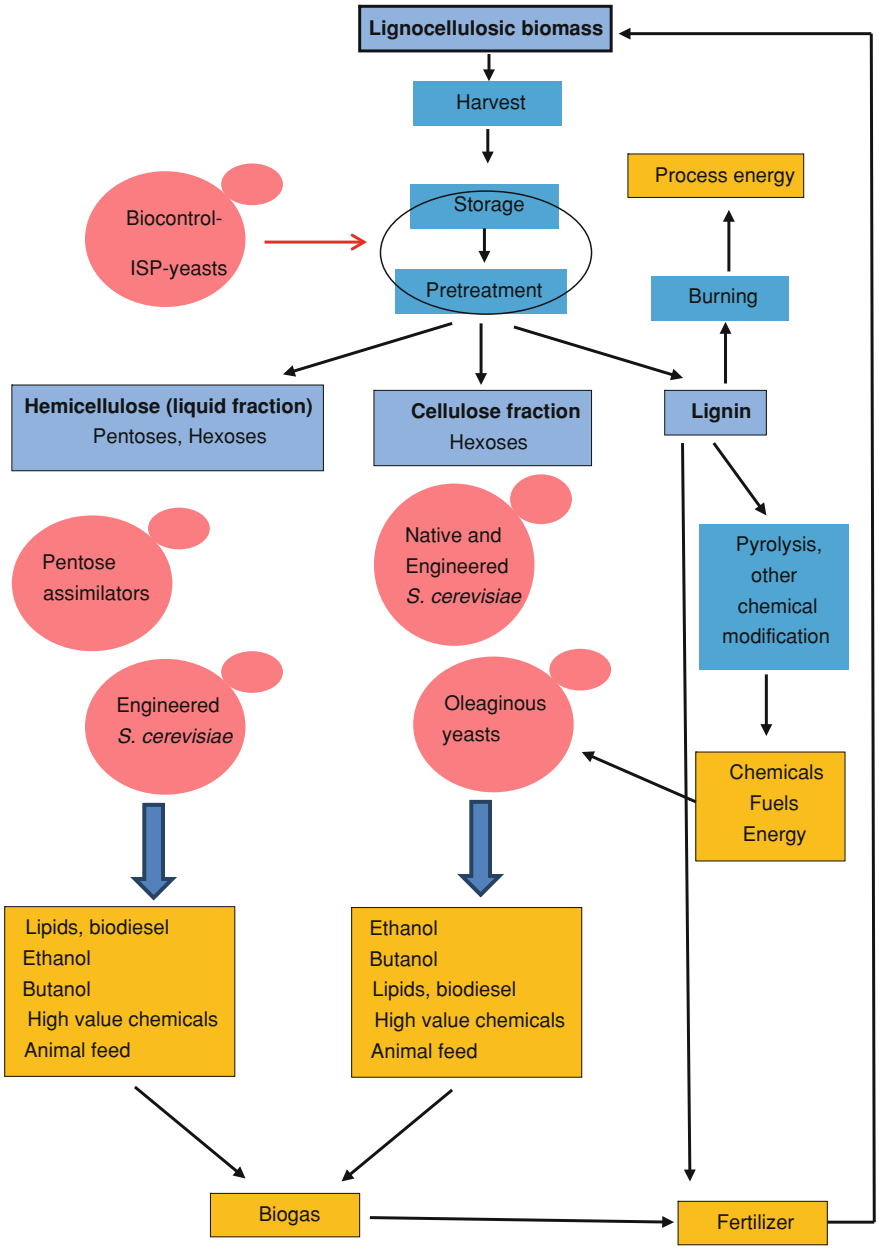
9.15 Outlook: Yeasts in a Biofuel/Biochemicals Refinery

The above-mentioned examples illustrate that, based on knowledge of physiology appropriate yeasts can be selected, processes can be optimised and metabolic pathways can be manipulated, which leads to substantial improvements in

producing the desired biofuels. However, it should be noted that currently there is almost no commercial scale biofuel production from second-generation raw materials, except biogas production, which is not yeast based. As mentioned above, high costs are among the major obstacles to commercialising second-generation biofuel production (Stephen et al. 2012; Cheng and Timilsina 2011). Integration of the different steps of handling lignocellulose biomass is a way of improving the process economy. Biomass handling for biofuel production includes growth, harvest, storage, pretreatment, fermentation, handling side and residual products, and if possible, generating value out of them (Vanholme et al. 2013; Liguori et al. 2013). Several of these partial processes can make use of the metabolic capacities of yeasts (Fig. 9.6).

Storage of biomass is an essential process, as biomass is seasonally produced, whereas it is highly desirable that biofuel production facilities run continuously. Therefore, it is necessary to preserve the harvested biomass until use. The most frequently used and safest means of biomass preservation is drying; however, especially in temperate climates, this can require a substantial input of energy (Olstorpe and Passoth 2011). In the case of lignocellulose material such as straw, which is usually passively dried in the field, excessively high moisture contents can result in losses of produced biomass (Nilsson 2000). For the handling of feed biomass, several methods of biopreserving moist biomass have been developed (Olstorpe and Passoth 2011; Zheng et al. 2011). When these methods were applied to preserve raw materials for biofuel production, yeast-based biopreservation of the moist biomass not only saved the energy that would have been consumed for drying, but also made the biomass more accessible for the subsequent pretreatment. Biopreservation of wheat straw with a yeast able to partially degrade hemicellulose (*S. stipitis*) had a positive effect on the pretreatment efficiency (Passoth et al. 2009, 2013). Integration of storage and pretreatment can thus streamline the pretreatment input in the process. This is one of the most critical issues in obtaining sustainable biofuel production, as pretreatment contributes to high costs, requires a major input of energy and releases inhibitors (Sassner et al. 2008). Consolidated bioprocessing, in which enzymatic degradation of the biomass and ethanol production are combined, represents another opportunity to reduce the impact of pretreatment (Hasunuma and Kondo 2012).

Handling fermentation residues from lignocellulose-based ethanol production is an important issue for the total process. Fermentation residues are nutrient rich and have a high COD value, and their cleaning represents a major cost factor (Wilkie et al. 2000). Biogas production is one opportunity for generating value out of the costly residues. Interestingly, some studies indicated that biogas production from lignocellulose was positively influenced when the material was fermented to ethanol and the residues were introduced into the biogas process. The total energy output from a combined ethanol/biogas production was higher than biogas production alone, and the biogas production rate was also enhanced. In this way, ethanol production acted like a pretreatment for the biogas process (Dererie et al. 2011; Kreuger et al. 2011). Residues from biogas production, in turn, have very


Lignocellulosic biomass
Harvest
Storage
Pretreatment
Biocontrol-
ISP-yeasts
Process energy
Burning
Hemicellulose (liquid fraction)
Pentoses, Hexoses
Cellulose fraction
Hexoses
Lignin
Pentose
assimilators
Native and
Engineered
S. cerevisiae
Pyrolysis,
other
chemical
modification
Engineered
S. cerevisiae
Oleaginous
yeasts
Chemicals
Fuels
Energy
Lipids, biodiesel
Ethanol
Butanol
High value chemicals
Animal feed
Ethanol
Butanol
Lipids, biodiesel
High value chemicals
Animal feed
Biogas
Fertilizer

◀ **Fig. 9.6** Integrative approach for generating biofuels and chemicals from non-food (lignocellulose) biomass and the role of yeasts in such a process. After harvest, biomass has to be preserved until pretreatment, which includes thermochemical and enzymatic processing. Biocontrol yeasts can be used for low-energy biopreservation of the biomass. Certain yeasts (ISP yeasts) can even de-stabilise the material during storage, and by this, integrate storage and pretreatment (ISP). After thermochemical treatment, most of the hemicellulose is present in the liquid fraction, whereas the cellulose together with the lignin is present in the solid fraction. If necessary, both can be separated by further treatment steps. Enzymatic treatment releases monosaccharides from the polysaccharides. The sugars can be converted into biofuels, chemicals and animal feed using appropriate yeasts. Residues from yeast fermentations can be converted into biogas. Lignin that cannot be degraded to ethanol or biogas can be burned to obtain process energy, or it can be converted into chemicals and biofuels by chemical processes (pyrolysis). Residues from pyrolysis can be transformed into lipids using oleaginous yeasts. Biogas residues and lignin are excellent fertilisers to produce new biomass

good potential as fertilisers and can thus close the loop to generate new biomass for biofuel and food production (Odlare et al. 2011).

Residues from first-generation raw material ethanol production are frequently used as animal feed. For instance, in the EU, feeding fermentation residues to animals may result in saving 0.7 Mio ha maize cultivation area for animal feed production (Özdemir et al. 2009). Utilisation of the fermentation residues as animal feed in a corn-based ethanol production process contributed to about one-sixth of the total energy output and was essential to obtain a positive energy balance for the whole process (Hill et al. 2006). However, residues from lignocellulose-based fermentation may not be suitable for direct incorporation into animal feed, due to inhibitors and lignin-derived compounds that influence palatability. Recently, the extraction of proteins from lignocellulose to use them as animal feed or even human food has been suggested (Chiesa and Gnansounou 2011). As discussed above, biofuel production from first-generation raw materials may raise ethical concerns (Buyx and Tait 2011) and even lignocellulosic feedstock may compete with food production. Thus, feed and food production from side streams of biofuel production may be a way to overcome potential food versus fuel debates. Moreover, as animal feed production in particular is one of the major consumers of fossil resources and arable land in agriculture, feed generation from biofuel production will substantially improve the overall environmental balance of the entire process (Graham-Rowe 2011).

In a future biofuel refinery, different compounds will be generated according to corresponding demands. The pentose fraction can primarily be used to produce: biodiesel using naturally pentose-assimilating oleaginous yeasts; bioethanol, using engineered *S. cerevisiae*; or yeast biomass for animal feed. The hexose fraction is the preferred substrate for ethanol and butanol production; lipid generation by oleaginous yeasts or engineered *S. cerevisiae* is also possible. Apart from this, high value compounds, e.g. platform chemicals for the pharmaceutical or cosmetic industries can be co-generated, which will significantly improve the total economic basis of the biofuel process (Nielsen et al. 2013; Zhang et al. 2011). Lignin

is difficult to degrade due to its complex structure. It is typically burned to generate process energy. However, it is also possible to convert it into valuable chemicals (Zhu and Pan 2010). Pyrolysis is one method to obtain low molecular weight chemicals from lignin and other compounds of lignocellulose, and during this process, several residues, including carboxylic acids, are generated. These acids can be converted into lipids by oleaginous yeasts (Lian et al. 2012). When used as fertiliser, lignin plays an important role for the carbon balance of soil (Jarecki and Lal 2003).

This chapter illustrates that biofuel-related yeast research has undergone an impressive development: based on the bulk of knowledge about yeast physiology, culture conditions and metabolic pathways, cells have been manipulated to optimise production of the desired biofuel. The ongoing efforts to obtain strains producing sufficient amounts of biofuels or platform chemicals in an industrial environment, and in integrating the processing steps of a biorefinery, are, in their turn, boosting scientific developments towards understanding yeast and thus eukaryotic physiology. In this way, biofuel research can be seen as an excellent example of mutual positive effects when combining fundamental science with an emerging technology development.

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