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Engineering yeasts for xylose metabolism

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Technologies for the production of alternative fuels are receiving increased attention owing to concerns over the rising cost of petrol and global warming. One such technology under development is the use of yeasts for the commercial fermentation of xylose to ethanol. Several approaches have been employed to engineer xylose metabolism. These involve modeling, flux analysis, and expression analysis followed by the targeted deletion or altered expression of key genes. Expression analysis is increasingly being used to target rate-limiting steps. Quantitative metabolic models have also proved extremely useful: they can be calculated from stoichiometric balances or inferred from the labeling of intermediate metabolites. The recent determination of the genome sequence for *P. stipitis* is important, as its genome characteristics and regulatory patterns could serve as guides for further development in this natural xylose-fermenting yeast or in engineered *Saccharomyces cerevisiae*. Lastly, strain selection through mutagenesis, adaptive evolution or from nature can also be employed to further improve activity.

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Current Opinion in Biotechnology 2006, **17**:320–326

This review comes from a themed issue on
Energy biotechnology
Edited by Jonathan R Mielenz

Available online 18th May 2006

0958-1669/$ – see front matter
Published by Elsevier Ltd.

DOI 10.1016/j.copbio.2006.05.008

Introduction

Rising concerns over the cost of petroleum and the prospect of global warming are driving the development of technologies for the production of alternative fuels such as ethanol [1]. The long-term source of ethanol is from plant matter (biomass) through the fermentation of biomass carbohydrates to ethanol. The conversion of biomass to useable energy is not economical, however, unless hemicellulose is used in addition to the cellulose [2]. Xylose is the second most abundant carbohydrate in nature and its commercial fermentation to ethanol could provide an alternative fuel source for the future.

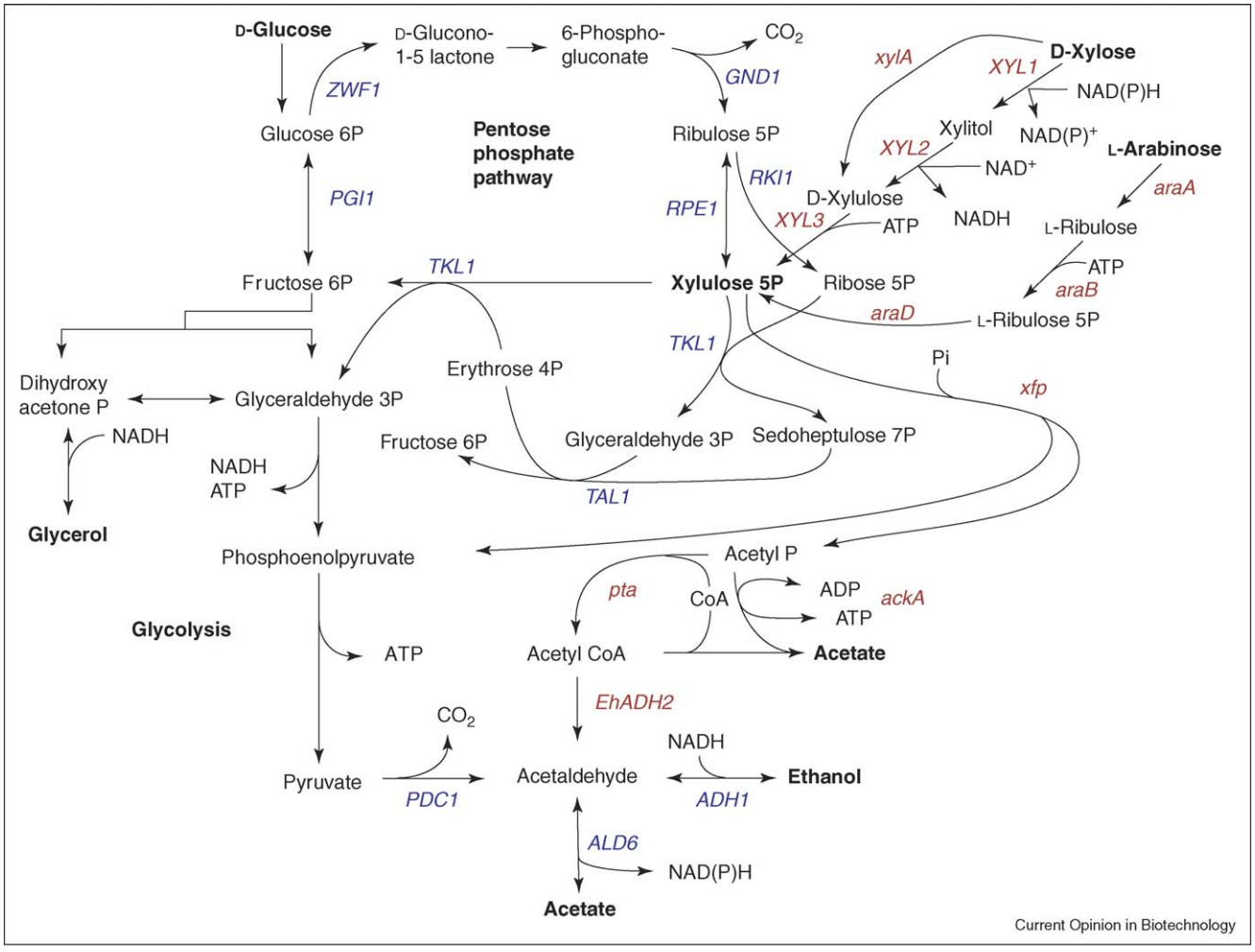
Microbes such as yeasts and bacteria are essential for the fermentation of xylose [3,4]. The larger sizes, thicker cell walls, better growth at low pH, less stringent nutritional requirements, and greater resistance to contamination give yeasts advantages over bacteria for commercial fermentations. *Saccharomyces cerevisiae* does not naturally use xylose as a substrate, however, and must be engineered to both transport and ferment xylose. Engineering can also improve the fermentative activities of some native xylose-metabolizing yeasts such as *Pichia stipitis*. A number of different approaches have been used to engineer yeasts for this purpose, including modeling, flux analysis and expression analysis followed by the targeted deletion or altered expression of key genes. In this review we consider some of the different approaches used to engineer yeast for xylose metabolism and discuss recent advances in this area.

The pentose phosphate pathway (PPP), which is the biochemical route for xylose metabolism, is found in virtually all cellular organisms where it provides D-ribose for nucleic acid biosynthesis, D-erythrose 4-phosphate for the synthesis of aromatic amino acids and NADPH for anabolic reactions. The PPP is thought of as having two phases. The oxidative phase converts the hexose, D-glucose 6P, into the pentose, D-ribulose 5P, plus CO_2 and NADPH. The non-oxidative phase converts D-ribulose 5P into D-ribose 5P, D-xylulose 5P, D-sedoheptulose 7P, D-erythrose 4P, D-fructose 6P and D-glyceraldehyde 3P. D-Xylose and L-arabinose enter the PPP through D-xylulose (Figure 1). In bacteria the conversion of D-xylose to D-xylulose goes by way of xylose isomerase (*xylA*). In yeasts, filamentous fungi and other eukaryotes, this proceeds via a two-step reduction and oxidation, which are mediated by xylose reductase (*XYL1*, Xyl1p) and xylitol dehydrogenase (*XYL2*, Xyl2p), respectively. The cofactor requirements of these two reactions affect cellular demands for oxygen, as explained in the text.

Modeling

Metabolic engineering is most effective when guided by systematic biochemical models to integrate the intrinsic variables and extrinsic changes. Models basically consist of biochemical pathways and information about cell physiology. In one example, models were used to identify the reactions that could be targeted to alleviate the problems associated with excess NADH accumulation during growth on xylose. Recombinant *S. cerevisiae* engineered to express the xylose reductase (*PsXYL1*) and xylitol dehydrogenase (*PsXYL2*) from *P. stipitis* has very low growth and fermentation rates on xylose. This is due in part to cofactor and metabolite imbalances. Xyl1p has a higher affinity for NADPH than for NADH, whereas

Figure 1

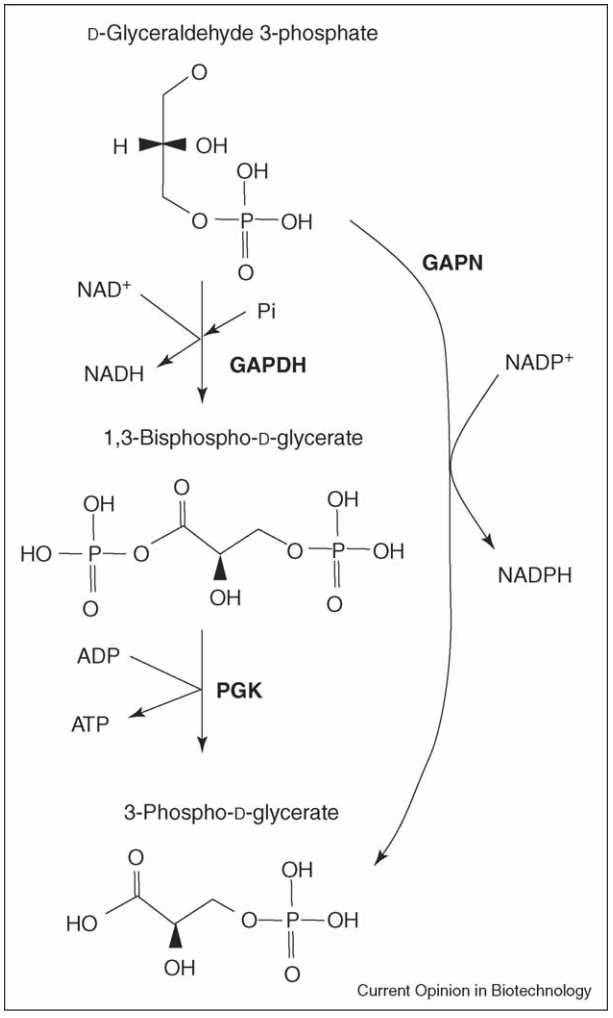


Engineering pentose metabolism in yeasts. The pentose phosphate pathway (PPP) in yeasts consists of the oxidative phase, which consists of glucose 6-phosphate dehydrogenase (*ZWF1*) and 6-phosphogluconate dehydrogenase (*GND1*), and the non-oxidative phase, which is carried out by D-ribulose-5-phosphate 3-epimerase (*RPE1*), ribose-5-phosphate ketol-isomerase (*RKI1*), transketolase (*TKL1*) and transaldolase (*TAL1*). Phosphoglycerate isomerase (*PGI1*) completes the cycle. The PPP has been engineered with the heterologous expression of D-xylose reductase (*XYL1*), xylitol dehydrogenase (*XYL2*), D-xylulokinase (*XYL3*), xylose isomerase (*xylA*), L-arabinose isomerase (*araA*), L-ribulokinase (*araB*), and L-ribulose-5-phosphate 4-epimerase (*araD*) for the assimilation of D-xylose and L-arabinose. Phosphoketolase (*xfp*) provides a bypass to the PPP, which incorporates inorganic phosphate into xylulose-5-phosphate, resulting in the production of acetyl phosphate and phosphoenolpyruvate. The former can be converted into acetate and ATP via acetate kinase (*ackA*). Alternatively, it can be converted into ethanol via phosphotransacetylase (*pta*), acetaldehyde dehydrogenase (*adhE*) and alcohol dehydrogenase (*ADH1*). Normal glycolysis also produces acetaldehyde via pyruvate decarboxylase (*PDC1*). Acetaldehyde is oxidized to acetate and NADPH by aldehyde dehydrogenase (*ALD6*). Yeast genes are shown in italic caps; bacterial genes in lower case italics.

Xyl2p uses only NAD^+, which leads to an excess accumulation of NADH that cannot be recycled through respiration under oxygen-limited conditions. *S. cerevisiae* normally compensates by producing glycerol, but during the assimilation of xylose this is not sufficient and xylitol accumulates. Xylose assimilation increases with aeration [5], which can relieve the accumulation of NADH [6].

Metabolic models guided Grotkjaer *et al.* [6] to delete the gene encoding $NADP^+$-dependent glutamate dehydrogenase (*GDH1*) and to overexpress NAD^+-dependent *GDH2* to shift reductant demand. This increased specific Xyl1p activity with NADH, and boosted ethanol production by 25%. However, this approach requires cell growth [7], and the presence of amino acids in the medium can repress the function of this enzyme. Bro *et al.* [8••] used a genome-scale metabolic flux model to minimize the production of glycerol and to maximize ethanol yield. Of the 3500 metabolic reactions tested in their model, the authors identified 56 that improved both growth and ethanol yield. One of the best strategies was the heterologous expression of NADP-dependent non-phosphorylating

Figure 2



Bypassing glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and phosphoglycerate kinase (PGK) with NADP-dependent non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase (GAPN) results in reduced production of NADH and ATP.

glyceraldehyde 3-phosphate dehydrogenase (GAPN), which converts 3-phosphoglyceraldehyde into 3-phosphoglycerate with the concomitant conversion of $NADP^+$ to NADPH. This bypasses phosphoglycerate kinase (PGK1), which reduces the ATP yield, but also increases cell yield by increasing the supply of NADPH (Figure 2). *S. cerevisiae* engineered in this manner produced 50% less glycerol and 3% more ethanol than the control strain.

Flux analysis

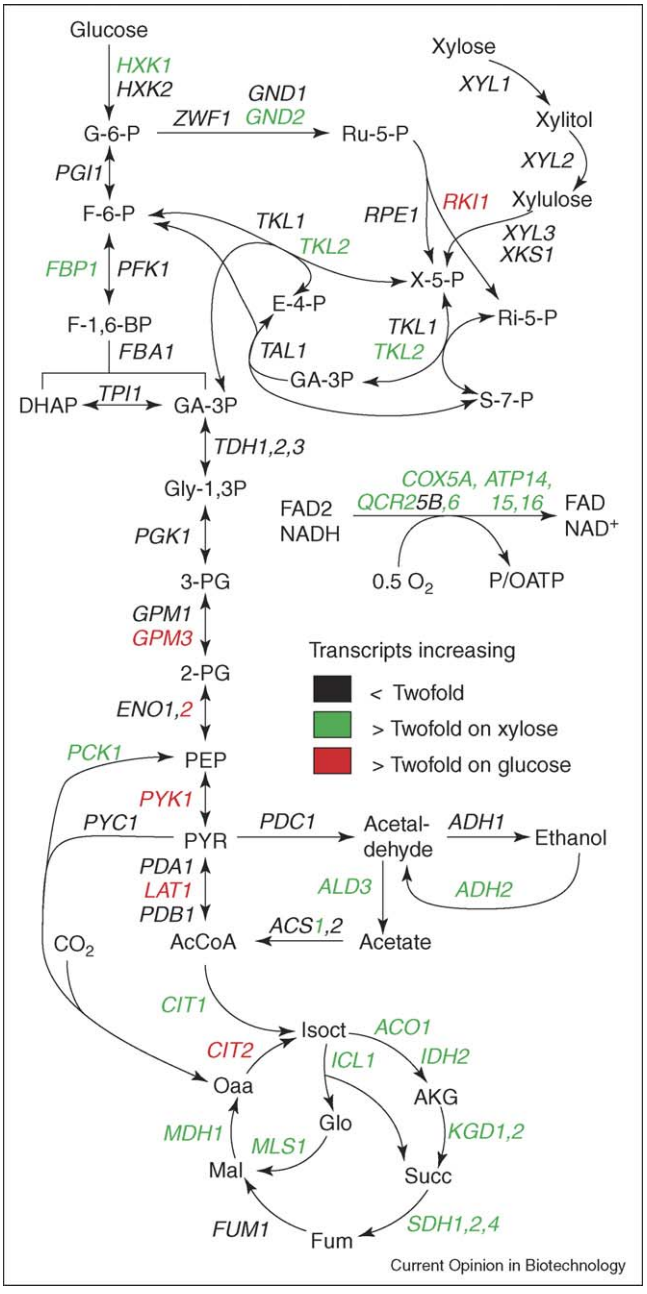
Flux balances can be used to calculate the partitioning of metabolites among the various pathways using stoichiometric metabolic matrices based on known reactions. Flux balances show the fraction of each metabolite flowing into various branches through the pathway and the rates at each branch point are derived from the overall rates of substrate uptake, cell growth and product formation [9]. Fractional ^{13}C labeling has been employed to measure the steady-state concentrations of intermediate metabolites by analyzing the distribution of ^{13}C label in the cellular amino acids [10]. A model of engineered *S. cerevisiae* central metabolism accurately predicted that ethanol yields would increase with aeration up to a maximum value, and that ethanol yield would increase with a decreasing phosphate/oxygen (P/O) ratio [5]. In experiments to test the model, however, xylitol production did not fall to zero as ethanol production attained its peak. A petite mutant generated from the engineered *S. cerevisiae* strain YSX3 showed significantly higher ethanol production from xylose but could not grow on it, adding evidence to support the crucial role of respiration in the metabolism of xylose [11••]. Petite mutants are yeasts deficient in mitochondrial functions. They typically form small colonies because their diminished respiration limits its formation of ATP. *P. stipitis* uses the non-oxidative PPP to produce more than half of its phosphoenolpyruvate (PEP), whereas production of PEP from pentoses is not observed in *S. cerevisiae* by ^{13}C flux analysis [12•]. This confirms the dependence of *S. cerevisiae* on glycolysis, whereas *P. stipitis* cycles a large fraction of glucose through the PPP. The metabolism of *P. stipitis* in batch culture was fully respirative, whereas *S. cerevisiae* was respiro-fermentative.

Expression analysis

The main challenge of global expression analysis using microarray technologies is analyzing the results. *S. cerevisiae* YSX3 grown on glucose or xylose under aerobic or oxygen-limited conditions showed significant changes in levels of more than 600 transcripts [13••]. Following gene classification by function [14], changes were most apparent in the 165 genes associated with energy production. Transcripts associated with the tricarboxylic acid (TCA) cycle and respiration were often at their highest levels during growth on xylose under oxygen-limited conditions (Figure 3). This observation suggested the presence of a signal mechanism that senses intracellular redox balance.

Under carbon-limited conditions in continuous culture, *S. cerevisiae* TMB3001 engineered for xylose metabolism evolved into a population exhibiting faster growth on D-xylose [15••]. One isolate (C1) was capable of slow anaerobic growth on D-xylose (growth rate of 0.012 h^{-1}). Global gene expression analysis revealed greatly increased transcript levels for a series of *GAL* genes. The GAL genes are involved in glucose-repressible galactose metabolism, and their gratuitous induction indicates the probable loss of glucose repression [16]. Other glucose-repressed transcripts such as *PYK2*, *HXK2* and *HXT16* were also elevated in the mutant. Less than 4% of the xylose entered the PPP in the TMB3001 parent, but about 20% entered the PPP in the C1 mutant.

Figure 3



Changes in transcript levels of *S. cerevisiae* YSX3 grown on glucose or xylose under aerobic or oxygen-limited conditions. Gene names follow the standard yeast convention. Red indicates a greater than twofold relative increase on glucose; green indicates greater than twofold increase on xylose [13••].

Xylose transport

S. cerevisiae takes up xylose through its glucose transporters even though their affinity for this sugar is very low. Moreover, competition with glucose restricts xylose assimilation [17], so heterologous expression of a specific xylose transporter could be very useful. Nobre and Lucas deposited the sequence of a putative xylose permease from *Debaryomyces hansenii* in the Protein Data Bank (PDB) in 2003, but it was not characterized. *Candida intermedia* possesses glucose-repressible, high-affinity and constitutive facilitated diffusion xylose uptake systems [18], which suggests the presence of specific xylose transporters [19]. Recently, Leandro *et al.* [20••] cloned and characterized two transporters from this yeast. One of these transporters, *GXF1*, is a glucose/xylose facilitator with a K_m for xylose of approximately 0.4 mM; the other, *GXS1*, is a glucose/xylose symporter. Because sugar uptake via proton symport requires more energy than facilitated diffusion, the facilitator protein would probably be more efficient under oxygen-limited or anaerobic conditions where ATP production is restricted.

Other approaches to alleviate redox imbalance

To alleviate redox imbalance, Jeppsson *et al.* [21•] expressed a mutated Xyl1p that has a higher K_m for NADPH than the wild-type enzyme. This increased ethanol yield to 0.4 g/g xylose and decreased xylitol production. The endogenous xylose reductase of *S. cerevisiae* (GRE3) increased xylitol production from xylose in the presence of Xyl1p; in the absence of Xyl1p it increased xylose uptake [22]. Watanabe *et al.* [23•] increased the thermostability and changed the coenzyme specificity of Xyl1p from NAD^+ to $NADP^+$ by introducing a zinc-binding site.

Verho *et al.* [24••] showed that *Ambrosiozyma monospora* ferments L-arabinose to ethanol through a series of oxidoreductase reactions that form L-arabinitol, L-xylulose, xylitol and D-xylulose. In most fungi, L-xylulose reductase is a strictly NADPH-dependent enzyme, but in *A. monospora* this step is NADH-dependent. The net effect of this is to reduce NADH accumulation.

S. cerevisiae could produce xylitol and trace amounts of ethanol when *GRE3* and the gene encoding an endogenous sorbitol dehydrogenase, *SOR1*, were overexpressed, but cells grew faster and accumulated less xylitol with the *P. stipitis* genes [25].

Evolutionary adaptation and anaerobic growth

The anaerobic rumen fungus *Piromyces* sp. E2 reportedly uses xylose isomerase (XylA) for the assimilation of xylose [26]. The cloned gene from *Piromyces* sp. ATCC 76762 shows very close identity to *xylA* of the intestinal bacteria *Bacteroides thetaiotamicron* and *Bacteroides fragilis*. When the clone was expressed in *S. cerevisiae*, transformants grew and produced ethanol slowly (specific growth rate 0.005 h^{-1}). After strain selection and engineering the growth rate on xylose increased to 0.09 h^{-1}, and following selective pressure under anaerobic continuous cultivation on xylose a strain capable of consuming 0.9 g xylose/g cells;h^{-1} was obtained [27,28,29••].

Karhumaa *et al.* [30••] have described the evolutionary adaptation of an *S. cerevisiae* strain (TMB3050) transformed with *xylA* from *Thermus thermophilus*. Four genes encoding enzymes of the non-oxidative phase of the PPP were also overexpressed in the same strain. Following continuous anaerobic cultivation, the adapted strain exhibited anaerobic growth on xylose. The authors showed that *xylA* is essential for the growth of *S. cerevisiae* on xylose, as it increases the conversion of xylose to xylulose, which can then be used in the downstream metabolic steps. *S. cerevisiae* CEN.PK2 *gre3*Δ transformed with *T. thermophilus xylA* was able to grow on xylose, whereas the same cells without *xylA* could not. Growth on xylose was limited by XylA activity, because replacing *xylA* with *P. stipitis XYL1* and *XYL2* increased the aerobic growth rate on xylose fourfold [30••].

Evolutionary adaptation of *S. cerevisiae* TMB3001 by continuous culture on xylose with glucose or galactose under microaerobic conditions enriched for two mutant subpopulations. Clone C1 used glucose and xylose simultaneously, but glucose utilization was about 50% slower than for the parent [31••]. Comparison of several engineered industrial strains of *S. cerevisiae* showed that the mutagenized, selected and evolved polyploid strain TMB3400 produced the most ethanol [32•,33]. Small amounts of glucose must be included in the medium to enable the emergence of faster growing mutants [34].

Several research groups have tried to use expression analysis, enzymatic assays and flux balance analysis to identify enzymes for anaerobic growth and rapid fermentation on xylose [12•,13••,15••,34]; however, the multiple changes observed have proven difficult to interpret.

Other PPP enzymes

Jin *et al.* [35] transformed a *P. stipitis* gene library into a recombinant *S. cerevisiae* strain carrying *P. stipitis XYL1* and *XYL2*. Of the 16 transformants recovered that grew on xylose, 10 carried vectors with *XYL3*, thereby showing that this activity is essential for xylose utilisation. A second round of transformation obtained 15 fast-growing transformants, all of which carried *P. stipitis TAL1*. Unlike overexpression of *S. cerevisiae TAL1*, *P. stipitis TAL1* did not cause growth inhibition on glucose.

Jin [36] recently showed that when *P. stipitis XYL3* is deleted, the resulting strain will still grow on xylose. This indicates the presence of a bypass reaction in this cell, but the *xyl3* mutant mainly produces xylitol. Unlike *S. cerevisiae*, *P. stipitis* induces respiro-fermentative metabolism largely in response to oxygen limitation [37].

Phosphoketolases that convert D-xylulose 5-phosphate into acetyl phosphate and glyceraldehyde 3-phosphate are found in pentose-metabolizing lactobacilli and a few yeasts [38] (Figure 1). Sondregger *et al.* [39••] engineered the expression of phosphoketolase (*xfp*) from *Bifidobacterium lactis* in *S. cerevisiae* under the control of the *HXT7* constitutive promoter. The resulting strains attained 30% lower biomass yields during growth on glucose, lower specific rates of xylose uptake, lower formation of xylitol and markedly higher accumulation of acetate. When genes for phosphotransacetylase (*pta*) and the bifunctional *Entamoeba histolitica ADH2* [40] were overexpressed together with *xfp*, ethanol production increased 25% — mainly at the expense of xylitol accumulation. When the gene encoding acetaldehyde dehydrogenase (*ALD6*) was deleted, a strain with 20% higher ethanol production and a 40% higher fermentation rate was obtained [39••]. Acetate kinase (AckA) is essential for anaerobic growth of *Escherichia coli* on xylose [41].

Another novel accomplishment with *S. cerevisiae* has been the engineering of L-arabinose metabolism through the overexpression of *Bacillus subtilis araA* along with *E. coli araB* and *araD* [42].

Xylanase and cellulase expression

Most xylanases produce xylobiose and xylotriose as the major oligosaccharides, and exocellobiohydrolases produce cellobiose. Simultaneous saccharification and fermentation must therefore use yeasts that assimilate these oligomers. The native xylose-fermenting yeast *P. stipitis* has genes for seven β-glucosidases and one endoxylanase, facilitating oligosaccharide utilization for this organism (see the JGI *P. stipitis* genome portal: www.jgi.doe.gov). Amino acid supplements enhance the expression of a heterologous endoxylanase in *P. stipitis* [43]. *S. cerevisiae* does not possess native xylanase activity, but its expression can be engineered in several ways [44].

Novel xylose-fermenting yeasts

P. stipitis, Pichia segobiensis, Candida shehatae, Pachysolen tannophilus and a few other yeasts constitute a small group that will ferment xylose directly to ethanol. Although they are still poorly understood, work by Suh, Blackwell and others has greatly improved knowledge of their natural origins by isolating more than 650 yeasts from microflora in the hindgut of beetles [45]. Of these, at least 200 were previously characterised, which is equivalent to almost 30% of all the currently recognized yeast species. *Enteroramus dimorbhus*, a novel xylose-fermenting yeast belonging to the same clade as *P. stipitis* is found frequently in the hindgut of *Odontotaenius disjunctus*, which feeds on fungi in white-rotted hardwood [46]. Many more new species remain to be isolated and characterized from beetles [47,48•], and it seems likely that new xylose-fermenting yeasts will emerge from these studies. Metabolic engineering of xylose fermentation has proceeding more readily since completion of the *P. stipitis* genome and the development of a new transformation system [49].

Conclusions

Interest in the use of yeasts for the commercial fermentation of xylose to ethanol is steadily increasing in light of rising petrol prices and concern over global warming. Several approaches have been successfully employed to engineer xylose metabolism. Modeling is a powerful tool for targeting metabolic changes, and many more model-driven attempts to alter the NADH/NADPH balance can be expected. Likewise, expression analysis is a powerful tool, but its retrospective use often reveals more changes than can be interpreted. Approaches that combine expression analysis with targeted deletions, overexpression or reverse genetics can elicit more definitive results. Adaptive evolution can obtain strains that use xylose more rapidly, but better growth does not equate with more ethanol; hence, the emphasis on anaerobic growth. However, unless stringent anaerobic conditions are employed, trace amounts of oxygen can allow slow oxygen-dependent growth. Evolutionary adaptation in chemostat cultures is not possible unless the cells are growing, hence the need for some fermentable sugar, which also complicates selection. Genomic and expression analysis of *P. stipitis* along with new strains from nature should continue to drive this field forward. The eventual goal is a yeast that is capable of efficiently fermenting glucose, xylose and other minor sugars to ethanol, and progress is being made on multiple fronts.

Acknowledgements

This research was supported in part by NIH grant GM067933-03 to TWJ.

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