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MINI-REVIEW

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Metabolic engineering for improved fermentation of pentoses by yeasts

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Abstract The fermentation of xylose is essential for the bioconversion of lignocellulose to fuels and chemicals, but wild-type strains of *Saccharomyces cerevisiae* do not metabolize xylose, so researchers have engineered xylose metabolism in this yeast. Glucose transporters mediate xylose uptake, but no transporter specific for xylose has yet been identified. Over-expressing genes for aldose (xylose) reductase, xylitol dehydrogenase and moderate levels of xylulokinase enable xylose assimilation and fermentation, but a balanced supply of NAD(P) and NAD(P)H must be maintained to avoid xylitol production. Reducing production of NADPH by blocking the oxidative pentose phosphate cycle can reduce xylitol formation, but this occurs at the expense of xylose assimilation. Respiration is critical for growth on xylose by both native xylose-fermenting yeasts and recombinant *S, cerevisiae*. Anaerobic growth by recombinant mutants has been reported. Reducing the respiration capacity of xylose-metabolizing yeasts increases ethanol production. Recently, two routes for arabinose metabolism have been engineered in *S. cerevisiae* and adapted strains of *Pichia stipitis* have been shown to ferment hydrolysates with ethanol yields of 0.45 g g^{-1} sugar consumed, so commercialization seems feasible for some applications.

Introduction

Fermentation of xylose to ethanol is driven by political, economic and technical considerations. For example, using biomass as a feedstock for renewable fuel production can substantially reduce the accumulation of greenhouse gasses (McMillan 1997; Claassen et al. 1999; Wyman 1999; Kheshgi et al. 2000), so a recent European Union directive proposed that biofuels should represent 2% of total transportation fuel consumption by 2005 and 5.75% by 2010 (Roca and Olsson 2003). Increased domestic use of agricultural commodities can increase income for farmers, so the agricultural policies of the United States, Brazil and the European Union created nascent ethanol industries for the commercial production of ethanol from grains, sugar cane and other feedstocks. Opportunities for the bioconversion of harvest and processing residues are increasing along with these markets. For example, as grain hull, corn cobs, corn stover and sugar cane bagasse byproducts increase, ethanol production from the waste streams becomes feasible (Saha et al. 1998; Saha and Bothast 1999). Xylose is a major constituent of these and other renewable biomass feedstocks, but its efficient utilization—which is essential for commercial bioconversion (Hinmann et al. 1989; Olsson and Hahn-Hägerdal 1996)—presents a technical barrier. Lignocellulosic crop residues comprise more than half of the world′s agricultural phytomass (Smil 1999) and significant fractions of the total can be recovered without competing with other uses (Lynd 1996; Wyman 1999). Xylose constitutes about 17% of the total dry weight in woody angiosperms and ranges up to 31% in herbaceous angiosperms (Pettersen 1984; Hespell 1998). One source of xylose is the sulfite-pulping of hardwood (Lawford and Rousseau 1993). Depending on the substrate and reaction conditions, dilute acid pretreatments of lignocellulosic residues can recover 80–95% of the xylose from the feedstock (Chen et al. 1998; Kim et al. 2001; Aguilar et al. 2002).

Native strains of *Saccharomyces cerevisiae* do not use xylose as a carbon source. *Candida utilis* or ″torula yeast″ will grow on xylose, but this yeast is strictly aerobic and does not produce ethanol. In the early 1980s, following the discovery that *S. cerevisiae*, *Schizosaccharomyces pombe* and other yeasts can ferment D-xylulose to ethanol (Wang and Schneider 1980; Wang et al. 1980), intensive screening efforts rapidly revealed that some can convert xylose

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to ethanol directly under aerobic or oxygen-limiting conditions (Schneider et al. 1981; Jeffries 1982; Slininger et al. 1982. Attention focused on *Pachysolen tannophilus*, *C. shehatae* (Du Preez and van der Walt 1983) and *Pichia stipitis*, which are the best native xylose-fermenting yeasts known (Toivola et al. 1984; Du Preez et al. 1986). Many improvements have been made in the genetic engineering of yeasts and bacteria for the fermentation of xylose and arabinose to ethanol and other products such as lactic acid. However, the bioconversion of pentoses to ethanol still presents a considerable economic and technical challenge (Jeffries and Shi 1999; Aristidou and Penttilä 2000; Hahn-Hägerdal et al. 2001).

The objective of this review is to assess the current state of microbial strain development for the fermentation of pentose sugars. This is a very active field. From January 1999 to June 2003, more than 60 articles on yeast appeared in press. We try to emphasize the latest work and refer the reader to reviews of the earlier literature (Jeffries 1983, 1985; Prior et al. 1989; Jeffries and Kurtzman 1994; Gong et al. 1999; Ho et al. 1999; Jeffries and Shi 1999; Aristidou and Penttilä 2000; Flores et al. 2000; Jeffries and Jin 2000; Ostergaard et al. 2000; Dequin 2001; Hahn-Hägerdal et al. 2001; Galbe and Zacchi 2002).

Metabolic engineering of yeasts

Yeast strain development focuses on the genetic engineering of *Saccharomyces cerevisiae*, but *P. stipitis* has also been modified for xylose fermentation. Metabolic engineering can alter sugar transport, assimilation, the pentose phosphate pathway, glycolysis, the terminal steps of fermentation and the relatively complicated interplay between respiration and fermentation that determine the intracellular redox balance. While a few of the changes enable xylose utilization in *S. cerevisiae*, most have marginal effects. No one factor or enzymatic step is rate-limiting, but some are critical. Even though genes for xylose assimilation are present in *S. cerevisiae*, they are not expressed at a sufficient level to enable significant sugar assimilation. Engineering of *P. stipitis* has been more limited, but some principles have been established with this yeast.

Xylose transport

Sugar uptake limits xylose utilization in both *S. cerevisiae* and *P. stipitis*. In *S. cerevisiae*, the *HXT* family of sugar transporters mediates glucose uptake (Kruckeberg 1996; Boles and Hollenberg 1997). Hxt1–Hxt7 and Gal2 exhibit counter-transport when individually expressed in a *hxt1-7* null mutant of *S. cerevisiae*, which indicates that they function by facilitated diffusion (Maier et al. 2002). Kinetic studies with ^{14}C glucose can distinguish high- and low-affinity uptake systems. Xylose uptake competes with glucose uptake, indicating that they share transport components (Meinander and Hahn-Hägerdal 1997). *S. cerevisiae* takes up xylose by both low- and high-affinity glucose transport systems (Lee et al. 2002), but after incubation with xylose only the high-affinity system is detected. Cultivation of the xylose-fermenting *S. cerevisiae* FPL-YSX3 on xylose under aerobic or oxygen-limiting conditions strongly induces (5- to 50-fold) the high-affinity transporters *HXT2*, *HXT6* and *HXT7* and the moderate affinity transporter *HXT5* (Buziol et al. 2002; Jin 2002). Hxt5 is produced under slow growth conditions (Diderich et al. 2001; Verwaal et al. 2002). With YSX3 cells cultivated in xylose medium, the low-affinity transporters *HXT1* and *HXT3* are expressed at 2–5% of the level observed with cells grown on glucose (Jin 2002). These results suggest that engineered *S. cerevisiae* mainly uses the high-affinity system for xylose transport.

Glucose strongly inhibits the transport of xylose by both high- and low-affinity systems (van Zyl et al. 1993). The native *S. cerevisiae* glucose transporters exhibit a significantly lower affinity for xylose (K_m=49–300 mM) than for glucose (K_m=1–28 mM; Kötter and Ciriacy 1993; Lagunas 1993; Van Zyl et al. 1999). Therefore, glucose and xylose are consumed simultaneously only under glucose-limited conditions (Meinander and Hahn-Hägerdal 1997). *S. cerevisiae* (TMB3201)—which is completely deficient in all 18 monosaccharide transporters (Wieczorke et al. 1999) but has a functional xylose utilization pathway (Hamacher et al. 2002)—cannot take up or grow on xylose. When various *HXT* genes are reintroduced, Hxt4, Hxt5, Hxt7 and Gal2 promote xylose uptake (Hamacher et al. 2002).

Native xylose-metabolizing yeasts have two kinetically distinct xylose transport systems. The low-affinity system is shared with glucose, while the high-affinity system is specific for xylose (Hahn-Hägerdal et al. 2001). Both of these systems are tightly coupled to energy metabolism (Kilian and van Uden 1988; Does and Bisson 1989). Three genes, *SUT1*, *SUT2* and *SUT3*, encode glucose transporters in *P. stipitis* (Weierstall et al. 1999). Sut2 and Sut3 are highly similar to the *S. cerevisiae* glucose transporter family and the Sut2 and Sut3 transporters have a higher affinity for glucose than for xylose. Transcription of *SUT1* is induced in *P. stipitis* independently of the oxygen supply. *SUT2* and *SUT3* are expressed only under aerobic conditions, but independently of the carbon source. Disruption of *SUT1* eliminates the low-affinity xylose transport system in *P. stipitis*. Xylose uptake in *P. stipitis* grown on glucose has high-affinity (K_{m1}=3.2 mM) and low-affinity (K_{m2}=80 mM) components. In Δ*sut1* cells grown under oxygen-limiting conditions—when *SUT2* and *SUT3* are not expressed—xylose transport is still active, which suggests that specific xylose transporters other than Sut1, Sut2 and Sut3 are present. However, Weierstall et al. (1999) were not able to identify any additional cross-hybridization signals for *HXT*- or *SUT*-related genes in *P. stipitis*.

Xylose isomerase

The initial metabolic engineering of *S. cerevisiae* for xylose assimilation attempted the heterologous expression of bacterial xylose isomerase (XI). This approach was reasonable, given that *S. cerevisiae* can grow on and ferment xylulose, but significant XI activity was not attained in the transformed cells (Jeffries and Shi 1999; Hahn-Hägerdal et al. 2001). Probably, this is attributable to improper folding of the protein (Sarthy et al. 1987), but even if small amounts of active XI are formed, xylitol is a competitive inhibitor (Smith et al. 1991). Expression of a XI from the thermophilic bacterium *Thermus thermophilus* achieved the heterologous production of an active enzyme in *S. cerevisiae* (Walfridsson et al. 1996) and the genetic background has been modified to reduce xylitol production (Walfridsson et al. 1996; Träff et al. 2001). However, the temperature required for moderate *T. thermophilus* XI activity is well above the maximum growth temperature of *Saccharomyces*, so researchers from this same laboratory have used directed evolution to obtain a XI with 9-fold higher activity constants at 60°C and much higher inhibition constants for xylitol (Lönn et al. 2002). Very recently, Harhangi et al. (2003) introduced a eukaryotic XI, the *AraA* gene, from the anaerobic fungus species into *S. cerevisiae* and enabled slow xylose assimilation by this route (Kuyper et al. 2003). Whether the use of XI will prove successful could depend on other factors. At equilibrium, energetics of the isomerization between xylose and xylulose favors xylose formation by 83:17 (Jeffries 1985), so some other driving force is necessary to promote this reaction.

Xylose reductase and xylitol dehydrogenase

As early as 1983, researchers concluded that the key to anaerobic assimilation of xylose by native yeasts such as *Pachystolon tannophilus* was the presence of an aldose (xylose) reductase (XR) that could accept either NADH or NADPH as a cofactor (Bruinenberg et al. 1983a, 1983b, 1984; Verduyn et al. 1985a, 1985b; Bruinenberg 1986). This hypothesis was based on a study of aerobic xylose utilization by *C. utilis*. Even though this yeast can rapidly ferment glucose, fermentative activity ceases immediately after transfer to xylose. The XR of *C. utilis* exclusively uses NADH as a cofactor. In comparison, at least one XR of *P. tannophilus* can use either NADH or NADPH (Verduyn et al. 1985a). The same is true of *Pichia stipitis* (Verduyn et al. 1985b) and *C. shehatae* (Ho et al. 1990); and all of these can metabolize xylose anaerobically—even though they do not grow under those conditions (Wijsman et al. 1985). Because the assimilation of xylose requires two oxidoreductase steps and because all oxidoreductase reactions following these are balanced, researchers hypothesized that, if no transhydrogenase were present to regenerate NAD^+ and NADPH, xylose assimilation under anaerobic conditions would quickly halt.

When the XR of *P. stipitis* is cloned and expressed in *S. cerevisiae* (Amore et al. 1991; Takuma et al. 1991; Tantirungkij et al. 1993, 1994; Billard et al. 1995; Handumrongkul et al. 1998), the resulting transformants produce xylitol if some other carbon source is present to provide a reductant (Hallborn et al. 1991, 1994). When glucose is used as a co-substrate, xylose assimilation and xylitol production are reduced, presumably because of competition for transport (Thestrup and Hahn-Hägerdal 1995). *XYL1* does not limit xylose assimilation in *P. stipitis* (Dahn et al. 1996). To produce ethanol, it is necessary to have a system that can oxidize xylitol to xylulose while reducing acetaldehyde under oxygen-limiting conditions.

Isolation of the first two genes for xylose assimilation from *P. stipitis* led to the initial development of *S. cerevisiae* strains able to metabolize xylose (Kötter et al. 1990; Amore et al. 1991). *S. cerevisiae* transformed with these two genes can use xylose oxidatively and produce xylitol without the addition of a co-metabolizable carbon source. Increasing xylitol dehydrogenase (XDH) activity relative to XR (XR:XDH=0.6) produces less xylitol and more ethanol than when XR is present in greater abundance (Walfridsson et al. 1997). When *XYL2* is strongly expressed, xylulose is secreted—indicating that xylulokinase (XK) activity limits xylose metabolism in these cells (Jin and Jeffries 2003).

Xylulokinase

Much has been reported about *S. cerevisiae* engineered to express XK. Chang and Ho cloned D-xylulokinase from *Pachysolen tannophilus* and *S. cerevisiae* as early as 1988 (Chang and Ho 1988; Deng and Ho 1990) and were the first to report a sequence for the *S. cerevisiae* XK gene in a patent (Ho and Tsao 1993). The complete *S. cerevisiae* gene, *XKS1*, was obtained in the yeast genome project (Rodriguez-Pena et al. 1998). The protein coded for by the original Ho and Tsao sequence has been reported as inactive (Eliasson et al. 2000a). D-xylulokinase activity limits the metabolism of D-xylulose in *S. cerevisiae* (Chang and Ho 1988; Deng and Ho 1990) when *S. cerevisiae* xylulokinase is overexpressed along with *Pichia stipitis XYL1* and *XYL2* in *Saccharomyces* sp. 1400, which is a fusant product of *S. diastaticus* and *S. uvarum* (Moniruzzaman et al. 1997; Ho et al. 1998). *Saccharomyces* sp. 1400 (pLNH33) can ferment a mixture of 53 g glucose l^{-1} and 56 g xylose l^{-1} to give an ethanol concentration of 50 g l^{-1} within 36 h. (Krishnan et al. 1999). This is the highest ethanol yield and fermentation rate from glucose/xylose mixtures reported for a recombinant *Saccharomyces* to date.

S. cerevisiae CEN.PK 113-7A, a strain with a well defined genetic background, has also been developed as a xylose-fermenting yeast. *XYL1*, *XYL2* and *XKS1* were each integrated into the chromosome under the control of the *S. cerevisiae* PGK1 promoter to obtain a stable transformant designated *S. cerevisiae* TMB3001 (Eliasson et al. 2000b)

Table 1 Kinetic parameters for xylose fermentation by yeasts

Species/strain	Description	Conditions	Carbon source	Yield (g g^{-1})			Ethanol productivity (g l^{-1}·h^{-1})	Reference
				Ethanol	Xylitol	Biomass		
Pichia stipitis								
CBS 7126	Wild type	Respiro-fermentative	Xylose (50 g l^{-1})	0.43	0	0.12	0.9	(Du Preez et al. 1986)
FPL-UC7	Parental strain of Shi21	Fermentative	Xylose (80 g l^{-1}) in complex medium	0.38	0	0.17	0.41	(Shi et al. 1999)
FPL-Shi21	Disruption of *PsCYC1*	Fermentative	Xylose (80 g l^{-1}) in complex medium	0.46	0	0.09	0.43	(Shi et al. 1999)
FPL-UC7	Parental strain of Shi31	Respiro-fermentative	Xylose (80 g l^{-1}) in complex medium	0.25	0.03	0.16	0.12	(Shi et al. 2002)
FPL-Shi31	Disruption of *PsAOX1*	Respiro-fermentative	Xylose (80 g l^{-1}) in complex medium	0.31	0.03	0.13	0.15	(Shi et al. 2002)
Saccharomyces cerevisiae								
1400(pLNH32)	*XYL1*, *XYL2*, *XKS1*	Fermentative	Glucose (90 g l^{-1}) and xylose (40 g l^{-1}) in complex medium	0.46	0.17		1.15	(Ho et al. 1998)
1400(pLNH32)	*XYL1*, *XYL2*, *XKS1*	Fermentative	Xylose (50 g l^{-1}) in complex medium	0.3	0.08		0.33	(Ho et al. 1998)
TMB3001	*XYL1*, *XYL2*, *XKS1*	Aerobic batch fermentation	Glucose (50 g l^{-1}) and xylose (50 g l^{-1}) in minimal medium	0.23	0.08	0.034	0.12	(Zaldivar et al. 2002)
A	Industrial strain	Aerobic batch fermentation	Glucose (50 g l^{-1}) and xylose (50 g l^{-1}) in minimal medium	0.42		0.09	0.11	(Zaldivar et al. 2002)
A4	*XYL1*, *XYL2*, *XKS1* in A4	Aerobic batch fermentation	Glucose (50 g l^{-1}) and Xylose (50 g/l^{-1}) in minimal medium	0.27	0.27	0.04	0.13	(Zaldivar et al. 2002)
A6	*XYL1*, *XYL2*, *XKS1* in A4	Aerobic batch fermentation	Glucose (50 g l^{-1}) and xylose (50 g l^{-1}) in minimal medium	0.27	0.32	0.046	0.13	(Zaldivar et al. 2002)
TMB3001	*XYL1*, *XYL2*, *XKS1*	Anaerobic continuous culture	Glucose (20 g l^{-1}) in minimal medium	0.30 (0.41)		0.084	0.38 (0.52)	(Eliasson et al. 2000b)
		Anaerobic continuous culture	Glucose (15 g l^{-1}) and xylose (5 g l^{-1}) in minimal medium	0.30 (0.39)	0.03	0.077	0.31 (0.41)	(Eliasson et al. 2000b)
		Anaerobic continuous culture	Glucose (10 g l^{-1}) and xylose (10 g l^{-1}) in minimal medium	0.29 (0.36)	0.09	0.089	0.21 (0.26)	(Eliasson et al. 2000b)
		Anaerobic continuous culture	Glucose (5 g l^{-1}) and xylose (15 g l^{-1}) in minimal medium	0.26 (0.35)	0.12	0.088	0.11 (0.15)	(Eliasson et al. 2000b)
H1691	*XYL1*, *XYL2*, *XKS1*	Anaerobic batch fermentation	Xylose (50 g/l^{-1}) in minimal medium	0.09	0.41	0	0.03	(Toivari et al. 2001)
		Micro-aerobic batch fermentation	Xylose (50 g l^{-1}) in minimal medium	0.12	0.42	0.03	0.05	(Toivari et al. 2001)
		Aerobic batch fermentation	Xylose (50 g l^{-1}) in minimal medium	0.06	0.06	0.16		(Toivari et al. 2001)
H158	*XYL1*, *XYL2*, *XKS1*	Anaerobic batch culture	Xylose (80 g l^{-1}) in minimal medium	0	0.53		0	(van Zyl et al. 1999)
			Xylose (80 g l^{-1}) in complex medium	0	0.66		0	(van Zyl et al. 1999)
			Xylose (50 g l^{-1}) and glucose (20 g l^{-1}) in complex medium	0.1	0.61		0.07	(van Zyl et al. 1999)
			Xylose (50 g l^{-1}) and raffinose (20 g l^{-1}) in complex medium	0.08	0.67		0.06	(van Zyl et al. 1999)
H158-pXks	*XYL1*, *XYL2*, *XKS1*	Fermentative	Xylose (80 g l^{-1}) in defined medium	0.15	0		0.02	(Johansson et al. 2001)
			Xylose (80 g l^{-1}) in complex medium	0.20	0.03		0.03	(Johansson et al. 2001)
CEN.PK-pXks			Xylose (80 g l^{-1}) in defined medium	0.13	0.25		0.03	(Johansson et al. 2001)

Table 1 (continued)

Species/strain	Description	Conditions	Carbon source	Yield (g g^{-1})			Ethanol productivity (g l^{-1}·h^{-1})	Reference
				Ethanol	Xylitol	Biomass		
			Xylose (80 g l^{-1}) in complex medium	0.17	0.36		0.04	(Johansson et al. 2001)
TMB3101	xylA in CEN.PK2-1C	Anaerobic batch culture	Xylose (50 g l^{-1}) in minimal medium	0	0.57		0	(Träff et al. 2001)
TMB3103	xylA, XKS1	Anaerobic batch culture	Xylose (50 g l^{-1}) in minimal medium	0	0.52		0	(Träff et al. 2001)
TMB3102	xylA with *GRE3* knockout	Anaerobic batch culture	Xylose (50 g l^{-1}) in minimal medium	0.22	0.31		0.15	(Träff et al. 2001)
TMB3104	xylA, *XKS1* with *GRE3* knockout	Anaerobic batch culture	Xylose (50 g l^{-1}) in minimal medium	0	0.31		0	(Träff et al. 2001)
TMB3001	*XYL1*, *XYL2*, *XKS1*	Anaerobic batch culture	Xylose (50 g l^{-1}) in minimal medium	0.31	0.29		0.20	(Jeppsson et al. 2002)
		Anaerobic continuous culture (D=0.06 h^{-1})	Glucose (20 g l^{-1}) and Xylose (20 g/L) in minimal medium	0.30	0.43			(Jeppsson et al. 2002)
		Anaerobic continuous culture (D=0.12 h^{-1})	Glucose (20 g l^{-1}) and xylose (20 g l^{-1}) in minimal medium	0.31	0.37			(Jeppsson et al. 2002)
TMB3255	*ZWF1* knockout in TMB3001	Anaerobic batch culture	Xylose (50 g l^{-1}) in minimal medium	0.41	0.05		0.04	(Jeppsson et al. 2002)
		Anaerobic continuous culture (D=0.06 h^{-1})	Glucose (20 g l^{-1}) and xylose (20 g l^{-1}) in minimal medium	0.39	0.13			(Jeppsson et al. 2002)
		Anaerobic continuous culture (D=0.12 h^{-1})	Glucose (20 g l^{-1}) and xylose (20 g l^{-1}) in minimal medium	0.366	0.131			(Jeppsson et al. 2002)
FPL-YSX3	XYL1, XYL2, XYL3	Aerobic fermentation	Xylose (40 g l^{-1}) in minimal medium	0.09	0.33	0.07	0.05	(Jin et al. 2003)
FPL-YSX3	*XYL1*, *XYL2*, *XYL3*	Oxygen-limited fermentation	Xylose (40 g l^{-1}) in complex medium	0.12	0.55		0.06	(Jin 2002)
FPL-YSX3P	Respiration-deficient mutant of FPL-YSX3	Oxygen-limited fermentation	Xylose (40 g l^{-1}) in complex medium	0.29	0.46		0.11	(Jin 2002)

which, like *S. cerevisiae* sp. 1400, is able to ferment mixtures of glucose and xylose to ethanol, albeit with slightly lower yields (cf. Ho et al. 1999; Krishnan et al. 1999). Overexpression of *XKS1* clearly enhances xylose utilization under aerobic conditions, but xylose utilization declines by almost an order of magnitude with decreased aeration.

Intracellular ATP levels and the ratio of ATP:ADP are also dramatically lower in the recombinant yeast (Toivari et al. 2001). Overexpression of *XKS1*, *XYL1* and *XYL2* increased ethanol yield in two different strains of *S. cerevisiae*, but it also decreased total xylose consumption by 50–80% (Johansson et al. 2001; Table 1).

D-xylulokinase is not expressed significantly in native *S. cerevisiae* (Deng and Ho 1990), but it is essential for xylulose metabolism because *xks1* mutants do not grow on xylulose. At the same time, overexpression of *XKS1* can decrease growth on xylulose (Rodriguez-Pena et al. 1998). Richard et al. (2000) and later Johansson et al. (2001) suggested that overexpression of *XKS* combined with unlimited access to xylulose might lead to toxicity, due to ATP depletion in a manner similar to that observed with unregulated glucose uptake in *Saccharomyces tps1*/*ggs1* mutants (Hohmann et al. 1993, 1996; Thevelein and Hohmann 1995; Teusink et al. 1998). Jin et al. (2002) expressed the *P. stipitis* gene for D-xylulokinase, *XYL3*, in a *S. cerevisiae* strain that was engineered for high levels of *XYL1* and *XYL2* (Jin et al. 2003). The *XYL3* XK gene product is more specific for D-xylulose; and it has much lower activity against D-ribulose than the *S. cerevisiae* XK (Richard et al. 2000). When the *P. stipitis* XK activity is low, cell growth on xylose is not significantly affected. When *P. stipitis* XK is strongly overexpressed, aerobic growth is significantly inhibited on xylose. Aeration increases the toxicity of *XYL3* or *XKS1* overexpression. Xylulokinase activity is higher in cells grown on glucose, but no growth inhibition is observed, which indicates that the inhibitory effect is specific to xylose uptake. Ethanol production and growth are optimal when low levels of *XYL3* are expressed from the native promoter. This suggests that the effect of overexpressing XK when D-xylulose is fully accessible is similar to substrate-accelerated cell death.

Deletion of *XKS1* completely blocks xylitol formation from xylose, but leads to arabitol accumulation and increases ethanol production (Eliasson et al. 2000a). Deletions within the *PGI1* promoter that reduce phosphoglucose isomerase activity by one to two orders of magnitude increase the accumulation of fructose-6-phosphate and result in about 15% higher ethanol yield. *tps1* and *tps2* mutants accumulate sugar phosphates and increase ethanol yield by 20–30%. *gnd1* mutants show 30% higher ethanol yield, but *rpe1* mutants hardly assimilate xylulose at all—as one might expect, because both ribulose-5-phosphate and xylulose-5-phosphate are necessary to form ribose-5-phosphate and the other intermediates of the pentose phosphate pathway (PPP; Fig. 1).

While we can learn a great deal about the mechanics of xylose fermentation by genetically manipulating laboratory strains, commercialization of the xylose fermentation will necessitate the use of strains that can grow vigorously, ferment rapidly and tolerate acids or other inhibitory compounds (Hahn-Hägerdal et al. 2001). Many industrial *Saccharomyces* strains have been developed that have these characteristics. In fermenting a mixture of glucose and xylose (50 g l^{-1} each), two industrial yeast strains expressing the xylose assimilation pathway produced more ethanol and consumed more xylose than an engineered laboratory strain (TMB 3001). However, the differences in ethanol production arose almost entirely

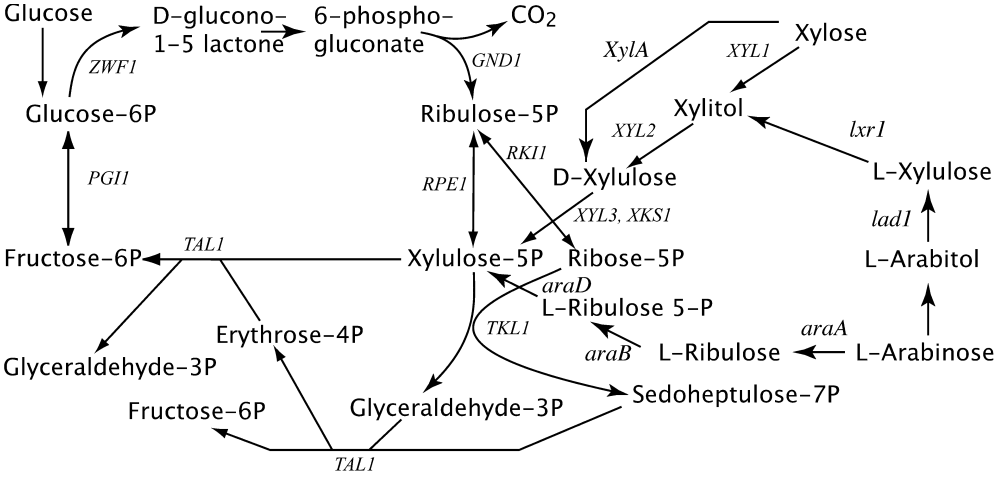

Fig. 1 The pentose phosphate cycle in *Saccharomyces cerevisiae* engineered for xylose and arabinose assimilation. Reduction of L-arabinose to L-arabitol is mediated by aldose reductase. *araA Bacillus subtilis*L-arabinose isomerase, *araB*L-ribulokinase, *araD*L-ribulose-5-phosphate 4-epimerase, *GND1 S. cerevisiae* phosphogluconate dehydrogenase, *lad1 Trichoderma reesei*L-arabinitol 4-dehydrogenase, *lxr1 T. reesei*L-xylulose reductase, *PGI1 S. cerevisiae* glucose-6-phosphate isomerase, *RKI1 S. cerevisiae* ribose-5-phosphate isomerase, *RPE1 S. cerevisiae* ribulose-phosphate 3-epimerase, *TAL1 S. cerevisiae* transaldolase, *TKL1*, *S. cerevisiae* transketolase, *XKS1 S. cerevisiae*D-xylulokinase, *XYL1 Pichia stipitis* xylose (aldose) reductase, *XYL2 P. stipitis* xylitol dehydrogenase, *XYL3*, *P. stipitis*D-xylulokinase, *XylA Piromyces* xylose isomerase, *ZWF1 S. cerevisiae* glucose-6-phosphate 1-dehydrogenase

during glucose consumption rather than during the xylose consumption phase (Zaldivar et al. 2002).

Pentose phosphate pathway

Kötter and Ciracy hypothesized that the excessive production of xylitol by *S. cerevisiae* genetically engineered with *XYL1* and *XYL2* was limited by the dual cofactor capacity of the *P. stipitis* XR, by excessive activity of the oxidative PPP in *S. cerevisiae* and by insufficient capacity of the non-oxidative PPP (Kötter and Ciriacy 1993). Overexpression of the *P. stipitis* gene for transketolase (*TKL1*) in a *S. cerevisiae* strain expressing heterologous *XYL1* and *XYL2* greatly reduced growth of the transformant on xylose minimal medium (Metzger and Hollenberg 1994). Strains overexpressing the *P. stipitis* gene for transaldolase (*TAL1*), *XYL1* and *XYL2* grew faster than strains expressing *XYL1* and *XYL2* alone (Walfridsson et al. 1995). However, the plasmid burden due to the overexpression of *TAL1*, *XYL1* and *XYL2* reduced the growth rate of the transformant relative to the host strain (Bao et al. 1997; Meinander et al. 1999).

Redox balance

The production of xylitol by recombinant *S. cerevisiae* is thought to originate from an overabundance of NADPH relative to NADH for the initial xylose assimilation step. To reduce xylitol production and thereby increase ethanol yield, Jeppson et al. (2002) overexpressed *XKS1* in *S. cerevisiae gnd1* and *zwf1* backgrounds. The Δ*zwf1* mutant greatly increased ethanol yield by producing 0.41 g ethanol g^{-1} xylose consumed, as compared with 0.31 g g^{-1} by the parent strain. The Δ*gnd1* mutant also showed increased ethanol yield to (0.38 g g^{-1}). However, both mutants showed reduced rates of xylose uptake, which indicates that NADPH production is necessary for xylose assimilation in *S. cerevisiae*.

Xylitol production by native xylose-metabolizing yeasts varies a great deal with species and aeration conditions. *Pachysolen tannophilus*, *C. shehatae* and *Pichia stipitis* all produce xylitol to varying extents (Du Preez et al. 1984; Sanchez et al. 2002). *P. stipitis* is notable for its very low xylitol production and high ethanol yields. Ethanol production decreases and xylitol production increases in *P. stipitis* when the primary alcohol dehydrogenase is deleted (Cho and Jeffries 1998). This suggests that ADH competes with XDH for reductant—presumably NADH—in *P. stipitis*. In this organism, the main ADH is induced as oxygen availability decreases (Cho and Jeffries 1999; Passoth et al. 2003).

Lowering the cytosolic NADPH concentration while regenerating NAD^+ from NADH could reduce xylitol production (Richard et al. 2003). This might be accomplished by expressing a cytosolic transhydrogenase. *S. cerevisiae* does not possess transhydrogenase activity (Bruinenberg et al. 1985), so Nissen et al. (1997) expressed a transhydrogenase gene from *Azotobacter vinlandii* in *S. cerevisiae* and then measured the intracellular concentrations of the NAD(P) and NAD(P)H cofactors. The concentrations of the nicotinamide cofactors in the glucose-grown control cells were as follows:

$$\underset{0.23}{NADP^+} + \underset{0.44}{NADH} \longleftrightarrow \underset{1.21}{NADPH} + \underset{2.87}{NAD^+} \quad \mu mol\, g^{-1}\ \text{biomass} \tag{1}$$

Expression of the transhydrogenase increased the production of 2-oxoglutarate and glycerol and shifted the intracellular ratio of $(NADPH/NADP^+):(NADH/NAD^+)$ from 35 to 17 (Nissen et al. 1997). These results indicated that the thermodynamic equilibrium for the transhydrogenase reaction lies in the direction of NADH formation. So it seems unlikely that this approach will be useful in the absence of other energy-consuming reactions.

The XR encoded by *P. stipitis XYL1* has K_m=3.2 μM for NADPH and K_m=40 μM for NADH (Rizzi et al. 1988). The production of NADPH by glucose-6-phosphate dehydrogenase occurs largely on demand in response to the intracellular concentrations of NADPH (Michal 1999). Taken together, these three factors mean that the *P. stipitis* XR will always favor consumption of NADPH over NADH. Native *P. stipitis* does not produce significant amounts of xylitol, whereas recombinant *S. cerevisiae* expressing the *P. stipitis XYL1* produces abundant xylitol, so some factor other than XR must be responsible for enabling cofactor balance in *P. stipitis*.

Respiration

Respiration plays a critical role in the metabolism of xylose by both native and engineered yeasts. The exact nature of the requirement is not fully understood, because oxygen appears to function differently in enabling growth and fermentation. Native xylose-metabolizing yeasts or genetically engineered *S. cerevisiae* (Eliasson et al. 2000b) can metabolize xylose to ethanol in the absence of oxygen, but oxygen is required for yeasts to grow on xylose (Ligthelm et al. 1988), and for optimal ethanol production, low aeration rates are required (Toivari et al. 2001). Anoxia kills *C. shehatae* when it is cultivated on xylose, but not when cultivated on glucose. This suggests that there is a fundamental difference in the oxygen requirements for the metabolism of these two sugars (Kastner et al. 1999).

Disruption of *CYC1* in *P. stipitis* blocks much of its capacity for ATP production and results in a petite colony morphology, even though this is a petite-negative yeast. *P. stipitis* Δ*cyc1* mutants grow slowly on glucose or xylose, but unlike wild-type *P. stipitis*, they do not grow on glycerol or xylitol. Volumetric ethanol production rates of *P. stipitis* Δ*cyc1* mutants on xylose are similar to the

parent, but because cell yields are much lower, the specific fermentation rate is about 50% higher with Δ*cyc1* mutants (Shi et al. 1999). Loss of *CYC1* restricts cell growth but respiration capacity remains high because *P. stipitis* also possesses an alternative salicylhydroxamic acid-sensitive terminal oxidase, *STO1* (Shi et al. 2002). Disruption of *STO1* increases ethanol production from xylose but does not affect cell growth, which indicates that electron transfer to Sto1 does not generate ATP.

Anaerobiosis

One of the biggest challenges for commercialization of the yeast xylose fermentation is obtaining growth on xylose under anaerobic conditions. The cost of aerating bioreactors for ethanol production is prohibitive by current practice. As was first noted by Wang and Schneider in 1980, *S. cerevisiae* and other yeasts can grow on xylulose under aerobic conditions (Wang and Schneider 1980), but even though they can ferment xylulose, they do not grow on this sugar under anaerobic conditions (Maleszka and Schneider 1984). Mitochondrial function appears to be necessary for the growth of yeasts on xylose or xylulose. Ethanol production from xylose, xylitol or xylulose is enhanced by aeration in most yeasts (Maleszka and Schneider 1982); and in some species, such as *C. tropicalis*, aeration is essential for ethanol production (Jeffries 1981).

Eliasson et al. (2000b) were the first to report the anaerobic production of ethanol from xylose by recombinant *S. cerevisiae* TMB3001. However, this yeast grew on xylose only in the presence of oxygen; and glucose was included in all fermentations to enable continuous cultivation. Xylose was co-utilized with glucose under anaerobic conditions. Xylose uptake varied inversely with its concentration, but even at the highest xylose concentration, (15 g xylose l^{-1}, 5 g glucose l^{-1}) only 12% of the xylose was consumed. The anaerobic ethanol yield on xylose was estimated at 0.21 g g^{-1}, assuming a constant ethanol yield on glucose. Glucose appears to be required for the anaerobic metabolism of xylose, because these authors were not able to maintain a steady state when cultures were grown on xylose alone (Eliasson et al. 2000b).

To overcome this limitation, Sonderegger and Sauer (2003) maintained *S. cerevisiae* TMB3001 in continuous culture under progressively more restrictive oxygen limitations. Starting from continuous aerobic cultivation on a mixture of xylose and glucose and progressing to anaerobic cultivation on xylose alone, the authors obtained two cell populations. Clones taken from the larger population grew anaerobically on xylose but showed impaired growth on glucose. Clones taken from the smaller population were incapable of anaerobic growth but produced more ethanol from xylose than the parental strain.

In a separate research effort, Wahlbom et al. (2003) also used continuous cultivation under aerobic, oxygen-limited and anaerobic conditions to obtain improved mutants of recombinant *S. cerevisiae* with higher capacities for xylose fermentation. However, even the best of these mutants showed only about one-third of the aerobic maximum growth rate and two-thirds of the ethanol productivity obtained with *P. stipitis* CBS 6054 on xylose (Wahlbom et al. 2003).

C. shehatae can use glucose and xylose simultaneously in a chemostat, but aeration is required for cell growth (Kastner et al. 1998). *C. shehatae* cells, cultivated aerobically on D-glucose and D-xylose, undergo one doubling or less following a shift to anoxia. Cell viability declines nine times faster in D-xylose than in D-glucose fermentations. Anaerobic growth does not occur on either D-glucose or D-xylose (Kastner et al. 1999).

Heterologous expression of *S. cerevisiae URA1* in *P. stipitis* was reported to confer anaerobic growth, but this apparently required an uncharacterized mutational event in the host cell background, because transformed cells required more than 100 h before initial growth was noted (Shi and Jeffries 1998).

For commercial purposes, it may be possible to cultivate either native or engineered yeasts anaerobically on glucose followed by a respiro-fermentative phase on the residual xylose. Glucose and xylose are almost always obtained as mixtures from lignocellulose hydrolysates, so by properly engineering cell recycle loops, it should be possible to obtain high-yield conversions under anaerobic or oxygen-limited conditions.

Metabolite flux and transcriptome profiling

Metabolic flux analysis and flux estimates based on ^{13}C-labeling experiments (Christensen et al. 2002) have been used to estimate metabolite levels of *S. cerevisiae* and *P. stipitis* grown on glucose and xylose. Intracellular metabolite levels are higher in industrial yeast strains than in laboratory strains engineered for xylose utilization (Zaldivar et al. 2002). *P. stipitis* derives at least 58% of its phospho-enol-pyruvate (PEP) through the non-oxidative PPP, whereas *S. cerevisiae* uses the non-oxidative PPP for the biosynthesis of less than 4% of its PEP (Fiaux et al. 2003). A flux balance analysis (FBA) showed that the maximum ethanol yield from xylose in yeast is 0.46 g g^{-1} rather than 0.51 g g^{-1}, because of the cofactor difference between XR and XDH. Metabolic FBA also predicted that there is an optimal aeration level for ethanol production and that xylitol accumulation decreases with higher aeration. Both of these predictions have been confirmed by measuring product yields at various aeration rates (Jin 2002).

Transcriptome profiling methods, such as RT-PCR and microarray experiments, were applied to monitoring the differential expression of genes between glucose and xylose fermentation by recombinant *S. cerevisiae*. Of the 5,944 genes detected under oxygen-limited culture conditions, 386 (6.6%) showed differential expression when cells were grown on xylose, as compared with glucose. As

expected, most of those genes fell in the energy-production category. Expression levels of genes coding for glycolytic, fermentative and pentose phosphate enzymes did not change greatly. However, expression of the genes encoding tricarboxylic acid cycle and respiratory enzymes greatly increased when cells were grown on xylose (Jin 2002).

Protoplast fusion

Protoplast fusion is widely used to improve the fermentative properties of industrial yeasts. By complementing multiple auxotrophic markers, it is possible to obtain stable hybrids between closely related species. In an attempt to increase yeast strains with ethanol tolerance and the ability to ferment xylose, *S. cerevisiae* has been fused with auxotrophic strains of *C. shehatae* or *P. stipitis* (Gupthar 1992), but mononucleate fusants quickly segregate into their parental type strains (Yoon et al. 1996). Other researchers report fusants between *S. cerevisiae* and *P. stipitis* that show the capacity for xylose fermentation and an ability to ferment glucose in the presence of 6% ethanol (Kordowska-Wiater and Targonski 2001). Given the instability of the hybrids, this will probably not lead to commercial yeast strains.

Xylitol production

Native xylose-metabolizing yeasts, such as *Pachysolen tannophilus*, *C. shehatae*, (Du Preez et al. 1984), *C. boidinii* (Vandeska et al. 1996; Winkelhausen et al. 1996), *Hansenula polymorpha* (Sanchez et al. 1998) and *C. guillermondii* (Rodrigues et al. 2002), all produce xylitol to greater or lesser degrees depending on the pH, oxygen availability and other culture conditions. *Pichia stipitis* is notable because it produces relatively little xylitol (Sanchez et al. 2002). When its genes for Adh are disrupted, xylitol production increases dramatically (Cho and Jeffries 1998); and when the D-xylulokinase gene is disrupted, *P. stipitis* produces a mixture of xylitol and arabitol (Jin et al. 2002). *S. cerevisiae* cells engineered for xylose utilization tend to produce xylitol (Kötter and Ciriacy 1993; Hallborn et al. 1994; Tantirungkij et al. 1994; Walfridsson et al. 1995; Eliasson et al. 2000b). If many copies of *XYL1* are integrated into the genome, *S. cerevisiae* transformants are stable and can produce xylitol in sequential batch or continuous culture (Kim et al. 1999). By increasing the expression of *XYL2* relative to *XYL1*, it is possible to decrease xylitol secretion (Jin et al. 2003).

Arabinose utilization

The utilization of L-arabinose is particularly important in the conversion of corn hulls to ethanol (Saha et al. 1998). Corn fiber consists of about 20% starch, 14% cellulose and 35% hemicellulose; and L-arabinose makes up approximately 28% of the hemicellulosic fraction (Park et al. 2001). An extensive screen of 116 yeasts that can grow on L-arabinose showed that four strains, *C. auringiensis*, *C. succiphila*, *Ambrosiozyma monospora* and *Candida* sp. (YB-2248) could produce some ethanol (4.1 g l^{-1} or less) directly from arabinose (Dien et al. 1996). While the production rates were very low, these studies showed for the first time that yeasts can directly convert L-arabinose to ethanol (Fig. 2).

The pathways for L-arabinose and D-arabinose metabolism are distinct in yeasts. *P. stipitis* will grow very

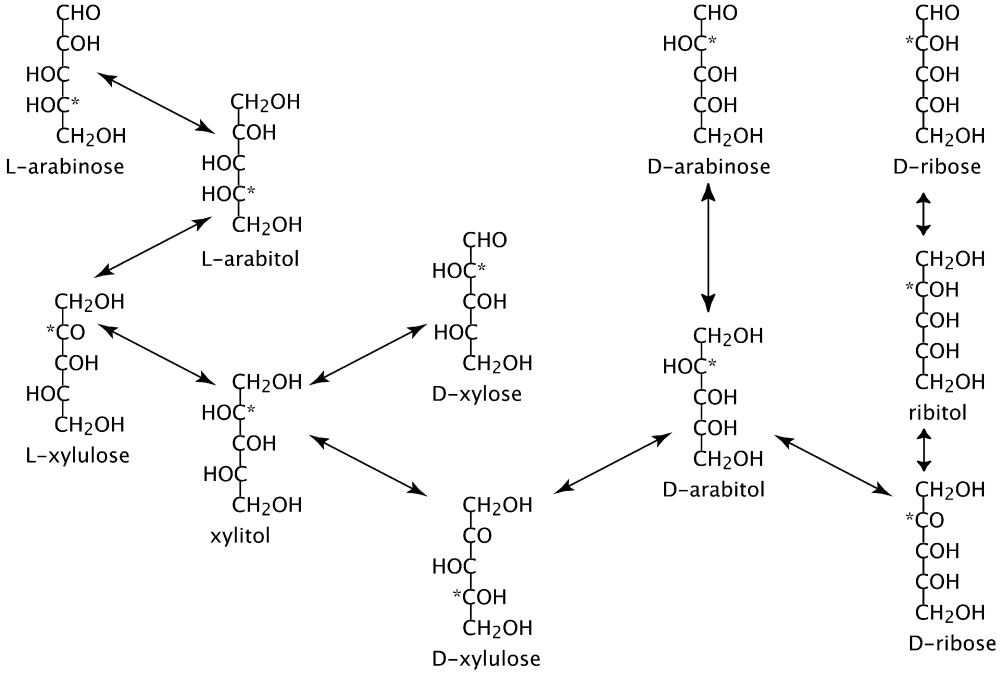

Fig. 2 Interconversion of pentoses and pentitols by NAD (PH)-mediated oxidoreductases

slowly on L-arabinose, but it does not ferment this sugar. A mutant of *P. stipitis* that was unable to metabolize L-arabinose could grow on D-arabinose (Shi et al. 2000). Complementation of this mutant with *XYL2* restored growth on L-arabinose. This showed that the pathway used by *P. stipitis* for L-arabinose metabolism is similar to that used by *Aspergillus niger* (Witteveen et al. 1989).

Relatively little is known about L-arabinose uptake by yeasts. In *C. shehatae*, it appears to be mediated by proton symport (Lucas and van Uden 1986). L-Arabinose is similar in structure to D-galactose (Rees 1977) and its transport in *S. cerevisiae* is mediated by *GAL2* (Kou et al. 1970). In *Kluyveromyces lactis*, the *LAC12* gene codes for an inducible lactose permease that is similar in structure to the *Escherichia coli* xylose-H^+ and arabinose-H^+ transporters (Chang and Dickson 1988). D-Arabinitol dehydrogenase (Hallborn et al. 1995) functions in an alternative pathway that connects D-xylulose to D-ribulose (Jin et al. 2002).

Fungi metabolize L-arabinose through five enzymes, aldose (xylose) reductase, L-arabinitol 4-dehydrogenase (*lad1*; Richard et al. 2001), L-xylulose reductase (*lxr1*; Richard et al. 2002), xylitol dehydrogenase and D-xylulokinase. Overexpression of *lad1* and *lxr1* along with *XYL1*, *XYL2* and *XKS1* enabled *S. cerevisiae* to grow on and ferment L-arabinose (Richard et al. 2003). Ethanol production occurred at a very low rate. About 0.1 g of ethanol was formed by 4 g of cells in 70 h under anaerobic conditions. Under aerobic conditions, the ethanol formed from L-arabinose would probably be re-assimilated.

In a different approach to engineer *S. cerevisiae* for L-arabinose fermentation, Sedlak and Ho (2001) expressed three genes of the araBAD operon from *E. coli* in *S. cerevisiae*. They reported activity with all three enzymes, but the transformant did not produce ethanol from L-arabinose (Sedlak and Ho 2001). Becker and Boles (2003) were more successful in this approach. Unlike Sedlak and Ho, they were not able to obtain activity through the heterologous expression of *E. coli araA*, which codes for L-arabinose isomerase, but they were able to express the *araA* gene from *Bacillus subtilis* and this—along with the heterologous expression of *E. coli araB* and *araD* plus overexpression of *S. cerevisiae GAL2*—gave rise to a yeast strain that could grow slowly on L-arabinose. After more than 200 h of cultivation, a transformant arose that could grow on L-arabinose, with a doubling time of about 8 h. They were able to identify two mutational events—one in the bacterial L-ribulokinase that reduced affinity for L-ribulose and one in the yeast genome that increased transaldolase expression. Together, these enabled growth on and fermentation of L-arabinose. The resulting strain produced up to 0.08 g ethanol g^{-1} biomass h^{-1}. This represents a major breakthrough in the metabolic engineering of arabinose metabolism in yeast.

Cellulase and xylanase expression

Native strains of *P. stipitis* produce xylanases (Ozcan et al. 1991) that enable the fermentation of xylan directly to ethanol, but the yields are very low (Lee et al. 1986). By increasing xylanase production either through mutation (Basaran et al. 2000) or heterologous expression (Morosoli et al. 1993; Den Haan and Van Zyl 2001), it is possible to enhance the xylan fermentation rate. Yeast β-xylosidase is probably most important for the fermentation of xylobiose and xylotriose, because these are the most conspicuous products of endoxylanase xylanase activity and they are also formed during the acid hydrolysis of xylan. Several researchers have heterologously expressed xylanases in *S. cerevisiae* (Den Haan and Van Zyl 2001; La Grange et al. 2001).

Spent sulfite and hydrolysate fermentation

In the final analysis, the ability of an organism to ferment sugars in hemicellulosic hydrolysates determines the success of metabolic engineering efforts. Acetic acid and toxic phenolic products from lignocellulose can inhibit growth of yeasts in hydrolysates. However, post-hydrolysis treatments can reduce toxicity and strains can be selected for resistance. The most cost-effective hydrolysate treatment—calcium over-liming—is also one of the oldest. Over-liming is used to prepare sulfite waste liquors (Nigam 2001b) and acid hydrolysates of hardwood (Nigam 2001a). Unfortunately, acid hydrolysis and over-liming produce large amounts of calcium sulfate that must be removed. The toxicity of hydrolysates can be significantly reduced by adding laccase to polymerize the free phenolic compounds (Jönsson et al. 1998). A laccase cloned from *Trametes versicolor* has been expressed at a high level in *S. cerevisiae* (Larsson et al. 2001). *S. cerevisiae* TMB 3001 grew better in laccase-treated hydrolysate than in untreated hydrolysate, but it showed almost no utilization of xylose. In a comparison of *P. stipitis* with *C. shehatae*, the latter yeast was better able to ferment hydrolysates (Sreenath and Jeffries 2000). In simultaneous saccharification and fermentation, yields of 0.47 g g^{-1} substrate were obtained (Sreenath et al. 1999, 2001). Wild-type strains of *C. shehatae* can ferment rice straw autohydrolysates to ethanol with yields of 0.45 g ethanol g^{-1} sugar consumed and they can produce 0.37 g g^{-1} from acid prehydrolysates (Abbi et al. 1996). The ethanol productivity of *P. stipitis* in wood hydrolysates can be improved by up to 2-fold by selecting for resistant strains (Nigam 2001a).

Prospects for future progress

Metabolic engineering enables *S. cerevisiae* to ferment D-xylose and L-arabinose to ethanol and it improves the capacity of native xylose-fermenting yeasts, such as *P. stipitis*. The improvement obtained with any one change

has been incremental. Co-production of xylitol, low ethanol production rates, requirements for oxygen and co-metabolizable carbon sources remain problems with recombinant *S. cerevisiae*. Most trials use haploid laboratory strains rather than industrial yeasts for their genetic backgrounds and trials with mixed sugar hydrolysates are not often reported. Mutagenesis and strain selection has improved xylose utilization in recombinant strains, but most mutations have not been characterized. In some instances, multiple genes have been altered through deletion or overexpression, but rarely have expression levels been manipulated. There are, therefore, many opportunities to obtain further improvements by learning more about the factors that limit xylose utilization under anaerobic conditions, by selecting better genetic backgrounds for heterologous expression, by expressing multiple genes at optimal levels and by combining the various beneficial traits into single strains. While strain improvement will probably continue for several years, ethanol production rates and yields are becoming practicable for some commercial applications.

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