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Yeast metabolic engineering for hemicellulosic ethanol production

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Efficient fermentation of hemicellulosic sugars is critical for the bioconversion of lignocellulosics to ethanol. Efficient sugar uptake through the heterologous expression of yeast and fungal xylose/glucose transporters can improve fermentation if other metabolic steps are not rate limiting. Rectification of cofactor imbalances through heterologous expression of fungal xylose isomerase or modification of cofactor requirements in the yeast oxidoreductase pathway can reduce xylitol production while increasing ethanol yields, but these changes often occur at the expense of xylose utilization rates. Genetic engineering and evolutionary adaptation to increase glycolytic flux coupled with transcriptomic and proteomic studies have identified targets for further modification, as have genomic and metabolic engineering studies in native xylose fermenting yeasts.

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Introduction

Bioconversion of lignocellulose to ethanol must occur at high rate, in good yield, and to concentrations that are economically recoverable. While readily achieved with starch, these goals are much more difficult with cellulose and hemicellulose. For cellulose, the major barrier is enzymatic saccharification. For hemicellulose, it is the use of glucose, xylose, mannose, galactose, arabinose, and rhamnose, in the presence of acetic and ferulic acids along with various degradation products from thermochemical pretreatment. While most hexoses are readily phosphorylated as soon as they enter the cell, hemicellulosic sugars must go through several biochemical steps before phosphorylation (Figure 1). Eukarya and bacteria use two distinct pathways each for the assimilation of D-xylose and L-arabinose. Most yeast metabolic engineering for ethanol production from xylose has focused on improving sugar uptake and the initial assimilation steps.

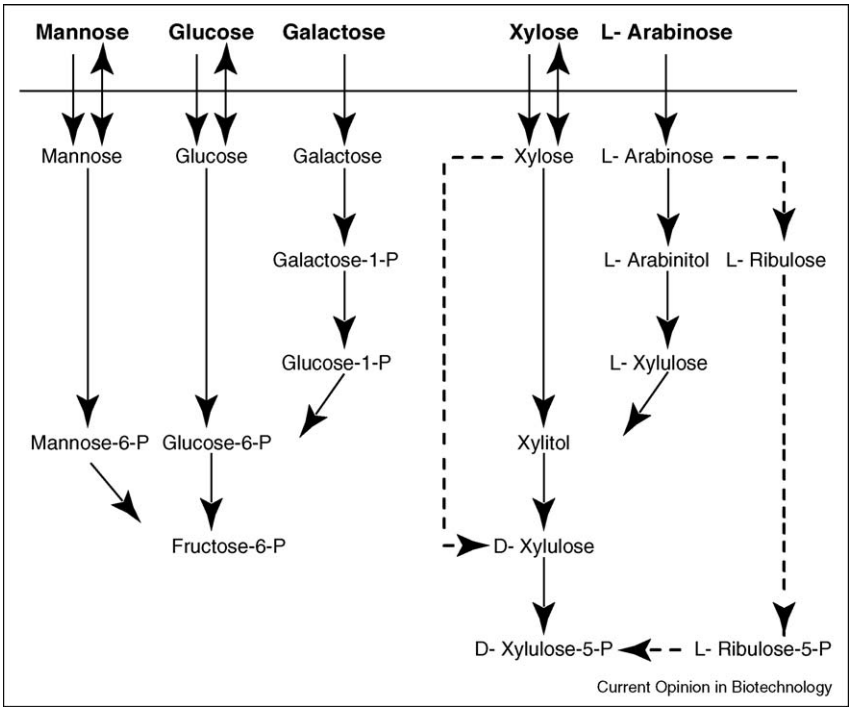
Xylose transport

Saccharomyces cerevisiae takes up xylose poorly owing to low affinity of its native nonspecific hexose-transport system for xylose, which are around 130–880 mM, or about 10–100 times higher than for glucose (Table 1) [1•]. Moreover, native transporters in *S. cerevisiae* are not properly regulated to facilitate xylose uptake [2•]. A recent metabolic model that combines induction of sugar transporters with the kinetic characteristics of the various proteins predicts that xylose transport by *S. cerevisiae* is maximal when the glucose concentration is near zero since the presence of glucose represses the high affinity hexose transporters that are responsible for xylose assimilation [3]. Xylose transport has little effect on the rate of xylose utilization when the levels of xylose reductase (XR) are limiting, but it affects utilization in cells with higher XR levels. Enhanced xylose transport also has a strong positive effect on *S. cerevisiae* cells engineered for assimilation through overexpression of *Piromyces* xylose isomerase (XI) [4•]. Better sugar transporters could be more effective.

The glucose transporters of *Pichia stipitis*, Sut1-3, were previously shown to transport glucose and other monosaccharides including xylose. When Sut1 was expressed in a xylose-utilizing *S. cerevisiae*, it enhanced xylose fermentation to ethanol [5]. Leandro *et al.* have cloned and characterized a glucose/xylose facilitator (Gxf1) and a glucose/xylose proton symporter (Gxs1) from *Candida intermedia* [6]. Both of these transporters show 10-fold higher affinity for glucose than xylose (Table 1). Expression of Gxs1 is affected by the heterologous expression of Gxf1 as well as the glucose concentration. When expressed together, or when the symporter was expressed in a background containing other hexose facilitators, symport activity was not detected. The authors hypothesized that yeasts limit expression of these energy-driven enzymes when their activity is dispensable [7•].

Expressing the Gxf1 facilitator had no observable effect in a xylose engineered *S. cerevisiae* background at high xylose concentrations, but it increased growth significantly at low concentrations [8]. Studies of engineered transporters have not been confined to yeast genes. *Trxlt1*, from a *Trichoderma reesei* library conferred growth on xylose, but not glucose indicating that it may be specific for xylose [1•], and heterologous expression of two xylose

Figure 1



Assimilation pathways for hexoses and pentoses. Mannose, glucose, and galactose are phosphorylated immediately after uptake by active (single arrow) or facilitative (double arrow) sugar transport. Pentoses must go through one or more metabolic steps before phosphorylation particularly with the oxidoreductase pathways found in yeasts (solid lines). The bacterial pathways (dotted lines) are shorter.

transporters from *Arabidopsis thaliana*, increased xylose consumption 40–46%, while increasing the xylose uptake rate approximately 2.5-fold [9].

Engineering xylose isomerase

The eukaryotic pathways for xylose and arabinose metabolism use oxidoreductases coupled to different cofactor requirements while the corresponding bacterial pathways use isomerases [4•]. XI avoids the cofactor imbalance that could impede metabolite flux, but at equilibrium xylose is clearly favored over xylulose. Homologs of bacterial XI from the anaerobic fungi, *Orpinomyces* and *Piromyces* have been successfully expressed in *S. cerevisiae* after 25 years of attempts in various laboratories [4•,10••]. Expression of the *Orpinomyces* gene enabled an initial growth rate twice that of the original *Piromyces* XI transformant. Even so, cells transformed with this gene consumed less than 5 g of xylose in 200 hours [11•]. Further metabolic engineering and adaptation increased the growth rate and ethanol yield of this strain to levels comparable to the best *Piromyces* XI strain (Table 2) [10••,12•].

It is not entirely clear whether the XI pathway is an improvement over the more conventional engineering of *S. cerevisiae* in which xylose reductase (XR) and xylitol dehydrogenase (XDH) are overexpressed [10••,13]. *S. cerevisiae* engineered with the XR/XDH pathway had much higher aerobic growth and anaerobic xylose

Table 1

Kinetic parameters of yeast glucose/xylose transporters

Transporters	K_m (mM)		V_{max} (nmol min^{-1} mg dw^{-1})		Reference
	Glucose	Xylose	Glucose	Xylose	
ScHxt1	107 ± 49^a	880 ± 8^b	50.9 ± 3.7^a	750 ± 94^b	[48]^a [1•]^b
ScHxt2	2.9 ± 0.3^a	260 ± 130^b	15.6 ± 0.9^a	340 ± 10^b	[48]^a [1•]^b
ScHxt4	6.2 ± 0.5^a	170 ± 120^b	12.0 ± 0.9^a	190 ± 23^b	[48]^a [1•]^b
ScHxt7	1.3 ± 0.3^a	130 ± 9^b	11.7 ± 0.3^a	110 ± 7^b	[48]^a [1•]^b
CiGxf1	2.0 ± 0.6	48.7 ± 6.5			[6]
CiGxs1	0.012 ± 0.004	0.4 ± 0.1			[6]

Table 2

Growth rate and fermentation characteristics of engineered *S. cerevisiae* strains

Strain	Strain description	Aerobic growth rate (hour ^{1})	Xylose (g l ^{1})	Xylose consumption rate (g g DCW ^{1} hour ^{1})	Ethanol yield (g g xylose ^{1})	Reference
TMB3057	XR-XDH strain overexpressing XKS1 and PPP Δ*gre3*	0.16	50	0.13	0.33	[14••]
TMB3066	*Piromyces* XI strain overexpressing XKS1 and PPP Δ*gre3*	0.02	50	0.05	0.43	[14••]
RWB202-AFX	*Piromyces* XI evolved isolate	0.03	20	0.21	0.42	[4•,10••]
INVSc1/ pRS406XKS/ pILSUT1/ pWOXYLA	*Orpinomyces* XI strain overexpressing *XKS1* and *SUT1*	0.025	50	NR	0.39	[11•,12•]
ADAP8	Evolved *Orpinomyces* XI strain overexpressing *XKS1* and *SUT1*	0.113	20	NR	0.37 (30°C)/0.43 (35°C)	[12•]

consumption rates than the XI strain, but the ethanol yield was lower (Table 2). Both engineered lab strains were inferior to an industrial strain on hydrolysate [14••,15]. In hydrolysates, the XR/XDH pathway may have an advantage by detoxifying 5-hydroxymethyl-furfural (HMF) [16]. High levels of XR and XDH activity are important during xylose utilization [17,18] and a higher level of XDH than XR reduces xylitol formation [19]. Endogenous levels of xylulokinase (XK) in *S. cerevisiae* are too low for efficient growth and ethanol production and moderate overexpression of XK is optimal [10••,18] while high levels can be detrimental [10••,20,21].

Engineering XR and XDH

Different requirements for XR and XDH activities (NADPH versus NAD) can lead to cofactor imbalances if routes are not available for their regeneration [10••,13,22••,23••]. Xylitol accumulation clearly results from lower XK, XI, and alcohol dehydrogenase (ADH) activities. All three of these changes impede flux, and the latter two also affect redox balances, but none directly affect the cofactor imbalance between NADPH demand and NADH supply. Altering the cofactor specificity for XR and/or XDH could accomplish this objective, albeit while decreasing the driving force realized from NADH oxidation by other routes (Table 3).

One of the keys to successful protein engineering is to alter affinity (K_m) for a substrate without significantly reducing the turnover number (K_{cat}) so that preference or specificity as measured by (K_{cat}/K_m) increases while maintaining a high catalytic efficiency. *P. stipitis* XR has about a twofold higher affinity for NADPH than for NADH [24,25••]. Watanabe reported that the intracellular ratio of NADPH/NADH is about 3.0 [23••], so both the cofactor affinity of the enzyme and the cofactor concentration favor the use of NADPH. By changing one residue in the putative phosphate pocket that binds NADPH, the resulting enzyme exhibits an NADH/NADPH activity ratio of 23:1. The K_m for NADH dropped by 45% while the K_m for NADPH went up more than 1000-fold, the K_m for xylose doubled and the K_{cat} with NADH stayed about the same. Thermal stability also increased. *S. cerevisiae* transformed with *P. stipitis XYL1* (R276H) showed a 20% increase in ethanol and 52% decrease in xylitol production [23••,26]. While these modifications were based on rational observations, semi-rational combinatorial active site saturation (CASTing) is an empirical method. In this approach selected amino acid residues were targeted with saturating mutagenesis in several successive rounds with high-throughput screening in each round to identify the best mutant. This led to a much more radical modification of the same region of *P. stipitis XYL1*. The best mutant, 2-2C12, carried four mutations: K270S, N272P, S271G, and R276F. It showed a NADH/NADPH activity ratio of about 13, and its K_{cat} with NADH was 73% of the wild type. The K_m for NADH, however, rose about 7-fold. Growth and fermentation characteristics were not reported [25••]. A *C. tenuis XYL1* K274R, N276D double mutant decreased xylitol production 52% while improving the ethanol yield 42% [27]. While the binding pockets of the *P. stipitis* enzyme for both cofactors are the same, the specific binding residues are different [28]. The cofactor imbalance also has been addressed by altering the preference of XDH to NADP. As in the case of XR, it is necessary to change the cofactor specificity without decreasing the overall activity or metabolite flux. The reported ratio of NAD/NADP is 4.9 [23••], so this reaction tends to favor NAD regardless of the enzymatic cofactor affinity.

A double mutant of *Gluconobacter oxydans* XDH showed completely reversed specificity for NADP. Equally importantly, the K_m for xylitol increased from 13.7 to 100 mM [29]. By substituting an XDH containing four mutations, xylitol production decreased 86% and ethanol production increased 41%. When this mutated enzyme was expressed in a background with high XK activity ethanol production increased and xylitol production

Table 3

Kinetic parameters for enzymes engineered with altered cofactor preference

Mutant	K_m NADH (μM)	K_m NADPH (μM)	k_{cat} NADH (min $^{-1}$)	k_{cat} NADPH (min $^{-1}$)	k_{cat}/K_m NADH (μM $^{-1}$ min $^{-1}$)	k_{cat}/K_m NADPH (μM $^{-1}$ min $^{-1}$)	K_m xylose NADH (mM)	K_m xylose NADPH (mM)	Specific xylose uptake rate (g/g DCW hour)	Ethanol yield (g g xylose $^{-1}$)	Reference
Xylose reductase											
P. stipitis											
WT	30.5	2.5	415	630	13.6	260	31.5	47.0	N.R	N.R	[23••]
R276H	17.0	1.7	408	16.0	24.4	9.3	45.5	53.2	N.R	N.R	[23••]
C. tenuis											
WT	138	3	660	780	4.78	260	142	96	0.06	0.34	[27,49]
274R/276D	41	128	720	1600	17.6	12.5	106	722	0.08	0.34	[27,49]

Mutant	K_m NAD^+ (μM)	K_m $NADP^+$ (μM)	k_{cat} NAD^+ (min $^{-1}$)	k_{cat} $NADP^+$ (min $^{-1}$)	k_{cat}/K_m NAD^+ (μM $^{-1}$ min $^{-1}$)	k_{cat}/K_m $NADP^+$ (μM $^{-1}$ min $^{-1}$)	K_m xylitol NAD^+ (mM)	K_m xylitol $NADP^+$ (mM)	Specific xylose uptake rate (g/g DCW h)	Ethanol yield (g g xylose $^{-1}$)	Reference
Xylitol dehydrogenase											
P. stipitis											
WT	381	170,000	1,050	110	2.76	0.006	21.7	–	0.19[a] 0.185[b]	0.34[a] 0.325[b]	[22••,31,50]
D207A/I208R/F209S/N211R	17,300	1,380	1,430	3,840	0.083	2.78	–	72.6	0.25[a]	0.36[a]	[31,50]
D207A/I208R/F209S	1,300	897	240	2,500	0.185	2.79	55.7	31.1	0.160[b]	0.261[b]	[22••,50]
S96C/S99C/Y102C/D207A/I208R/F209S	23,500	1,180	1,770	12,600	0.075	10.68	–	111	0.121[b]	0.223[b]	[22••,50]
G. oxydans											
WT	348	–	1,632	–	4.69	–	13.7	–			[29]
D38S/M39R	–	206	–	1,230	–	5.97	–	100			[29]

[a] Anaerobic.
[b] Low oxygen.

decreased [30]. This modified enzyme also increased the specific xylose consumption and specific ethanol production rates in hydrolysate [31]. Not all mutations proved to be positive, however, since an enzyme with six total residue mutations showed increased xylitol production because of low XDH activity [22••]. Fusion of XR and XDH has also been used in an attempt to get around cofactor imbalance. This resulted in a low ethanol yield, but the strain was capable of growth and ethanol production from xylose [32].

Other modifications

Overexpression of *TAL1* or downregulation of *PHO13* enables growth of *S. cerevisiae* that has been over engineered for XK expression [20]. Deleting *pho13* dramatically increases the capacities of engineered *S. cerevisiae* to grow on and ferment xylose [21]. Optimal expression of genes downstream of XK likewise increases ethanol production. In an XI strain, overexpressing several PPP genes significantly improved the growth rate [4•,10••]. Protoplast fusion between a thermotolerant *S. cerevisiae* and the xylose utilizing *C. shehatae* was reported to create a yeast capable of using xylose at 42°C which after mutation and selection showed a high fermentation efficiency. Evolutionary adaptation has also been used with *S. cerevisiae*. Some *S. cerevisiae* strains grow extremely slowly on xylose. Without heterologous expression breeding and selection yielded a strain showing improved growth on xylose. Consecutive anaerobic batch cultivation of *S. cerevisiae* on glucose, xylose, and arabinose reduced the selective pressure on any one pathway and improved mixed sugar fermentation [33].

'Omic' approaches to strain improvement

Transcriptome analysis of recombinant *S. cerevisiae* strains showing enhanced growth on xylose were examined for genes with altered expression levels in four yeasts. Out of 13 genes showing common changes, 5 proved advantageous. Upregulation of *SOL3* and *TAL1* and downregulation of *YLR042C*, *MNI1*, and *RPA49* each increased growth on xylose [34••]. The transcriptomes and proteomes of recombinant *S. cerevisiae* cultivated on xylose are not identical to those of cells grown on glucose or to those from derepressed cells, which implies that cultivation on xylose creates an intermediate metabolic state [35•]. A dynamic flux balance model based on a *S. cerevisiae* genome-scale metabolic network and simulated fed batch fermentations with glucose/xylose mixtures showed reasonable agreement with previously published batch fermentations, but the model was not sufficient to predict the performance of fed batch cultivations [36].

Pichia stipitis and other yeasts

Completion of the *P. stipitis* genome has revealed the presence of seven β-glucosidases, three endoglucanases, mannanases, xylanases, and numerous sugar transporters in this native xylose fermenting yeast [37••]. A cluster of genes coding for a novel L-rhamnose dehydrogenase degradation pathway was demonstrated [38–40], along with gene clusters for cellulose, maltose, galactose, urea, and iron metabolism [41]. Resequencing of the *P. stipitis* FPL-SHI21 (*cyc1*) strain, which was isolated after mutagenesis, selection, genetic engineering, and cultivation of its parent strain, CBS6054 over approximately seven years provided insight into how adaptation occurs at a molecular level [42]. Only 14 single nucleotide point mutations differed SHI21 from CBS6054. Three of these occurred in intergenic noncoding regions and the other 11 all gave rise to amino acid changes, most of which were nonconservative [42]. This is a very large fraction since most random mutations are either silent or give rise to changes that maintain function, and one can infer that the mutations conferred survival advantages under the highly selective conditions used in isolating the intermediate strains.

Despite the capacity of *P. stipitis* to ferment xylose to ethanol at nearly maximum yields with the production of very little xylitol, few recent metabolic engineering studies with this yeast have been published. Work has been done, however, to examine the effect of initial cell concentration and media composition [43] on ethanol production and inhibitor tolerance by the native organism and the production of ethanol when cultivated on hydrolysate [44].

Several studies have focused on metabolic engineering in the thermotolerant yeast *Hansenula polymorpha*. D-XK activities increased twofold and ethanol production increased in a strain of *H. polymorpha* expressing *Escherichia coli* XI and endogenous XK in a background lacking the native XR and XDH [45]. Expression of *P. stipitis* XR that had been engineered for decreased NADPH affinity along with the endogenous XDH and XK increased ethanol production and decreased xylitol production, thereby illustrating that enzymatic modifications shown to work in *S. cerevisiae* work for other yeasts as well [46]. Overexpression of pyruvate decarboxylase in *H. polymorpha* resulted in a threefold increase in ethanol production, albeit from rates and yields considerably below those achieved with *P. stipitis* and engineered *S. cerevisiae* strains [47].

Conclusions

Despite the efforts of several excellent research groups, development of yeast strains with sufficient activity and resilience to ferment hemicellulose hydrolysates remains elusive, but we have seen rapid progress resulting from biochemical and metabolic engineering guided by genomic and transcriptomic studies.

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