

# Alcoholic fermentation of carbon sources in biomass hydrolysates by *Saccharomyces cerevisiae*: current status

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**Abstract** Fuel ethanol production from plant biomass hydrolysates by *Saccharomyces cerevisiae* is of great economic and environmental significance. This paper reviews the current status with respect to alcoholic fermentation of the main plant biomass-derived monosaccharides by this yeast. Wild-type *S. cerevisiae* strains readily ferment glucose, mannose and fructose via the Embden–Meyerhof pathway of glycolysis, while galactose is fermented via the Leloir pathway. Construction of yeast strains that efficiently convert other potentially fermentable substrates in plant biomass hydrolysates into ethanol is a major challenge in metabolic engineering. The most abundant of these compounds is xylose. Recent metabolic and evolutionary engineering studies on *S. cerevisiae* strains that express a fungal xylose isomerase have enabled the rapid and efficient anaerobic fermentation of this pentose.

L-Arabinose fermentation, based on the expression of a prokaryotic pathway in *S. cerevisiae*, has also been established, but needs further optimization before it can be considered for industrial implementation. In addition to these already investigated strategies, possible approaches for metabolic engineering of galacturonic acid and rhamnose fermentation by *S. cerevisiae* are discussed. An emerging and major challenge is to achieve the rapid transition from proof-of-principle experiments under ‘academic’ conditions (synthetic media, single substrates or simple substrate mixtures, absence of toxic inhibitors) towards efficient conversion of complex industrial substrate mixtures that contain synergistically acting inhibitors.

**Keywords** Arabinose · Ethanol · Galacturonic acid · Hydrolysate · Rhamnose · Xylose

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## Introduction

Industrial and societal developments in the 20th century have to a large extent been shaped by petrochemistry. The ever increasing rate of exploration and exploitation of oil reserves has, for a long time, secured an abundant and cheap raw material for the production of bulk and fine chemicals as well as transport fuels. At the beginning of the 21st century, several factors are

contributing in creating an urgent need for alternatives to an economy that predominantly depends on fossil resources.

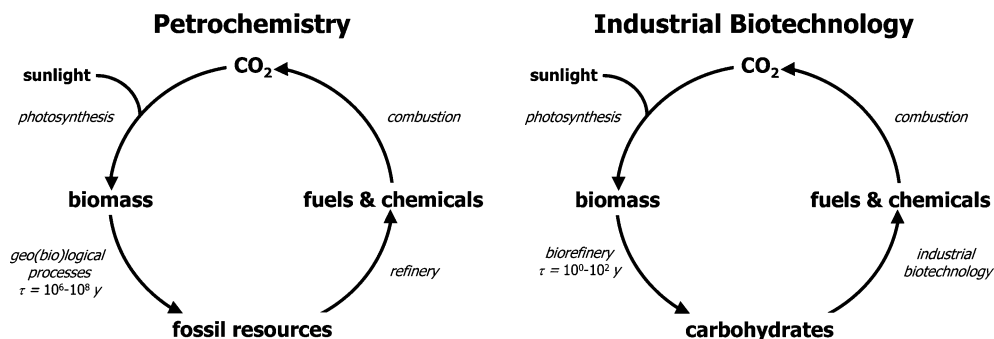
In the past decade, the rate at which new fossil resources are discovered has ceased to match or exceed the global rate of oil consumption (Greene et al. 2002). As a result, it is clear that oil consumption cannot continue indefinitely at its current pace. This realization is strengthened by the fact that oil consumption worldwide continues to increase, amongst others due to rapid development of the major Asian economies (Bigg et al. 2003). While these factors already are leading to an increasing price of crude oil, geopolitical issues generate short- and long-term uncertainties that require national governments to reconsider their exclusive dependency on (often foreign) oil reserves (Klare 2001; Lugar and Woolsey 1999).

A fourth issue related to the use of fossil resources as feedstocks for chemical production ties in directly with global cycling of the elements, the central theme of this special issue of Antonie van Leeuwenhoek. At first glance, petrochemical production appears to be a cyclic process driven by solar energy (Fig. 1, left). However, the time constants of the processes in this cycle are alarmingly different. Formation of fossil resources is a process that takes aeons, while humanity is depleting the limited oil reserves in a period of, at best, a few centuries. One of the net results of this imbalance in the ‘petrochemical carbon cycle’ (Fig. 1, left) is a rapid increase of the carbon dioxide concentration in the Earth’s atmosphere and oceans (Bigg et al. 2003). Since the beginning of the industrial revolution, the

CO<sub>2</sub> concentration in the atmosphere has steadily increased, with a strong acceleration since the second half of the 20th century. There is strong and growing concern that increased CO<sub>2</sub> levels affect the global climate via the well-publicized ‘greenhouse effect’ (Khandekar et al. 2005).

Plant biomass offers an attractive alternative, bypassing the need for fossil resources in chemical production and balancing the time constants of feedstock production and carbon dioxide fixation (Fig. 1, right). Transport fuels are a major product of the petrochemical industry. It would therefore be extremely attractive to have cost-effective, sustainable means of producing transport fuels from plant biomass. One of the most promising processes in this respect is the production of fuel ethanol. Ethanol can be blended with conventional fuels or used as such. Depending on the mixture used, modifications to conventional car motors are either limited or not required (Iogen Corporation 2005).

The concept of producing fuel ethanol from plant biomass is by no means new. In 1918, the British Government appointed an Alcohol Motor Fuel Committee that, among other tasks, was charged with investigating the infrastructural implications of a large expansion of industrial ethanol production (Holden et al. 1919). A few years later, a famous predecessor of Gijs Kuenen at the Delft University of Technology, Albert Jan Kluyver, remarked in his inaugural address: ‘The intensification of agriculture, and its development along lines of modern industry will thus, in the future, become more and more the order of the day. This will mean that the chemical industry will



**Fig. 1** The carbon cycle applied to consumer goods and fuels. Left: Petrochemical material streams. Right: Industrial biotechnological material streams

be provided primarily with a few staple agricultural products whose value derives largely from their content of one or more of the three major groups of substances, the carbohydrates, oils and proteins. Particularly the carbohydrates—the sugars, starches and cell wall constituents—will become prominent as raw materials' (Kamp et al. 1959).

In the past decades consensus has been reached that, in the long run, sustainable and cost-effective production of ethanol from plant biomass should not only be based on the readily fermentable starch and sucrose fractions of plant carbohydrates, but also on the much more resistant lignocellulosic fractions. Again, this conclusion is hardly novel. Kressmann (1922) already stated: 'If the manufacturing cost of producing ethyl alcohol from wood can be reduced to the same figure or nearly the same figure as that for making it from grain or molasses, there will be a large margin in favour of producing the alcohol from wood waste'.

An important factor for the cost-effective production of ethanol from lignocellulosic feedstocks is the high-yield, high-rate fermentation of biomass hydrolysates to ethanol. The demands on the microorganisms that perform this reaction are more complicated than those for conventional ethanol production from hexoses or their disaccharides, which uses exclusively *Saccharomyces* yeasts. For example, hydrolysis of hemicellulose generates substantial amounts of pentose (C5) sugars that cannot be fermented by wild-type *Saccharomyces cerevisiae*. Additionally, plant hydrolysates contain numerous compounds that inhibit microbial growth (Klinke et al. 2004). Tolerance to these compounds, as well as to high concentrations of ethanol, is a prerequisite for efficient fermentation of biomass hydrolysates. Another important issue is the ability to grow under strictly anaerobic conditions. For example, many yeasts that ferment sugars (including pentoses) to ethanol, only do so when small amounts of oxygen are provided, while overaeration leads to increased respiration and suboptimal ethanol yields. Accurate dosage of oxygen in large-scale processes with viscous two-phase feedstocks is not only economically undesired, but also virtually impossible, thus underlining the importance of

the ability for strict anaerobic growth such as exhibited by *S. cerevisiae* (Andreasen and Stier 1953, 1954; Visser et al. 1990).

The scale of industrial ethanol production is such that it is almost impossible to maintain aseptic conditions. Contaminations can result in reduction of the ethanol yield and formation of inhibitory compounds. Consequently, the process should be operated under conditions that minimize the risk of contamination. In general, the combined use of low pH and high ethanol concentrations suffices to keep contaminations at bay in large-scale yeast-based ethanol production processes. Although impressive results have been realized with respect to metabolic engineering of *Escherichia coli* and *Zymomonas mobilis* for ethanol production from non-glucose sugars (Dien et al. 2003), their low tolerance to acid, ethanol and other inhibitors will make it difficult to avoid contamination with, for example, lactic acid bacteria (Thomas et al. 2001). Another challenge in bacterial systems is the potential contamination with bacteriophages. An additional future option may be based on thermophilic cellulolytic *Clostridia* (Demain et al. 2005; Lynd et al. 2002). Although considerable progress has been made with respect to genetic engineering of these bacteria for homoethanolic fermentation, industrial implementation is not yet within reach.

Based on its widespread, large-scale application for bioethanol production from C6-substrates, *S. cerevisiae* appears to be the most promising metabolic-engineering platform for bioethanol production from plant hydrolysates. The most important challenge in this respect is the expansion of the narrow range of fermentable sugars. Wild-type *S. cerevisiae* strains readily ferment glucose, mannose and fructose as well as the disaccharides sucrose and maltose via the Emden-Meyerhof pathway of glycolysis, while D-galactose is fermented via combined action of the Leloir pathway and glycolysis. Production of ethanol from other carbon sources in plant hydrolysates (D-xylose, L-arabinose, galacturonic acid and L-rhamnose) requires extensive metabolic engineering. The aim of this review is to give an update of the current status of research on this subject and to outline the challenges in the

conversion of *S. cerevisiae* into a biomass hydrolysate-fermenting organism.

## Composition of biomass hydrolysates

### Lignocellulosic biomass

There are a wide variety of potential feedstocks for fuel ethanol production. Fast-growing trees, grass, whole plants, industrial by-products, aquatic plants, waste products (including agricultural, paper industry and forestry waste), municipal and industrial waste streams, all represent examples of plant-derived lignocellulosic resources (Aristidou and Penttilä 2000; Galbe and Zacchi 2002; Howard et al. 2003; Lee 1997; Zaldivar et al. 2001). The nature and availability of lignocellulosic feedstocks in different parts of the world depends on climate and other environmental factors, agricultural practice and technological development (Claassen et al. 1999). Lignocellulose consists of an intermeshed and chemically bonded complex of three main polymers (Table 1): cellulose, hemicellulose, lignin and depending on the feedstock, pectin (Hahn-Hägerdal et al. 1991; Ingram et al. 1999; Perez et al. 2002; Zaldivar et al. 2001).

Cellulose, the major constituent of lignocellulose (Table 1), is a linear polymer composed of thousands of D-glucose subunits linked by  $\beta$ -(1–4)-glycosidic bonds. Linear cellulose typically occurs as parallel polymers with extensive hydrogen bonding (Lynd 1996). This structural conformation, as well as the close association with lignin, hemicellulose, starch, protein and minerals makes cellulose highly resistant to hydrolysis

**Table 1** Polymer composition of lignocellulose (data from Ingram et al. 1999)

Fraction	Content in lignocellulose	Major monomers
Cellulose	33–51%	Glucose
Hemicellulose	19–34%	Glucose, mannose, galactose, xylose and arabinose
Lignin	20–30%	Aromatic alcohols
Pectin	2–20%	Galacturonic acid and rhamnose

(Aristidou and Penttilä 2000; Klinke et al. 2004; Perez et al. 2002; Zaldivar et al. 2001).

Hemicellulose, the second major constituent of lignocellulose (Table 1) is a highly branched and complex heteropolymer that contains hexoses (D-glucose, D-galactose, D-mannose, L-rhamnose, L-fucose), pentoses (D-xylose and L-arabinose) and uronic acids (D-glucuronic acid and D-galacturonic acid). Hemicellulose composition is strongly dependent on the plant source. However, in contrast to cellulose, hemicellulose is easily hydrolyzed to its constituent monosaccharides (Aristidou and Penttilä 2000; Klinke et al. 2004; Perez et al. 2002; Zaldivar et al. 2001).

Lignin, constituting 10–20% of biomass dry weight (Table 1), is an aromatic polymer containing phenolic residues such as *trans*- $\rho$ -coumaryl alcohol, *trans*- $\rho$ -coniferyl alcohol and *trans*- $\rho$ -sinapyl alcohol (Hahn-Hägerdal et al. 1991; Ingram et al. 1999; Klinke et al. 2004; Perez et al. 2002; Zaldivar et al. 2001). While the lignin fraction does not contribute fermentable carbon sources, it is relevant as a potential source of microbial inhibitors (see below).

Although, in ‘average’ plant biomass, pectin is less prominent than cellulose and hemicellulose, some agricultural waste streams such as citrus peels and sugar beet pulp are extremely rich in pectin (Doran et al. 2000; Doran and Foster 2000; Grohmann et al. 1998; Grohmann and Bothast 1994). Pectins are complex and heterogeneous polymers that primarily act as hydrating and cementing agents for the cellulosic matrix of plant cell walls. The principal unit in pectin chains is  $\alpha$ -(1–4) linked galacturonic acid. The galacturonic acid residues can be esterified with methyl and acetyl groups (Baciu and Jördening 2004; Oosterveld 1997; Thakur et al. 1997). Additionally, pectin contains the branched polysaccharides rhamnogalacturonan I, rhamnogalacturonan II and xylogalacturonan (Micard et al. 1996; Oosterveld 1997; Zandleven et al. 2005).

### Pretreatment and hydrolysis

Conversion of lignocellulosic materials to fermentable sugars is an intensive process that involves a combination of pretreatment (chemical and mechanical) and hydrolysis (chemical and

enzymatic) (Galbe and Zacchi 2002). Pretreatment is required to increase the surface area of the feedstock, thereby rendering the lignocellulose accessible for hydrolysis (Klinke et al. 2004). Pretreatment methods include concentrated- or dilute-acid treatment, high-pressure steam explosion, ammonia-freeze explosion and wet oxidation (Galbe and Zacchi 2002; Klinke et al. 2004). These methods are most often used in combination, such as in the commonly used combination of dilute-acid hydrolysis (0.5–1.0% sulfuric acid) with high-pressure steam explosion. During this pre-treatment, hemicellulose is readily chemically hydrolyzed, while cellulose is essentially inert (Galbe and Zacchi 2002). Pretreatment of hemicellulose results in the release of monomeric sugars and in the production of a complex mixture of compounds that inhibit the bioconversion of these sugars (Ingram et al. 1999; Klinke et al. 2004; Mussatto and Roberto 2004). It has been proposed that the lignin fraction, which can be separated after pretreatment, may be used as an ash-free, solid fuel for the generation of heat and/or electricity (Galbe and Zacchi 2002).

Cellulose hydrolysis can either be done chemically or enzymatically using specific cellulolytic enzymes. Although chemical processes are more technically mature, they generally have greater environmental and personnel risks (Lynd 1996). Until recently, the cost of cellulose-hydrolyzing enzymes was prohibitive for commercial ethanol production from lignocellulosic biomass. However, over the past five years enormous progress has been made in the development of novel hydrolytic enzymes, amongst others in research efforts at Novozymes and Genencor that were subsidized by the USA National Renewable Energy Laboratories (<http://www.nrel.gov>). While further optimization of these enzymes and their integration with physical/chemical techniques for sugar release is needed, there appear to be no insurmountable barriers for their industrial implementation. For more information on naturally produced cellulases, such as for instance by the wood-rotting fungus *Trichoderma reesei*, we refer to Galbe and Zacchi (2002), Howard et al. (2003) and Perez et al. (2002). During enzymatic hydrolysis

incomplete conversion of the cellulose and build-up of cellobiose can occur due to inhibition by the end products. This can, for instance, be avoided by simultaneous hydrolysis and fermentation. For comparison of separate or simultaneous hydrolysis and fermentation we refer to excellent papers by Lynd (1996), Galbe and Zacchi (2002) and Ingram et al. (1999). In practice, it may not be necessary to completely hydrolyse polymers to monosaccharides. Wild-type *S. cerevisiae* strains readily ferment the disaccharides sucrose and maltose, and the ability to hydrolyse cellobiose, lactose and melibiose has been introduced by metabolic engineering and/or classical strain improvement (Domingues et al. 1999a, b; Ronnow et al. 1999; van Rooyen et al. 2005; Vincent et al. 1999).

Pectin hydrolysis can be accomplished by a mixture of enzymes, consisting of pectin methyl esterase, pectin acetyl esterase, endo-polygalacturonase, endo-pectin lyase, rhamnogalacturonan hydrolyase, rhamnogalacturonan lyase, rhamnogalacturonan acetyl esterase, arabinofuranosidase, endo-arabinose, endo-galactanase and  $\beta$ -galactosidase (Baciu and Jördening 2004; Oosterveld 1997). While enzyme costs are still an important issue, the recent developments in cellulose hydrolysis suggest that future cost-effective production of pectinolytic enzymes is feasible.

#### Monosaccharides in biomass hydrolysates

Depending on the feedstock, the resulting hydrolysates contain a large variety of sugars (Table 2). It is clear that glucose and xylose are often the predominant sugars. However, for economically efficient conversion to commodity chemicals like ethanol, the profit margins are small. This creates an incentive to also convert smaller carbohydrate fractions, such as arabinose, galacturonic acid and rhamnose, to ethanol. Conversion of such other sugars becomes even more important when side streams of existing processes that are enriched for these compounds are used to produce ethanol. In the following paragraphs, an overview will be presented of the current status of the utilization of these various sugars by *S. cerevisiae*. In addition, we will briefly address the challenges posed by the complex mixture of inhibitory

**Table 2** Composition (major sugars) of common agricultural lignocellulosic feedstocks (adapted from Grohmann and Bothast 1994; Lee 1997; <http://www.ecn.nl/phyllis/>)

	Corn stover	Wheat straw	Bagasse	Cotton gin	Sugar beet pulp	Switch grass
Carbohydrate (%)						
Glucose	34.6	32.6	39.0	37.1	24.1	31.0
Mannose	0.4	0.3	0.4	1.1	4.6	0.2
Galactose	1.0	0.8	0.5	2.4	0.9	0.9
Xylose	19.3	19.2	22.1	9.4	18.2	0.4
Arabinose	2.5	2.4	2.1	2.3	1.5	2.8
Uronic acids	3.2	2.2	2.2	NA	20.7	1.2
Non-carbohydrate (%)						
Lignin	17.7	16.9	23.1	28.8	1.5	17.6
Extractives	7.7	13.0	3.8	7.7	NA	17.0
Ash	10.4	10.2	3.7	10.5	8.2	5.8

compounds that may originate during the preparation of hydrolysates (see below).

### Fermentation of hexoses by *Saccharomyces cerevisiae*

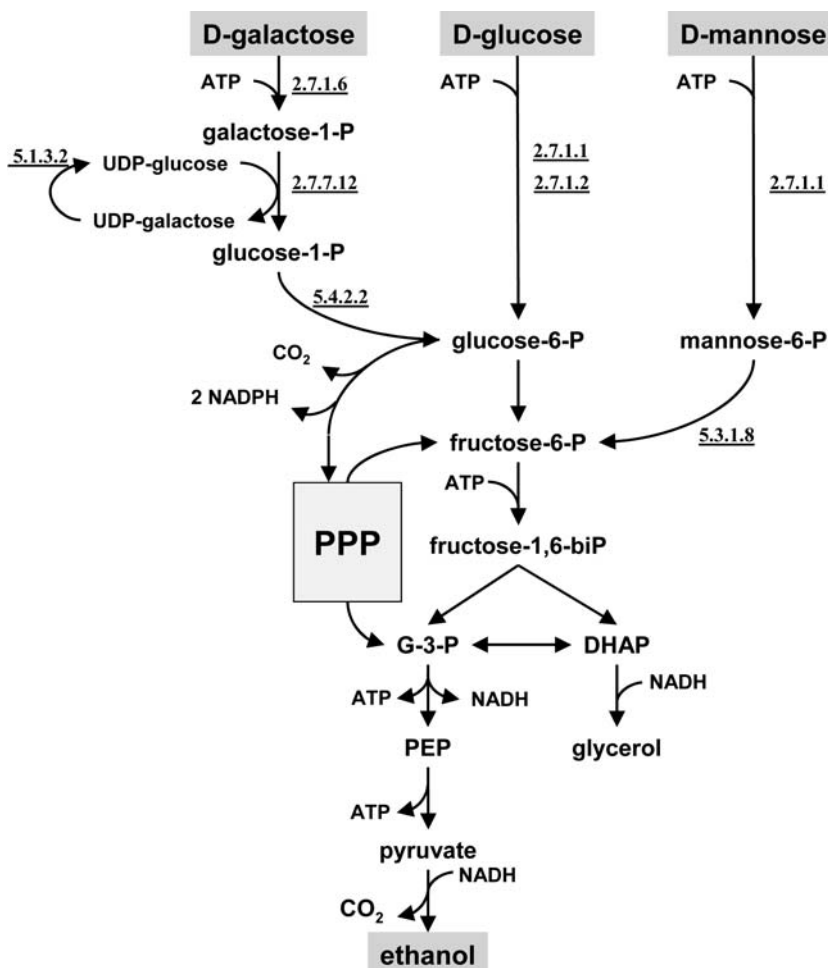
Wild-type *S. cerevisiae* ferments glucose, the dominant sugar in all plant hydrolysates, at high rates even under anaerobic conditions. The anaerobic ethanol production rate in defined media is as high as 30 mmol g biomass<sup>-1</sup> h<sup>-1</sup> at 30°C (Bakker et al. 2000). *S. cerevisiae* contains an elaborate system for hexose transport. The 32 members of the HXT (hexose transporter) family in *S. cerevisiae* differ with respect to transcriptional and posttranscriptional regulation, substrate specificity and affinity for glucose (Boles and Hollenberg 1997; Kruckeberg 1996). However, since they all transport glucose via facilitated diffusion, glucose uptake only requires a concentration gradient across the plasma membrane. After uptake, glucose dissimilation proceeds via the Embden-Meyerhof glycolytic pathway. This pathway oxidizes glucose to two pyruvate, resulting in the net formation of two ATP per glucose (Fig. 2). In anaerobic, fermentative cultures of *S. cerevisiae*, the NADH formed by glyceraldehyde-3-phosphate dehydrogenase is reoxidized via alcoholic fermentation. This essential redox balancing involves the combined activity of pyruvate decarboxylase and alcohol dehydrogenase. But obviously glucose is not the only carbohydrate present in the hydrolysates. In

order to ferment such non-glucose carbohydrates with *S. cerevisiae*, three key criteria have to be met: (i) presence of a functional transporter in the plasma membrane, (ii) presence of enzyme(s) that couple metabolism of the carbohydrate to the main glycolytic pathway and (iii) maintenance of a closed redox balance.

Mannose and fructose are two isomers of glucose that occur in all plant-derived biomass hydrolysates and that can be fermented by all wild-type *S. cerevisiae* strains. The general observation that yeast capable of fermenting glucose can also ferment fructose and mannose, is known as the Kluyver rule (Kluyver 1914). Both mannose and fructose are transported by all the different members of the HXT family, although the  $K_m$  value is generally higher than that for glucose (Reifenberger et al. 1997). After phosphorylation by hexokinase, mannose-6-phosphate is isomerized to fructose-6-phosphate by phosphomannose isomerase, encoded by the *PMI40* gene. Hexokinase is also responsible for phosphorylation of fructose to fructose-6-phosphate, which is subsequently metabolized through glycolysis. As mannose and glucose compete for the same hexose transporters, kinetics of mixed-substrate utilization is determined by their relative and absolute concentrations in hydrolysates.

Galactose, another sugar that can be fermented by *S. cerevisiae*, is first taken up by a dedicated member of the HXT family, the galactose permease Gal2p, and subsequently converted into glucose-6-phosphate via the Leloir pathway (Leloir 1951; Melcher 1997)





**Fig. 2** Hexose catabolism of *Saccharomyces cerevisiae*. Underlined EC numbers represent enzymes/steps present in *S. cerevisiae* metabolism. The gene names encoding the various enzymes are given in parentheses in the legend of this figure. Glucose catabolism: 2.7.1.1, hexokinase (*HXK1/HXK2*); 2.7.1.2, glucokinase (*GLK1*); Galactose catabolism: via the Leloir pathway: 2.7.1.6, galactokinase (*GAL1*); 2.7.7.12, galactose-1-phosphate uridylyltransfer-

ase (*GAL7*); 5.1.3.2, UDP-glucose 4-epimerase (*GAL10*); 5.4.2.2 phosphoglucomutase (*GAL5/PGM2*). Mannose catabolism: 2.7.1.1, hexokinase I (*HXK1*); 5.3.1.8, mannose-6-phosphate isomerase (*PMI40*). G-3-P, Glyceraldehyde-3-phosphate; DHAP, dihydroxy-acetone-phosphate; PEP, phospho-enol pyruvate; PPP, Pentose phosphate pathway

(Fig. 2). This pathway, which links galactose catabolism to the main glycolytic pathway, consists of three reactions. After phosphorylation of galactose by galactokinase (Gal1p), galactose-1-phosphate uridylyltransferase (Gal7p) converts UDP-glucose and galactose-1-phosphate to UDP-galactose and glucose-1-phosphate. UDP-galactose is reconverted into UDP-glucose by the uridine-diphosphoglucose 4-epimerase (Gal10p, Douglas and Hawthorne 1964). Finally, glucose-1-phosphate is converted to

glucose-6-phosphate by phosphoglucomutase, major and minor isoforms of which are encoded by *PGM2* (also called *GAL5*) and *PGM1*, respectively (Fig. 2) (Johnston et al. 1987; Oh and Hopper 1990). In wild-type *S. cerevisiae* strains, growth rates on galactose are generally lower than those on glucose. Bro et al. (2005) have recently demonstrated that overexpression of *PGM2* caused a 70% increase of galactose consumption rates in aerobic batch cultures. It has not yet been investigated whether a similar

positive effect can be reached under anaerobic, fermentative growth conditions.

Expression and activity of the Leloir pathway in *S. cerevisiae* is positively controlled by galactose. Galactose induction of the GAL genes is initiated by ATP-dependent interaction of galactose with Gal3p, which then forms a complex with the negative regulator Gal80p (Platt and Reece 1998). This releases the positive transcriptional regulator Gal4p from Gal80p control and allows it to activate transcription of the *GAL1*, *GAL2*, *GAL7* and *GAL10* genes that contain the upstream activation sequences (UAS<sub>GAL</sub>) in their promoter regions (Leuther and Johnston 1992; Wu et al. 1996). Presence of glucose causes a virtually complete transcriptional repression of the GAL genes and thereby effectively shuts down galactose metabolism (Johnston et al. 1994). This repression is mediated by Mig1p, the non-phosphorylated form of which binds to upstream repression sequences (URS<sub>GAL</sub>) found in several of the GAL genes, including the transcriptional activator Gal4p (Nehlin et al. 1991). Additionally, Gal6p has been proposed to control degradation of the GAL transcripts (Ostergaard et al. 2001). Besides transcriptional regulation by glucose, the Gal2p permease is subject to glucose catabolite inactivation (Horak and Wolf 1997).

The glucose concentration in plant hydrolysates will generally lead to complete repression of galactose metabolism, thus preventing simultaneous consumption of glucose and galactose. Whereas fast, sequential utilization of different carbohydrates is not necessarily a problem in industrial ethanol production, the long lag phase that separates glucose and galactose consumption in mixed-substrate cultures of wild-type *S. cerevisiae* strains is a problem that needs to be addressed. Interestingly, a natural isolate of *S. cerevisiae* has recently been reported to prefer galactose over glucose (Keating et al. 2004). Moreover, Ostergaard et al. (2001) have demonstrated that combined deletion of *GAL6*, *GAL80* and *MIG1* in a laboratory *S. cerevisiae* strain led to a partial co-consumption of glucose and galactose in aerobic batch cultures. This indicates that metabolic engineering can

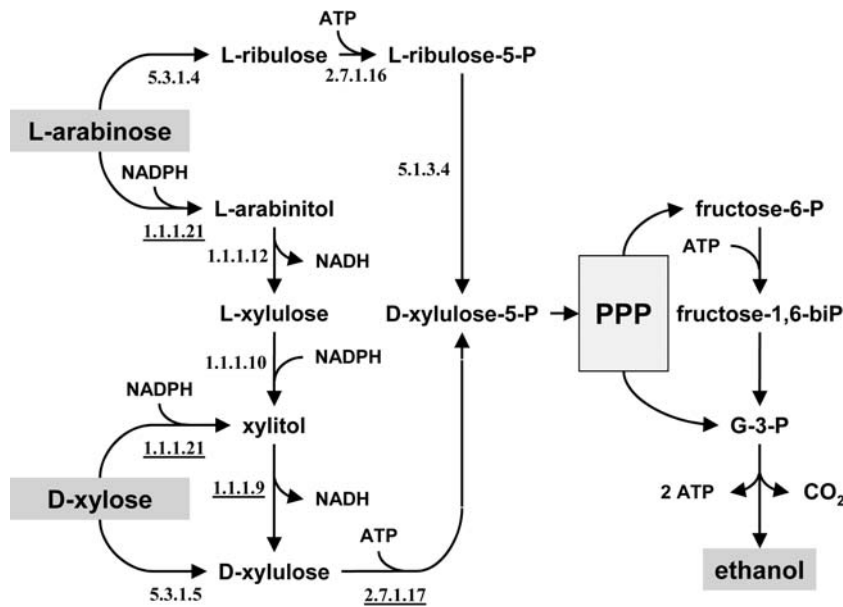
contribute to improved kinetics of mixed-substrate utilization.

## Xylose fermentation

In plant biomass hydrolysates, the pentose sugar xylose ('wood sugar') is the major monosaccharide that cannot be fermented by wild-type strains of *S. cerevisiae*. Although *S. cerevisiae* can neither ferment nor assimilate xylose, this is not a general characteristic of yeasts. The earliest observations on xylose fermentation by yeasts stem from the early 1980's. Yeasts like *Pachysolen tannophilus* (Slininger et al. 1982) and *Candida tropicalis* (Gong et al. 1981a) are able to ferment xylose to ethanol. In an extensive screening of 200 yeast strains able to grow on xylose, nineteen were found that produced 0.1–1.0 g l<sup>-1</sup> of ethanol under fermentative conditions from 20 g l<sup>-1</sup> xylose. Strains from the species *Brettanomyces nardenensis*, *Candida shehatae*, *Candida tenuis*, *P. tannophilus*, *Pichia segobiensis* and *Pichia stipitis* produced more than 1.0 g l<sup>-1</sup> (Toivola et al. 1984). Since xylose is a significant constituent of plant biomass it is not surprising that all these yeasts originate from plant-related sources.

Surprisingly, only a small number of facultatively fermentative yeasts that can metabolise xylose are able to ferment this sugar to ethanol. This apparent discrepancy is intrinsic to the metabolic pathway of xylose metabolism. This pathway, as first described in 1955 (Gunsalus et al. 1955) links xylose metabolism to the pentose-phosphate pathway by converting xylose into xylulose 5-phosphate (Fig. 3). In xylose-fermenting yeasts, two oxidoreductases are involved in this process, xylose reductase (XR) and xylitol dehydrogenase (XDH). As these oxidoreductases have different cofactor specificities, conversion of xylose into xylulose yields one NADP<sup>+</sup> and one NADH. NADPH and NAD<sup>+</sup> need to be regenerated in order to maintain redox balance. For NADPH this can be accomplished by diverting part of the fructose-6-phosphate produced into the oxidative part of the pentose-phosphate pathway. Under aerobic conditions the NADH can be reoxidized via the respiratory chain with molecular oxygen. However, under anaerobic conditions another electron





**Fig. 3** D-Xylose and L-arabinose catabolism in metabolically engineered *S. cerevisiae* strains. Underlined EC numbers represent enzymes/steps present in ‘wild-type’ *S. cerevisiae* metabolism. The gene names corresponding to the enzymes are given in parentheses in the legend of this figure. 1.1.1.21, aldose/xylose reductase (*GRE3/xyII*); 1.1.1.9, xylitol dehydrogenase (*XYL2/xyI2*); 2.7.1.17, xylu-

lokinase (*XKSI/xyI3*); 5.3.1.5, xylose isomerase (*xylA*); 1.1.1.12, arabinulose 4-dehydrogenase (*ladI*); 1.1.1.10, L-xylulose reductase (*lxrI*); 5.3.1.4, L-arabinose isomerase (*araA*); 2.7.1.16, L-ribulokinase (*araB*); 5.1.3.4, L-ribulose-5-phosphate 4-epimerase (*araD*). G-3-P, glyceraldehyde-3-phosphate; PPP, pentose phosphate pathway

acceptor is required to reoxidise the NADH, like acetoin (Scheffers 1966; Bruinenberg et al. 1983a) or furfural (Wahlbom and Hahn-Hägerdal 2002). If no such electron acceptor is available, the cell cannot maintain redox balance and will not ferment xylose under anaerobic conditions. A second solution to this redox imbalance would be to convert NADH into NADPH with a transhydrogenase. However, no such enzymes have been discovered in yeasts (Blank et al. 2005; Bruinenberg et al. 1983b) and introduction of a heterologous transhydrogenase in *S. cerevisiae* has demonstrated that in vivo conditions favour NADPH production (Nissen et al. 2001).

Why is it then that some yeasts such as *Pichia stipitis* and *Pachysolen tannophilus* are able to ferment xylose under anaerobic conditions? The answer is in the properties of the xylose reductase enzyme. In Table 3 the cofactor dependence of xylose reductase activity in several yeast strains is listed, thus revealing a clear correlation between the ability of their XR to utilise both NADH and NADPH and their ability to anaerobically

ferment xylose. This relationship was clearly established for *Pa. tannophilus* and *Pi. stipitis* (see Table 4, and Bruinenberg et al. 1984). The inherent drawback of this dual specificity XR is the production of xylitol. For every NADH that is reoxidized by XR one xylitol is produced as by-product. In Fig. 4 the theoretical yields of ethanol, xylitol and glycerol are given as a function of the ratio of in vivo fluxes through NADPH- and NADH-linked xylose reductase. The relations of these yields are derived from the following equations (Kuyper et al. 2004), assuming anaerobic conditions and formation of glycerol not taking place at ratios lower than 1.

$$\text{Ratio} = 0 : 6 \text{ xylose} \rightarrow 10 \text{ ethanol} + 10 \text{ CO}_2 + 10 \text{ ATP} \quad (1)$$

$$\text{Ratio} = 1 : 12 \text{ xylose} \rightarrow 9 \text{ ethanol} + 12 \text{ CO}_2 + 9 \text{ ATP} + 6 \text{ xylitol} \quad (2)$$

$$\text{Ratio} = \infty : 6 \text{ xylose} + 3 \text{ ATP} \rightarrow 3 \text{ ethanol} + 6 \text{ glycerol} + 6 \text{ CO}_2 \quad (3)$$

**Table 3** NADPH-linked and NADH-linked xylose reductase activities in batch cultures of various D-xylose-assimilating yeasts

Organism	CBS no.	Spec. act.			Xylose fermentation <sup>a</sup>
		NADH	NADPH	Ratio	
<i>Candida</i>	615	2	130	0.02	–
<i>tenuis</i>	2226	7	320	0.02	–
	2885	0 <sup>b</sup>	100	0	–
	4113	60	120	0.5	+
	4285	305	670	0.5	+
	4434	0 <sup>b</sup>	485	0	–
	4435	340	670	0.5	+
	4604	0 <sup>b</sup>	365	0	–
<i>Candida</i>	5813	210	480	0.4	+
<i>shehatae</i>					
<i>Candida</i>	621	0 <sup>b</sup>	75	0	–
<i>utilis</i>					

Cells were harvested at mid-exponential growth phase. Enzyme activities are expressed as  $\text{nmol min}^{-1} \text{mg prot.}^{-1}$ . Data taken from Bruinenberg et al. (1984)

<sup>a</sup> Results obtained in a fermentation test using a Durham vial: –: no gas production, ethanol less than  $0.3 \text{ g l}^{-1}$ ; +: gas production, ethanol higher than  $5.0 \text{ g l}^{-1}$

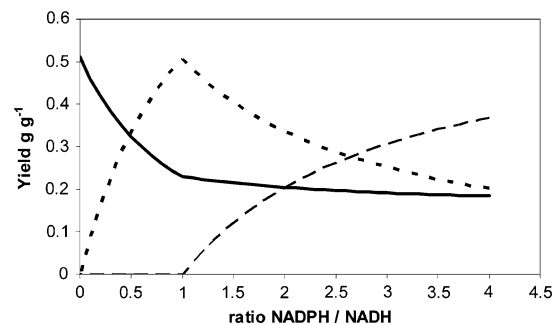
<sup>b</sup> not detectable

When the NADPH/NADH ratio equals zero, only NADH-linked reduction of xylose takes place and all xylose can be converted to ethanol because the conversion of xylose into xylulose is redox neutral (Eq. 1). At a ratio of one, one xylose must be converted with NADH-linked XR for every xylose converted with NADPH-linked XR (Eq. 2). With a ratio that is infinite, there is no NADH-linked XR activity and the excess NADH can only be reoxidized through the production of glycerol (Eq. 3). Since this equation

**Table 4** Activities of xylose reductase and rate of ethanol production from xylose-batch cultures switched to anaerobic conditions

	<i>C. utilis</i>	<i>Pa. tannophilus</i>	<i>Pi. stipitis</i>
$q_{\text{ethanol}}$	0	8	54
Xylose reductase			
NADH	0	9	310
NADPH	75	220	600
Ratio	0	0.04	0.5

$q_{\text{ethanol}}$  is expressed as  $\text{mg ethanol g cells}^{-1} \text{h}^{-1}$ . Enzyme activities were determined in cells harvested just prior to the shift from aerobic to anaerobic conditions and are expressed as  $\text{nmol min}^{-1} \text{mg protein}^{-1}$ . Data taken from Bruinenberg et al. (1984)



**Fig. 4** Calculated ethanol (—), xylitol (---) and glycerol (– –) yields during anaerobic catabolism of xylose as a function of the ratio of the fluxes via NADPH-linked and NADH-linked xylose reductase calculated from equations 1, 2, 3. Assumed is that glycerol formation does not occur below a ratio of 1, in other words: NADH is preferentially shuttled into xylitol formation instead of glycerol formation. Above a ratio of 1 there is a stoichiometric necessity for an alternative redox sink such as glycerol formation. At a ratio of 4.0 the ATP yield is zero

requires energy input, glycerol formation cannot solve the redox problem of the XR-XDH system.

Since *S. cerevisiae* is capable of metabolizing xylulose, albeit at very low rates (Hsiao et al. 1982; Wang and Schneider 1980), the first challenge in realizing efficient alcoholic fermentation of xylose by this yeast was the introduction of (a) heterologous enzyme(s) that convert xylose into xylulose, without causing cofactor imbalances. To quote the work of Bruinenberg et al. (1983a): ‘Efficient anaerobic fermentation of xylose to ethanol by yeasts apparently requires that the first two reactions of its metabolism be circumvented. Implantation of a xylose isomerase would offer a possibility. ... another option would be to select for a yeast possessing a xylose reductase and a xylitol dehydrogenase which are linked to the same coenzyme system, thus eliminating the production of excess NADH in the process of ethanol production.’

Both postulated options have, to a certain extent, been realized since then. Petschacher et al. (2005) were able to shift the cofactor preference of the xylose reductase of *Candida tenuis* from NADPH towards NADH by site-directed mutagenesis. Regrettably, this paper does not mention the xylose fermentation properties of the strains expressing the engineered enzyme. The introduction of a xylose isomerase (E.C. 5.3.1.5) in *S. cerevisiae* has been tried many times with

disappointing results (Blow et al. 1990; Fukazawa 1989; Gárdonyi and Hahn-Hägerdal 2003; Ho et al. 1984; Moes et al. 1996; Sarthy et al. 1987; Walfridsson et al. 1996). The lack of success of these heterologous expressions has been attributed to improper protein folding, posttranslational modifications, disulfide-bridge formation, and the internal pH of yeast. A notable exception in this line of research was the xylose isomerase from *Thermus thermophilus* (Walfridsson et al. 1996). This was the first xylose isomerase that was functionally expressed in *S. cerevisiae*. However, the enzyme activity of the isomerase at temperatures allowing yeast growth was not high enough to facilitate efficient xylose fermentation. Even when all enzymes involved in the conversion of xylulose to glycolysis intermediates were overexpressed the xylose isomerase activity ( $0.017 \text{ U mg}^{-1}$ ) was too low for efficient xylose metabolism (Karhumaa et al. 2005).

Because of the disappointing results of the xylose isomerase research, a lot of attention has been given to the expression of the *Pi. stipitis* XR and XDH in *S. cerevisiae* (for an excellent review see Jeffries and Jin 2004). Contrary to the xylose isomerases from *Archaea* and *Bacteria*, these eukaryotic genes could be functionally expressed in *Saccharomyces*, enabling this yeast to metabolise xylose (Kötter et al. 1990; Kötter and Ciriacy 1993). However, anaerobic xylose fermentation by engineered strains was inevitably accompanied by considerable xylitol production. For every NADPH used by XR, one NADH needs to be reoxidized, and the only way for these yeasts to do so is to produce xylitol. Table 5 presents ethanol and xylitol yields for different *Saccharomyces* strains engineered for xylose metabolism via the heterologous expression of XR and XDH. In this Table, three strains stand out in having both a low xylitol yield and a high ethanol yield. Strain TMB 3255 (Jeppsson et al. 2002) has a very low specific xylose consumption rate and will not be further discussed here. The other strains, 1400 (pLNH32) and 424A(LNH-ST), were constructed by Ho and co-workers at Purdue University and both have a reasonable ethanol yield and comparatively low xylitol production. The mechanism by which cofactor imbalances are prevented in these strains is unclear. A relevant factor in this respect may be

the large variation in XR cofactor dependency observed in *Candida tenuis* (see Table 3): the Purdue strains express the xylose reductase gene from CBS 5773 (Chen and Ho 1993), whereas the TMB strains from Lund express that from CBS 6054 (Eliasson et al. 2000; Walfridsson et al. 1995). The cultivation method may also help to explain the low xylitol production found with the Purdue strains. As outlined above, anaerobic cultivation of *S. cerevisiae* strains expressing both XR and XDH on xylose is expected to lead to substantial production of xylitol, unless an electron acceptor is present. In their papers Ho and co-workers describe a cultivation method that applies oxygen limitation by covering shake flasks with Saran wrap. It is conceivable that this system allows for sufficient diffusion of oxygen into the system to regenerate  $\text{NAD}^+$  via respiration instead of via xylitol production. On an industrial scale, this would require exact aeration of the reactors, which is too costly for a viable production process.

The recent discovery of the first fungal xylose isomerase (Harhangi et al. 2003) has been an important breakthrough in the field of xylose fermentation. This xylose isomerase from the obligately anaerobic fungus *Piromyces* sp.E2 was the first enzyme of its kind that could be functionally expressed in *S. cerevisiae* at high levels (Kuyper et al. 2003). The introduction of the XylA gene was sufficient to enable the laboratory strain CEN.PK113-7D to grow slowly on xylose as the sole carbon source under aerobic conditions (Table 6). Subsequently the XylA-expressing strain (RWB202) was subjected to evolutionary engineering, resulting in a strain (RWB 202-AFX) capable of anaerobic growth on xylose producing mainly ethanol,  $\text{CO}_2$ , glycerol and biomass and notably little xylitol (Kuyper et al. 2004). RWB 202-AFX performed nearly identical to the wild type grown on glucose, with the exception of a slightly reduced specific growth rate.

The ethanol production rate of RWB 202-AFX was still too low to be considered for industrial applications. To obtain a higher specific rate of ethanol production, a strain was constructed that, in addition to the XylA gene, overexpressed all genes involved in the conversion of xylose into intermediates of glycolysis. This strain, RWB 217,

**Table 5** Ethanol and xylitol yields of *Saccharomyces* yeast strains engineered for xylose utilization by introduction of heterologous xylose reductase and xylitol dehydrogenase genes

Strain	Description	Conditions	Yields		Reference
			Ethanol g g <sup>-1</sup>	Xylitol g g <sup>-1</sup>	
1400 (pLNH32)	<i>XYLI, XYL2, XKSI</i>	Fermentative batch	0.30	0.08	Ho et al. (1998)
H1691	<i>XYLI, XYL2, XKSI</i>	Anaerobic batch	0.09	0.41	Toivari et al. (2001)
H1691	<i>XYLI, XYL2, XKSI</i>	Micro-aerobic batch	0.12	0.42	Toivari et al. (2001)
H1691	<i>XYLI, XYL2, XKSI</i>	Aerobic batch	0.06	0.06	Toivari et al. (2001)
TMB 3001	<i>XYLI, XYL2, XKSI</i>	Anaerobic batch	0.31	0.29	Jeppsson et al. (2002)
TMB 3255	<i>XYLI, XYL2, XKSI, Δzwf1</i>	Anaerobic batch	0.41	0.05	Jeppsson et al. (2002)
FPL-Y SX3	<i>XYLI, XYL2, XYL3</i>	Respiro fermentative batch	0.12	0.27	Jin et al. (2003)
H2674	<i>XYLI, XYL2, XKSI</i>	Anaerobic batch	0.14	0.53	Verho et al. (2003)
H2673	<i>XYLI, XYL2, XKSI, GDPI</i>	Anaerobic batch	0.17	0.48	Verho et al. (2003)
H2723	<i>XYLI, XYL2, XKSI, Δzwf1</i>	Anaerobic batch	0.18	0.28	Verho et al. (2003)
H2684	<i>XYLI, XYL2, XKSI, Δzwf1, GDPI</i>	Anaerobic batch	0.34	0.34	Verho et al. (2003)
TMB3001C1	TMB3001 selected for anaerobic growth	Anaerobic batch	0.24	0.32	Sonderegger and Sauer (2003)
424A(LNH-ST)	<i>XYLI, XYL2, XKSI</i>	Fermentative batch	0.43	0.10	Sedlak and Ho (2004)

Gene names used: *XYLI* xylose reductase; *XYL2* xylitol dehydrogenase; *XKSI* and *XYL3* xylulokinase; *ZWFI* glucose phosphate dehydrogenase; *GDPI* Glyceraldehyde 3-phosphate dehydrogenase

overproduces the enzymes: xylulokinase (EC 2.7.1.17), ribulose 5-phosphate isomerase (EC 5.3.1.6), ribulose 5-phosphate epimerase (EC 5.3.1.1), transketolase (EC 2.2.1.1) and transaldolase (EC 2.2.1.2). In addition, the *GRE3* gene encoding aldose reductase was deleted to further minimise xylitol production (Kuyper et al. 2005a). The resulting strain could be cultivated under anaerobic conditions without any need for selection or mutagenesis and had at that time the highest reported specific ethanol production rate

(Table 6). Again, as with RWB 202-AFX, the metabolite profiles from anaerobic batch cultivation on xylose were very similar to those of the wild type on glucose. The largest by-products were biomass and glycerol, and since the production of these products is growth-linked (Albers et al. 1996; van Dijken and Scheffers 1986) the expectation is that both can be reduced by cultivating under conditions where maintenance energy requirement and nutrient limitation prevail.

**Table 6** Characteristics of xylose-fermenting strains overproducing the *Piromyces* xylose isomerase

Strain	Description	$\mu_{\max}$ h <sup>-1</sup>	Yield ethanol g g <sup>-1</sup>	Ethanol production g g <sup>-1</sup> h <sup>-1</sup>	Xylose consumption g g <sup>-1</sup> h <sup>-1</sup>	Yield xylitol g g <sup>-1</sup>
RWB 202	XylA	0.005 <sup>a</sup>	–	–	ND	ND
RW 202-AFX	Selection mutant of RWB 202	0.03	0.42	0.14	0.34	0.021
RWB 217	XylA + <i>XKSI</i> ↑ <i>TALI</i> ↑ <i>TKLI</i> ↑ <i>RPE1</i> ↑ <i>RKII</i> ↑ <i>gre3Δ</i>	0.09	0.43	0.46	1.06	0.003
RWB 218	Selection mutant of RWB 217	0.12	0.41	0.49	1.2	0.001

<sup>a</sup> measured under aerobic conditions

ND not determined

After the initial tests in synthetic medium with xylose as the carbon source, research was focused on utilization of mixed substrates. When RWB 217 was grown in anaerobic batch culture with 20 g l<sup>-1</sup> glucose and xylose each, an exponential glucose consumption phase was observed followed by a much slower, almost linear, xylose consumption phase. Even with this suboptimal xylose consumption the metabolite profiles of RWB 217 compared very well to those on xylose alone and the wild type on glucose. To further improve the xylose consumption characteristics in mixed-substrate batch cultures, RWB 217 was subjected to evolutionary engineering (Kuyper et al. 2005b). In a first stage, xylose-uptake kinetics were improved by cultivation for 85 generations in a xylose-limited chemostat. In a second stage, the long-term chemostat culture was used to inoculate a sequencing batch reactor

(SBR) on mixtures of glucose and xylose. After 35 cycles, a single colony streaked from this SBR culture was dubbed RWB 218. When this new strain was cultivated in anaerobic batch culture with 20 g l<sup>-1</sup> glucose and xylose each, it fermented all sugars within 24 h, an improvement of 20 h on RWB 217. On xylose alone RWB 218 has the highest reported specific anaerobic growth rate, xylose consumption and ethanol production rate to date (Table 6). The glucose and xylose uptake characteristics of RWB 218 had significantly changed compared to those of RWB 217. For both sugars, a doubling of the maximal uptake rate ( $v_{max}$ ) was observed, and in the case of xylose a ca. 25% decrease of  $K_m$  further contributed to improved uptake kinetics. These results lead us to conclude that the kinetics of xylose fermentation are no longer a bottleneck in the fermentation of xylose for the

**Table 7** Characteristics of (recombinant) arabinose-fermenting yeasts

Yeast strain	Culture conditions	Final ethanol concentration (g l <sup>-1</sup> )	Ethanol production (g g <sup>-1</sup> dry weight h <sup>-1</sup> )	Reference
<i>Candida</i> sp.	Microaerophilic	0.16		Gong et al. (1981b)
<i>Candida</i> sp. XF 217	Microaerophilic	0.68		Gong et al. (1981b)
<i>Ambrosiozyma monospora</i>	Microaerophilic, 30°C	1.8		Dien et al. (1996)
<i>C. aurangiensis</i>	Moderate aeration, 25°C	4.1		Dien et al. (1996)
	Microaerophilic, 30°C	1.4		
	Moderate aeration, 25°C	not detected		
<i>C. succiphila</i>	Microaerophilic, 30°C	2.3		Dien et al. (1996)
	Moderate aeration, 25°C	3.9		
<i>Candida</i> sp. (YB-2248)	Microaerophilic, 30°C	3.4		Dien et al. (1996)
	Moderate aeration, 25°C	1.7		
<i>C. arabinofermentans</i>	Microaerophilic	0.7–1.9		Kurtzman and Dien (1998)
<i>S. cerevisiae</i> pYaraDBA heterologous expression of <i>araB</i> , <i>araA</i> , and <i>araD</i> from <i>E. coli</i>	Aerobic	–	–	Sedlak et al. (2001)
<i>S. cerevisiae</i> H2561 expression of fungal L-arabinose pathway: <i>ladI</i> and <i>lxl1</i> from <i>Trichoderma reesei</i> ; <i>XYL1</i> and <i>XYL2</i> from <i>Pichia stipidis</i> ; <i>XKS1</i> from <i>S. cerevisiae</i>	Anaerobic	0.1	0.35×10 <sup>-3</sup>	Richard et al. (2003)
<i>S. cerevisiae</i> JBY25-4M heterologous expression of <i>B. subtilis</i> <i>araA</i> , <i>E. coli</i> <i>araB</i> and <i>araD</i> , and <i>S. cerevisiae</i> <i>GAL2</i>	Oxygen-limited	6	0.06–0.08	Becker and Boles (2003)



production of bioethanol. The challenge in xylose fermentation is now to successfully transfer strains and concepts from the laboratory to industrial conditions.

### L-Arabinose fermentation by *S. cerevisiae*

Although D-xylose is the most abundant pentose sugar in the hemicellulosic fraction of biomass, L-arabinose is also present in significant amounts. Various agricultural feedstocks contain 1.5–2.75% of L-arabinose (Table 7), thus establishing the importance of efficient conversion of L-arabinose to ethanol.

Wild-type *S. cerevisiae* strains cannot ferment or assimilate L-arabinose (Barnett et al. 1990). Although many yeasts are capable of assimilating L-arabinose aerobically, most are unable to ferment it to ethanol. In the 1980's, a few yeast species have been reported to produce small amounts of ethanol from L-arabinose (Table 7; Gong et al. 1981b). An extensive screening of 116 yeast strains resulted in the identification of four additional yeast species capable of arabinose fermentation: *Ambrosiozyma monospora*, *Candida aurangiensis*, *Candida succiphila*, and *Candida* sp. NRRL YB2248 (Dien et al. 1996). Although 101 species tested could convert L-arabinose to arabinitol, only 4% of the strains screened were able to ferment L-arabinose to ethanol, with a maximum ethanol concentration of 4.1 g l<sup>-1</sup> under microaerobic conditions or moderate aeration (Table 7). Moreover, ethanol production rates observed were very low. The final ethanol concentrations (Table 7) were reached not earlier than 7–14 days after inoculation. More recently, strains of the yeast species *Candida arabinofermentans* were reported to produce 0.7–1.9 g l<sup>-1</sup> ethanol from L-arabinose under microaerobic conditions (Table 7; Kurtzman and Dien 1998).

The observation that, apparently, L-arabinose fermentation is rare among yeast species, may be due to a redox imbalance in the fungal arabinose pathway. In this pathway, as described for *Penicillium chrysogenum* (Chiang and Knight 1960) and *Aspergillus niger* (Witteveen et al. 1989), L-arabinose is metabolized via the reactions

catalyzed by aldose (xylose) reductase, L-arabinitol 4-dehydrogenase, L-xylulose reductase, D-xylulose reductase and D-xylulokinase (Fig. 3). This pathway consists of two NAD<sup>+</sup>-linked oxidations and two NADPH-linked reductions, resulting in a redox cofactor imbalance under anaerobic conditions (Dien et al. 1996). In contrast to the strictly NADP(H)-dependent L-xylulose reductase described for the molds *P. chrysogenum* (Chiang and Knight 1960), *A. niger* (Witteveen et al. 1994), and *Trichoderma reesei* (Richard et al. 2002), the L-xylulose reductase of the yeast *Ambrosiozyma monospora* has been reported to be strictly NADH-dependent (Verho et al. 2004). It is unknown whether this is a general characteristic of this enzyme in L-arabinose-metabolizing yeasts.

Several studies have been aimed at metabolic engineering of *S. cerevisiae* for L-arabinose fermentation. Overexpression of all structural genes of the fungal L-arabinose pathway (*XYL1*, *lad1*, *lxr1*, *XYL2*, and *XKSI*; Table 7) resulted in the first *S. cerevisiae* strain capable of fermenting L-arabinose to ethanol. However, the engineered strain produced only 0.35 mg ethanol g<sup>-1</sup> h<sup>-1</sup> under anaerobic conditions (Richard et al. 2003). It seems probable that cofactor imbalances may have contributed to this very low ethanol production rate. Indeed, the authors suggest that the redox factor balance might be restored by a transhydrogenase activity that would interconvert NADH/NADP<sup>+</sup> and NAD<sup>+</sup>/NADPH. However, Nissen et al. (2000) showed that the in vivo cofactor concentrations in *S. cerevisiae* probably would not allow the reaction to proceed in the desired direction. Another way that has been proposed to ameliorate problems with cofactor imbalances is expression of the aldose reductase gene from *Pi. stipitis* (Rizzi et al. 1988) that also accepts NADH as cofactor, or the gene encoding NADH-dependent L-xylulose reductase (*ALX1*) from *Ambrosiozyma monospora* (Verho et al. 2004).

An alternative approach to construct an L-arabinose-fermenting *S. cerevisiae* strain is the overexpression of the bacterial L-arabinose pathway (Fig. 3). In the bacterial pathway, no redox reactions are involved in the initial steps of L-arabinose metabolism. Instead, the enzymes



L-arabinose isomerase, L-ribulokinase, and L-ribulose-5-phosphate 4-epimerase are involved converting L-arabinose to L-ribulose, L-ribulose-5-P, and D-xylulose-5-P, respectively. These enzymes are encoded by the *araA*, *araB*, and *araD* genes, respectively (Lee et al. 1986). A first attempt to express the *E. coli* *araA*, *araB*, and *araD* genes in *S. cerevisiae* was only partly successful. Although activities of all three enzymes were shown to be present in a yeast strain carrying the heterologous genes, the engineered strain only accumulated L-arabinitol and did not produce ethanol from L-arabinose (Sedlak and Ho 2001). Becker and Boles (2003), who followed essentially the same strategy, were more successful. In their work, the complete bacterial L-arabinose pathway—consisting of *E. coli* *araB* and *araD* and *Bacillus subtilis* *araA*—was introduced into *S. cerevisiae*, simultaneous with the overexpression of the yeast galactose permease gene (*GAL2*). Gal2p is known to transport L-arabinose (Kou et al. 1970). Although overexpression of the complete arabinose pathway did not result in immediate growth on L-arabinose as the sole carbon source, the growth rate of the transformants increased progressively after 4–5 days of incubation (Becker and Boles 2003). Eventually, an L-arabinose-utilizing strain was selected after several sequential transfers in L-arabinose medium. In addition to being able to grow aerobically on L-arabinose, the evolved *S. cerevisiae* strain produced ethanol from L-arabinose. The specific ethanol production rate under oxygen-limited conditions was 0.06–0.08 g g<sup>-1</sup> h<sup>-1</sup> (Table 7), and the ethanol yield was 60% of the maximum theoretical yield. Anaerobic fermentation of L-arabinose was not reported. The evolved strain appeared to have acquired a mutation in the L-ribulokinase gene (*araB*), resulting in a reduced activity of this enzyme. An enhanced transaldolase (*TALI*) activity was also reported to enhance L-arabinose fermentation. Overexpression of *GAL2* was found not to be essential for growth on L-arabinose, suggesting that other yeast sugar transporters can also transport L-arabinose (Becker and Boles 2003).

In our opinion, Becker and Boles (2003) have convincingly demonstrated that the overexpression of the bacterial L-arabinose pathway is the

most promising basis for constructing L-arabinose-fermenting *S. cerevisiae* strains. This approach circumvents the intrinsic redox imbalances associated with expression of the fungal pathway. However, while the concept seems entirely sound, additional research is required to achieve fast, efficient fermentation of L-arabinose under anaerobic conditions.

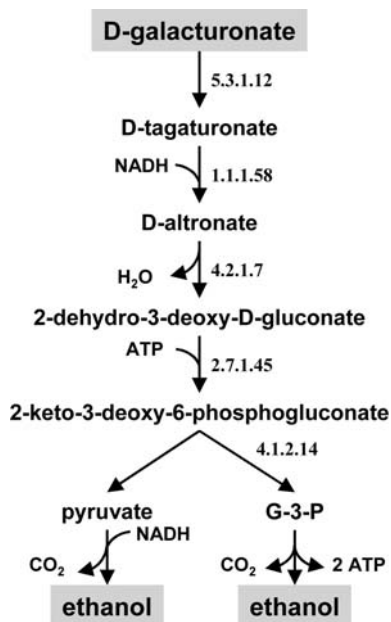
### Galacturonic acid

Galacturonic acid is a sugar acid with the same configuration as D-galactose, but with a carboxylic acid group at the C6 position. Reduction of galacturonic acid to the level of a hexose requires the input of two electron pairs, for instance via two NADH-dependent reduction steps. As a major constituent of pectin, D-galacturonic acid occurs in all plant biomass hydrolysates. Pectin-rich residues from citrus fruit, apples, sugar cane and sugar beets contain especially large amounts of D-galacturonic acid. If D-galacturonic acid can be converted to ethanol, this would increase the relevance of these abundantly available feedstocks for the production of fuels and chemicals (Doran et al. 2000; Doran and Foster 2000; Grohmann et al. 1998; Grohmann and Bothast 1994).

Although, interestingly, some *S. cerevisiae* strains contain an endogalacturonase activity (Blanco et al. 1994; Gainvors and Belarbi 1995; Radoi et al. 2005), they cannot assimilate galacturonic acid (Barnett et al. 1990). However, this is contradicted by Gainvors and Belarbi (1995). We have not found reports on plasma membrane transport of galacturonic acid by *S. cerevisiae*, nor could we identify *S. cerevisiae* genes with a clear homology to genes encoding key enzymes in galacturonic acid metabolism (data not shown). Metabolic engineering of *S. cerevisiae* for the production of ethanol from galacturonic acid will therefore probably have to address the functional expression of a plasma membrane galacturonic acid transporter as well as a heterologous pathway for galacturonic acid catabolism. The high degree of oxidation of galacturonic acid represents a special challenge in metabolic engineering of *S. cerevisiae* for ethanol production from this substrate.

Since the complete genome sequence is known for several yeasts and fungi that can grow on galacturonic acid, e.g. *Candida* and *Pichia* (Fellows and Worgan 1986; Fredlund et al. 2002; Kastner et al. 1999; Visser et al. 1990), it could be relatively straightforward to identify structural genes for galacturonic acid transporters by a transcriptome comparison of glucose- and galacturonic acid-grown cultures, provided such transporters are induced on galacturonic acid. Transport should preferentially be addressed before attempts are made to express a heterologous catabolic pathway, as this sequence of events allows direct screening for growth of transformants in later stages of the research.

The ability to utilize galacturonic acid is widespread among bacteria, which all seem to use essentially the same metabolic pathway (Doran et al. 2000; Fellows and Worgan 1986; Freeman and San Francisco 1994; Grohmann et al. 1998; Kilgore and Starr 1959; Serrat et al. 2004; Sirotek et al. 2004). In this bacterial pathway, D-galacturonic acid is converted to pyruvate and glyceraldehyde-3-phosphate via a five-step pathway



**Fig. 5** Bacterial D-galacturonate catabolism. 5.3.1.12, D-galacturonate isomerase; 1.1.1.58, altronate oxidoreductase; 4.2.1.7, altronate dehydratase; 2.7.1.45, 2-dehydro-3-deoxygluconokinase; 4.1.2.14, 2-keto-3-deoxy-6-phosphogluconate aldolase; G-3-P, glyceraldehyde-3-phosphate

(Fig. 5). Overall this results in the conversion of galacturonic acid, NADH and ATP into pyruvate, glyceraldehyde-3-phosphate and water.

Glyceraldehyde-3-phosphate can be converted to equimolar amounts of ethanol and CO<sub>2</sub> via standard glycolytic reactions, yielding 2 ATP. However, conversion of pyruvate to ethanol requires the oxidation of a second NADH. Some galacturonic acid-metabolizing bacteria produce ethanol, but the ethanol yields are generally low (Doran et al. 2000; Grohmann et al. 1998). Reported yields are 0.19 g ethanol g galacturonic acid<sup>-1</sup> for *E. coli* and 0.12 g ethanol g galacturonic acid<sup>-1</sup> for *Erwinia chrysanthemi* and *Klebsiella oxytoca* (Doran et al. 2000). All three bacteria produced large amounts of acetate (0.23–0.38 g acetate g galacturonic acid<sup>-1</sup>) (Doran et al. 2000). Clearly, a large fraction of the carbon in galacturonic acid was excreted in the form of compounds that are more oxidized than ethanol. The same problem is likely to occur upon introduction of the prokaryotic pathway into *S. cerevisiae*, at least when galacturonic acid is used as the sole carbon source. However, a considerably more positive scenario can be envisaged for ethanol formation from hydrolysates.

During anaerobic growth and anaerobic fermentation of sugars (hexoses, but also xylose by engineered xylose-fermenting strains) by *S. cerevisiae*, a significant fraction of the carbon is channelled into glycerol to compensate for oxidative, NADH-generating reactions in biosynthesis (Bakker et al. 2001; van Dijken and Scheffers 1986). In theory, introduction of the prokaryotic galacturonic acid fermentation route can create an alternative redox sink for the ‘excess’ NADH formed in biosynthesis. This would have two advantages. Firstly, the NADH derived from biosynthetic processes can be used to increase the ethanol yield on galacturonic acid to 2 mol ethanol per mol galacturonic acid, as the pyruvate formed from galacturonic acid can now be completely converted to ethanol. Secondly, since the sugar requirement for glycerol production is reduced, the ethanol yield on sugars will increase. The practical feasibility of this mixed-substrate scenario will depend on the actual galacturonic acid content of the feedstock. During anaerobic growth of *S. cerevisiae* on glucose, the

glycerol yield on glucose is ca. 0.2 mol glycerol mol glucose<sup>-1</sup>, corresponding to an equimolar production of NADH from biosynthetic reductions (Verduyn et al. 1990a, b). As 2 mol NADH can be reoxidized during the complete alcoholic fermentation of galacturonic acid via the prokaryotic pathway for galacturonic acid metabolism, the formation of 0.2 mol glycerol from glucose should be redox-equivalent to the conversion of 0.1 mol galacturonic acid. This indicates that an engineered *S. cerevisiae* strain that expresses a functional prokaryotic pathway for galacturonic acid metabolism should be able to efficiently convert galacturonic acid when this compound is present at a molar fraction of 10% or less relative to hexose sugars. It should be mentioned that growth in large-scale ethanol production processes often results in lower biomass yields and, consequently, less generation of 'biosynthetic' NADH.

Although a large number of yeasts and molds can use galacturonic acid as a carbon and energy source for growth, knowledge of the metabolic route for D-galacturonic acid catabolism in eukaryotes is surprisingly limited (Kuorelahti et al. 2005; Visser et al. 1988). It has been concluded that D-galacturonic acid is metabolized through glyceraldehyde and pyruvate (Visser et al. 1988). Kuorelahti et al. (2005) have recently identified a D-galacturonic acid reductase in the mold *Hypocrea jecorina*. This enzyme converts D-galacturonic acid and NADPH to L-galactonic acid and NADP<sup>+</sup>. It has been proposed that this enzyme may be involved in a pathway for galacturonic acid metabolism that is distinctly different from the bacterial route, and which would involve L-galactonic acid, 3-dehydro-L-gulonate, L-xylulose, xylitol, D-xylulose, and xylulose 5-phosphate as intermediates (Kuorelahti et al. 2005). The NADPH-dependence of the reductase may represent a complication in yeast metabolic-engineering strategies based on this pathway as it implies a need for balancing of the two different cofactor balances NADH/NAD<sup>+</sup> and NADPH/NADP<sup>+</sup>. This problem, which is reminiscent of the redox problems arising from the xylose reductase/xylitol dehydrogenase strategy for engineering xylose fermentation (see above) leads us to conclude

that the prokaryotic pathway offers the most promising approach for engineering *S. cerevisiae* for galacturonic acid metabolism.

While, as described above, there appears to be a good perspective for high-yield production of ethanol from galacturonic acid in feedstocks that contain low fractions of this compound, this strategy does not offer a promising metabolic engineering approach for fermenting pectin-rich feedstocks—unless the process infrastructure allows for blending with feedstocks that have a low galacturonic acid content.

### Rhamnose

The deoxyhexose L-rhamnose (6-deoxy-L-mannose, L-mannomethylose) is named after the plant it was first isolated from: the buckthorn (*Rhamnus*). In contrast to the situation for most sugars, L-rhamnose is much more common in nature than D-rhamnose. Although rhamnose is not frequently included in the analysis of plant biomass hydrolysates, it occurs in hydrolysates as a constituent of the rhamnogalacturonan part of pectin and of hemicellulose. Being a 6-deoxy sugar, L-rhamnose is more reduced than the rapidly fermentable sugars glucose and fructose.

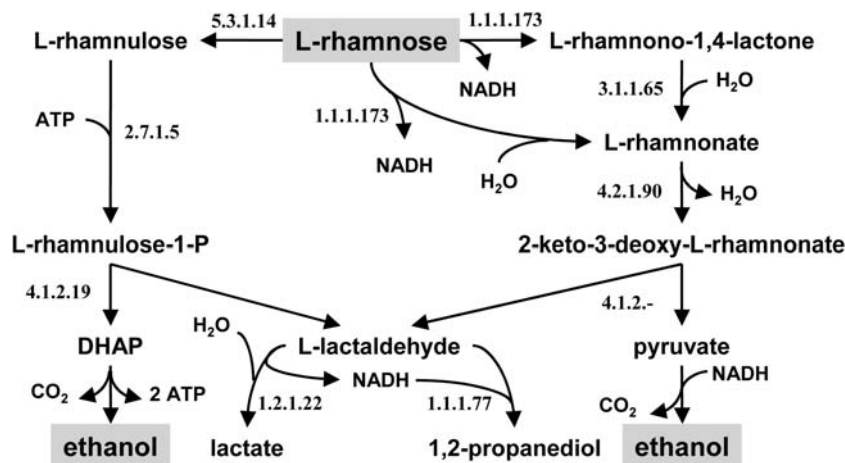
*S. cerevisiae* cannot grow on L-rhamnose as the sole carbon source (Barnett et al. 1990). Moreover, Yoon et al. (2003) showed the inability of immobilized *S. cerevisiae* to remove rhamnose from a carbohydrate stream under anaerobic conditions. Indeed, the *S. cerevisiae* genome does not reveal genes with a clear homology to genes encoding rhamnose-metabolizing enzymes (data not shown). Sugar-uptake studies with protoplasts and cells of glucose-pregrown *S. cerevisiae* have shown that L-rhamnose crosses the plasma membrane at an extremely low rate (ca. 2 μmol g<sub>biomass</sub><sup>-1</sup> h<sup>-1</sup>) (Heredia et al. 1968). Even though rhamnose is transported about 10,000-fold slower than glucose (Bakker et al. 2000; Heredia et al. 1968), its transport is almost exclusively facilitated via hexose transporters and free diffusion hardly occurs (Heredia et al. 1968). Clearly, metabolic engineering of *S. cerevisiae* for the production of ethanol from rhamnose will have to address two key aspects: the enhancement of

rhamnose transport across the plasma membrane and the introduction of a rhamnose-metabolizing pathway.

Two different strategies may be envisaged to achieve efficient uptake of L-rhamnose by *S. cerevisiae*. Firstly, after the introduction of an ATP-yielding pathway for L-rhamnose catabolism (see below), selection for growth on L-rhamnose can be used to investigate whether mutations in one or more of the hexose transporter genes in *S. cerevisiae* affect regulation and/or kinetic properties in such a way that it enables efficient L-rhamnose uptake. Alternatively, introduction of a heterologous rhamnose transporter can be considered. Although the rhamnose transporter of for instance *E. coli* is well characterized (Baldomá et al. 1990), functional expression of bacterial transporters in the yeast plasma membrane may be too difficult a proposition. A more promising strategy would involve the identification and expression in *S. cerevisiae* of an L-rhamnose transporter from a yeast known to utilise rhamnose (e.g. *Pi. stipitis*, currently being sequenced (<http://www.jgi.doe.gov/sequencing/DOEmicrobes.html>)). As proposed for galacturonic acid (see above) transcriptome analysis might be used to identify a rhamnose-transporter gene if such a gene is induced on rhamnose.

After its uptake, the next requirement for successful rhamnose fermentation is its conversion into intermediates of central metabolism. In 1937, Albert-Jan Kluyver (an illustrious predecessor of Gijs Kuenen), was the first to quantitatively study the metabolic fate of rhamnose (Kluyver and Schnellen 1937). By now, two pathways for L-rhamnose catabolism have been reported in microorganisms (Fig. 6). These pathways provide interesting starting points for engineering a rhamnose-utilizing route into *S. cerevisiae*.

The first catabolic pathway involves phosphorylated intermediates (Fig. 6) and is for instance used by *E. coli* (Wilson and Ajl 1955). In this pathway, L-rhamnose is first converted to L-rhamnulose by L-rhamnose isomerase (EC 5.3.1.14) (Takagi and Sawada 1964a; Wilson and Ajl 1957a). After subsequent phosphorylation by rhamnulokinase (EC 2.7.1.5), L-rhamnulose-1-phosphate is split into dihydroxy-acetone-phosphate (DHAP) and L-lactaldehyde by rhamnulose-1-phosphate aldolase (EC 4.1.2.19) (Sawada and Takagi 1964; Takagi and Sawada 1964b; Wilson and Ajl 1957b). DHAP can be normally processed by glycolysis, yielding 1 mol ethanol mol L-rhamnose<sup>-1</sup>. In *E. coli*, further metabolism of L-lactaldehyde depends on the redox state of the cells. L-Lactaldehyde can either be oxidized to



**Fig. 6** Bacterial (left) and fungal (right) L-rhamnose catabolism. 5.3.1.14, L-rhamnose isomerase; 2.7.1.5, rhamnulokinase; 4.1.2.19, rhamnulose-1-phosphate aldolase; 1.1.1.173, L-rhamnose dehydrogenase; 3.1.1.65, L-rham-

nono-1,4-lactonase; 4.2.1.90, L-rhamnonate dehydratase; 4.1.2.-, aldolase; 1.2.1.22, lactaldehyde dehydrogenase; 1.1.1.77, lactaldehyde reductase; DHAP, dihydroxy-acetone-phosphate

lactate by lactaldehyde dehydrogenase (EC 1.2.1.22), reduced to 1,2-propanediol by lactaldehyde reductase (EC 1.1.1.77), or processed via a redox-neutral mix of these two reactions (Baldoma and Aguilar 1988). After introduction of this pathway into *S. cerevisiae*, conversion of L-rhamnose to equimolar amounts of ethanol, lactaldehyde and CO<sub>2</sub> leads to the net generation of 1 ATP. This strategy would require the introduction of a transporter and three heterologous enzymes into *S. cerevisiae* (Fig. 6).

A second route for rhamnose degradation, which does not involve phosphorylated intermediates, was first described in the fungus *Aureobasidium pullulans* and is referred to as direct oxidative catabolism of rhamnose (Rigo et al. 1985). A similar pathway occurs in the yeasts *Pichia stipitis* and *Debaryomyces polymorphus* (Twerdochlib et al. 1994) and is initiated by the oxidation of L-rhamnose by NAD<sup>+</sup>-dependent L-rhamnose dehydrogenase (EC 1.1.1.173), yielding either L-rhamnono-1,4-lactone or the unstable rhamnono-1,5-lactone (Rigo et al. 1985; Twerdochlib et al. 1994). The 1,4 lactone is hydrolyzed to L-rhamnonate by L-rhamnono-1,4-lactonase (EC 3.1.1.65) (Rigo et al. 1985). The unstable 1,5-lactone has been reported to spontaneously hydrolyse to L-rhamnonate (Twerdochlib et al. 1994). L-Rhamnonate is subsequently dehydrated to 2-keto-3-deoxy-L-rhamnonate by L-rhamnonate dehydratase (EC 4.2.1.90) (Rigo et al. 1985; Twerdochlib et al. 1994). The product of this reaction is then cleaved into pyruvate and L-lactaldehyde by an aldolase (Rigo et al. 1985; Twerdochlib et al. 1994). In *Pi. stipitis* the thus formed L-lactaldehyde is converted to lactate and NADH by L-lactaldehyde dehydrogenase (1.2.1.22). Introduction of this ‘fungal’ pathway into *S. cerevisiae* should enable the conversion of L-rhamnose to equimolar amounts of ethanol, lactaldehyde and CO<sub>2</sub> without a net generation of ATP. This conversion would require the introduction of a transporter and four heterologous enzymes (including the 1,4-lactonase) into *S. cerevisiae* (Fig. 6).

At first sight, the absence of ATP formation in the direct oxidative pathway may appear beneficial, since ATP formation would enable growth of engineered *S. cerevisiae* on L-rhamnose. The

resulting biomass formation would go at the expense of the ethanol yield. However, the option to select for growth on L-rhamnose during strain construction and, potentially, during long-term industrial cultivation, provides a strong incentive for using the ‘bacterial’ pathway instead.

Both pathways described above result in the formation of L-lactaldehyde. In anaerobic, fermenting *S. cerevisiae* cultures, the most attractive means of converting lactaldehyde is its NADH-dependent reduction to 1,2-propanediol. Besides the presence, in *S. cerevisiae*, of a functional L-lactaldehyde reductase, this requires one extra NADH per lactaldehyde. During anaerobic growth on sugars, *S. cerevisiae* normally uses glycerol formation, at a cost of 1 ATP and a lower ethanol yield, as a sink for excess reduction equivalents (Bakker et al. 2001; van Dijken and Scheffers 1986). In mixed-substrate cultures, production of 1,2-propanediol from rhamnose might function as an alternative redox sink, thereby reducing the need for glycerol production from conventional sugars and, hence, further increasing the ethanol yield.

In our opinion, it should be possible to engineer *S. cerevisiae* such that it converts half of the carbon in L-rhamnose to ethanol and CO<sub>2</sub>, with the concomitant production of equimolar amounts of 1,2-propanediol. We anticipate that the glycerol-sparing effect described above may increase the net ethanol yield from L-rhamnose in hydrolysate fermentations to 2 mol mol<sup>-1</sup>. Our recommended approach is to introduce a fungal transporter for L-rhamnose, together with the three key enzymes of the ‘bacterial’ pathway for L-rhamnose catabolism and an L-lactaldehyde reductase.

## Inhibitor tolerance

### Inhibitors in plant hydrolysates

The harsh conditions that prevail during the chemical and physical pretreatment of lignocellulose result in the release of many substances that inhibit growth and ethanol production by *S. cerevisiae*. The number and identity of these toxic compounds varies with the nature of the raw



material and pretreatment conditions (see Klinke et al. 2004 for a recent review). There are two approaches to limit the impact of inhibitors on the fermentation process: (i) introduction of additional chemical, physical or biological process steps for removal or inactivation of inhibitors (López et al. 2004; Mussatto and Roberto 2004; Nichols et al. 2005; Palmqvist et al. 1997; Palmqvist and Hahn-Hägerdal 2000) and (ii) improvement of the tolerance of *S. cerevisiae* to the inhibitors. Physical and chemical methods of detoxification quickly become cost-prohibitive for an industry that operates with narrow profit margins. In contrast, biological methods focused on either in situ inactivation/metabolism of inhibitors or the development of more stress-resistant yeast, are relatively inexpensive. In this review, we will focus on options to improve tolerance of *S. cerevisiae* to the main inhibitors in plant biomass hydrolysates.

Three major categories of inhibitory compounds derived from physico-chemical pretreatment of lignocellulosic material can be identified: weak acids, furan derivatives, and phenols (Palmqvist et al. 1999). For example, poor fermentability of dilute acid-wood hydrolysates was primarily correlated to high levels of furfural, 5-hydroxymethylfurfural (5-HMF) and acetic acid (Taherzadeh et al. 1997). Therefore, these categories will be the major focus of review.

#### Common inhibitors in hydrolysates, effects and mechanisms

Of the many weak organic acids that occur in lignocellulose hydrolysates, acetic acid is most common to hemicellulose hydrolysates (Ingram et al. 1999), while hydroxycarboxylic acids like glycolic acid and lactic acid are also present when alkaline hydrolysis is performed (Sjöström 1991). Furthermore, formic acid is generated by degradation of sugars or lignin (Klinke et al. 2002) with levulinic acid commonly arising from 5-HMF degradation (Palmqvist et al. 1999).

As undissociated weak organic acids readily diffuse across biological membranes and the pH of the yeast cytosol is generally higher than that of the surrounding medium, internal hydrolysis of weak acids causes cytosolic acidification

(Pampulha and Loureiro-Dias 1989), which has to be compensated for by ATP-dependent proton pumping (Holyoak et al. 1996; Imai and Ohno 1995; Verduyn et al. 1992). In addition, intracellular accumulation of the anion, either by itself or in combination with intracellular acidification, can lead to toxic effects (Casal et al. 1996; Eklund 1983; Pampulha and Loureiro-Dias 1990; Russell 1992). Benzoate, for instance, has been implicated in inhibition of autophagy (Hazan et al. 2004) and acetate has been demonstrated to induce apoptosis (Ludovico et al. 2001). Moreover, hydrolysates contain many compounds that act synergistically with organic acids. Such synergistic effects have been demonstrated for various combinations of acids, phenols and furans (Nigam 2001; Palmqvist et al. 1999). Ethanol has also been reported to augment the toxicity of weak acids (Leão and Van Uden 1984; Pampulha and Loureiro-Dias 1989).

The predominant furan derivatives, furfural and 5-HMF, which are mainly derived from dehydration of pentose and hexose sugars during hydrolysis, are among the strongest inhibitors in lignocellulosic hydrolysates (Taherzadeh et al. 1997). Although furfural is more toxic than 5-HMF, both compounds act synergistically to suppress yeast growth (Liu et al. 2004). In general, furans have been implicated in a broad range of effects, including inhibition of cell growth and glucose utilization, reduction of enzymic activity and ethanol productivity, DNA damage and inhibition of protein and RNA synthesis (as cited in Liu et al. 2004). However, *S. cerevisiae* is capable of reducing furfural and 5-HMF to the less toxic furfuryl (Taherzadeh et al. 1999) and 5-hydroxymethyl furfuryl alcohols (Taherzadeh et al. 2000), respectively. When present alone, furfural is converted so efficiently that inhibitory effects decrease with increasing cell mass due to its rapid bioconversion (Navarro 1994). Increasing the biomass concentration via biomass recycling similarly reduced the impact of furans on ethanol productivity (Brandberg et al. 2005). Conversely, conversion rates of both compounds are decreased when they are present in combination (Taherzadeh et al. 2000) with furfural being converted at higher rates (Larsson et al. 1999) and HMF metabolism only proceeding



after the complete degradation of furfural (Taherzadeh et al. 2000).

Finally, various phenolic compounds that are derived from lignin degradation have a significant impact on fermentation of hydrolysates. Due to its abundance in hardwood hydrolysates (Jönsson et al. 1998), 4-hydroxybenzoic acid is often used as a model compound to study the effects of phenolics. Other phenols, including vanillin, vanillic acid, hydroxybenzaldehyde, syringaldehyde, catechol, resorcinol, and salicylic acid have also been studied (as cited in Palmqvist and Hahn-Hägerdal 2000). These compounds are toxic (Buchert et al. 1989) because they compromise the integrity of biological membranes (Heipieper et al. 1994).

#### Improving inhibitor tolerance of *S. cerevisiae*

The tolerance towards inhibitors in plant biomass hydrolysates differs considerably among *S. cerevisiae* strains (Sonderegger et al. 2004). Therefore, depending on the composition of the biomass hydrolysates and the complexity of the intended metabolic engineering strategy, it may be useful to select a host strain with an appropriate inhibitor tolerance. Where increased tolerance is required for successful industrial application, this can be approached via metabolic engineering, evolutionary engineering (Sauer 2001), or a combination thereof.

Increased furan resistance of *S. cerevisiae* has been realized by accumulation of furan-selective mutants via serial transfer in the presence of increasing furan concentrations (Liu et al. 2005). The resulting strains were capable of complete reduction of 60 mM HMF and 30 mM furfural to the corresponding alcohols. Furthermore, genetic engineering strategies to increase furan tolerance are likely to be proposed in the near future as Gorsich et al. (2005) have demonstrated that deletion of the pentose-phosphate-pathway genes, *ZWF1*, *GND1*, *RPE1* and *TLK1*, negatively affects furfural tolerance. Although NADPH, which is primarily generated via the pentose-phosphate pathway, is required for HMF reduction (Flores et al. 2000; Wahlbom and Hahn-Hägerdal 2002), furfural reduction has been linked to NADH (Wahlbom and

Hahn-Hägerdal 2002). However, it has been suggested that decreased NADPH generation reduces the overall reduction potential of the cell, thus leaving less NADH for furfural reduction (Gorsich et al. 2005). Similarly, Nilsson et al. (2005) have reported that the furan reduction capacity is key to performance of a yeast strain in lignocellulosic hydrolysates. This identifies both cofactor recycling and the capacity of the alcohol dehydrogenase(s) involved in furan reduction as key targets for metabolic engineering.

The toxicity of phenols has been successfully reduced by the addition of laccase (EC 1.10.3.2) to hydrolysates (Jönsson et al. 1998). Expression of the *Trametes versicolor* laccase gene into *S. cerevisiae* (Larsson et al. 2001a) resulted in decreased fermentation times on hydrolysates, while overexpression of a phenylacrylic-acid decarboxylase resulted in improved growth rate and ethanol productivity in dilute-acid hydrolysates (Larsson et al. 2001b). Contrarily, metabolic or evolutionary engineering studies to improve the tolerance of *S. cerevisiae* to the weak organic acids that are present in the hydrolysates are rare. However, successes in different backgrounds, such as for instance an acetate-tolerant (6–8 g l<sup>-1</sup>) *S. cerevisiae* strain selected for use in sour dough (Aarnio et al. 1991), could be extrapolated to hydrolysate fermentation.

Evidently, the vast array of inhibitors present in lignocellulose hydrolysates presents a formidable challenge for biologists and engineers alike. Although numerous detoxification strategies and yeast strains have been investigated, an ideal and generic solution has not yet been identified. Most likely, hydrolysate-specific combinations of detoxification methods with tailor-made yeast strains will play a major role in industrial research. Additionally, advances in pretreatment and separation technology may have a major impact by minimizing the formation of inhibitors.

#### Outlook

Given the economic and environmental importance of a closed carbon cycle in transport fuel production, construction of strains that efficiently convert all fermentable substrates in plant biomass

into ethanol is, perhaps, the single most important challenge in yeast metabolic engineering.

In terms of volume, xylose is the most abundant sugar in plant biomass hydrolysates that cannot be fermented by wild-type *S. cerevisiae* strains. The recent breakthroughs with xylose-isomerase-expressing strains (Kuyper et al. 2005b) illustrate a number of issues that, in our opinion, are of paramount importance for achieving fast and efficient alcoholic fermentation of additional compounds in a time-efficient manner. These include:

1. The metabolic-engineering strategy should enable a closed balance of relevant redox cofactors (NAD<sup>+</sup>/NADH and NADP<sup>+</sup>/NADPH).
2. Catalytic activity of heterologous enzymes and transporters in *S. cerevisiae* may require extensive screening. Whereas the discovery of the *Piromyces* xylose isomerase (Harhangi et al. 2003) was, to some extent, serendipitous, it underscores that screening of culture collections and/or metagenomics (Schloss and Handelsman 2003) approaches may be required for identification of enzymes with optimal properties.
3. Directed evolution of wild-type or metabolically-engineered microbial cultures (evolutionary engineering, Sauer 2001) is a powerful approach in scenarios where performance of microbial strains is directly linked to specific growth rate. As long as an engineered pathway for ethanol production results in a net ATP gain, this approach should be applicable to all substrates discussed above.

As demonstrated above, promising strategies are available for a further expansion of the substrate range of ethanol-producing *S. cerevisiae* strains, probably starting with the full optimization of the bacterial L-arabinose pathway (Becker and Boles 2003; Sedlak and Ho 2001). We are confident that a concerted, multidisciplinary effort in yeast metabolic engineering will result in rapid progress in the areas discussed in this review.

However important, proof-of-principle experiments under ‘academic’ conditions (synthetic media, single substrates or simple substrate

mixtures, absence of toxic inhibitors) merely represent the first step towards industrially applicable strains. Efficient conversion of complex substrate mixtures in the presence of synergistically acting inhibitors needs to be addressed before industrial implementation can be contemplated. Given the diversity of feedstocks as well as of methods for producing plant biomass hydrolysates, it appears prudent to integrate studies on hydrolysate preparation and strain optimization from an early stage onwards.

Once the crucial issues related to substrate range and inhibitor tolerance have been successfully addressed, a next key target for yeast-based ethanol research is likely to be the ‘consolidated bioprocessing’ approach (Lynd et al. 2005), i.e. the functional expression of cellulolytic enzymes and other hydrolases in ethanol-producing microbial strains. Despite the recent dramatic reductions of the costs of hydrolytic enzymes, by amongst others Genencor, Novozymes and NREL, it seems probable that the ultimate ‘cell factory’ for ethanol production will integrate fermentation with at least some of the major catalytic activities required for polymer degradation.

Its role in current large-scale ethanol production, genetic accessibility and robustness under process conditions make *S. cerevisiae* the most promising platform for process development and optimization in the coming decade. This statement should not be interpreted as overly conservative or, indeed, protective. We strongly advocate that, in addition to major public and private investments in *S. cerevisiae*-oriented bioethanol research, substantial support be given to alternative platforms such as cellulolytic thermophilic microorganisms.

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