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Novel strategies to improve co-fermentation of pentoses with D-glucose by recombinant yeast strains in lignocellulosic hydrolysates

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Economically feasible production of second-generation biofuels requires efficient co-fermentation of pentose and hexose sugars in lignocellulosic hydrolysates under very harsh conditions. Baker's yeast is an excellent, traditionally used ethanol producer but is naturally not able to utilize pentoses. This is due to the lack of pentose-specific transporter proteins and enzymatic reactions. Thus, natural yeast strains must be modified by genetic engineering. Although the construction of various recombinant yeast strains able to ferment pentose sugars has been described during the last two decades, their rates of pentose utilization is still significantly lower than D-glucose fermentation. Moreover, pentoses are only fermented after D-glucose is exhausted, resulting in an uneconomical increase in the fermentation time. In this addendum, we discuss novel approaches to improve utilization of pentoses by development of specific transporters and substrate channeling in enzyme cascades.

Keywords: bioethanol, co-fermentation of pentoses with glucose, sugar transport, substrate channeling

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Introduction

Global needs for renewable energy sources have stimulated efforts to metabolically engineer microorganisms capable of synthesizing alcohols by fermentation of plant biomass. Recent research particularly concentrates on the usage of lignocellulosic substrates such as crop wastes, forestry residues and municipal solid waste to avoid the consumption of food products for fuel synthesis. Lignocellulose is a complex mixture of polymers like cellulose, hemicellulose and lignin; cellulose consists of D-glucose chains, while hemicellulose additionally contains the pentose sugars D-xylose and, in only lower amounts, L-arabinose which together can make up 30–40% of total sugars. Efficient exploitation of the substrate would therefore require the ability of a microorganism to metabolize not only D-glucose but also the pentose sugars. However, the baker's yeast *Saccharomyces cerevisiae*, which is the most commonly used organism for bioethanol production, is naturally not able to metabolize L-arabinose and D-xylose. Several strategies have therefore been developed to enable yeast to convert these sugars into metabolic intermediates that can be funneled into the endogenous metabolism via the pentose phosphate pathway (PPP) (for a review, see refs. 1–4). In an approach favored by our laboratory, D-xylose is isomerized by a bacterial D-xylose isomerase (XI) to D-xylulose, which is subsequently phosphorylated by an endogenous D-xylulokinase, yielding D-xylulose-5-phosphate (Xul5P), an intermediate of the non-oxidative part of the PPP.[5] The conversion of L-arabinose to Xul5P is more complex; the pathway engineered in our laboratory involves codon-optimized L-arabinose isomerase from *Bacillus licheniformis* (*araA*), L-ribulokinase (*araB*) and L-ribulose-5-P 4-epimerase (*araD*) from *E. coli*.[6,7] Through a cascade of reactions in the PPP, Xul5P is converted into glycolytic intermediates D-fructose-6-phosphate (F6P) and D-glyceraldehyde-3-phosphate (GAP) which are finally fermented to ethanol. Despite great research effort that has been put into strain optimization by dozens of groups during the last two decades, the rate of pentose utilization by engineered yeast is still significantly

slower than the fermentation of D-glucose, even when xylulokinase (XK) and all enzymes of the non-oxidative part of the PPP, D-ribulose 5-phosphate isomerase (RKI), D-ribulose 5-phosphate epimerase (RPE), transketolase (TKL) and transaldolase (TAL), are strongly overexpressed (ref. 8; Dietz and Boles, unpublished results). One major limitation is imposed by the lack of specific and efficient pentose transporters. Other important bottlenecks seem to occur due to the drain of reaction intermediates by competing enzymatic reactions and pathways.

Development of Efficient and Specific Pentose Transporters

Pentose transport in *S. cerevisiae* is mediated by different members of the hexose transporter family e.g., Hxt7 for D-xylose and Gal2 for L-arabinose and D-xylose.[6,9] These transporters, however, have only a low affinity for pentoses and considerably limit the overall pentose utilization. Furthermore, the affinities for their respective hexose substrates D-glucose or D-galactose are higher than their affinities for pentoses, leading to competitive inhibition of pentose transport in the presence of hexoses as being present in lignocellulosic hydrolysates. This causes sequential rather than simultaneous consumption of hexoses and pentoses, which is undesirable from an economical as well as an operational standpoint. Improvements in D-xylose fermentation can be achieved by overexpression of pentose transporting hexose transporters, which also alleviates competitive inhibition to a small extent, but efficient co-fermentation is still not possible.[10]

As several approaches to express specific pentose transporters, that are not inhibited by D-glucose, in *S. cerevisiae* have failed,[11-13] our laboratory has recently developed a novel screening system to search for heterologous, specific pentose transporters or to engineer them from hexose transporters. In a D-xylose utilizing yeast strain glucose utilization was disrupted at its first step by deletion of the hexo-/glucokinase genes, resulting in D-glucose being no longer used as a carbon source but only acting as a mere inhibitor of pentose uptake (**Fig. 1**). In addition, all endogenous hexose transporter genes were deleted, enabling us to re-introduce individual sugar transporters.

This system is used to screen for improved, "D-glucose-resistant" D-xylose transporters, either native (e.g., from cDNA libraries) or after mutagenesis of sugar transporters like Hxt7 or Gal2. Additionally, evolutionary engineering approaches are possible—addition of increasing concentrations of D-glucose to D-xylose growth medium can be applied as an evolutionary growth pressure to force the culture to accumulate beneficial mutations in order to overcome the inhibition. Both strategies already led to first promising results in our laboratory. Sequence analysis revealed mutations at position T213 in Hxt7, a position that has also been identified by Kasahara[14] to be one of the key residues for D-glucose affinity. Our results imply that this residue is important for discrimination between D-glucose and D-xylose and mutations at this position impair D-glucose affinity more than D-xylose affinity. Based on our previously reported analyses[10] the newly engineered transporters should lead to significantly improved co-fermentation of D-xylose and D-glucose, and therefore faster fermentation rates of mixed-sugar hydrolysates.

Substrate Channelling Improves Pentose Fermentation Rates

Independently of the transport efficiency, pathway bottlenecks seem to occur due to the drain of reaction intermediates by competing pathways. For example, some promiscuous aldose-reductases (e.g., Gre3) are capable of reducing a part of the available D-xylose to D-xylitol, which cannot be efficiently metabolized and additionally has an inhibitory effect on the XI.[5] Moreover, as shown by our group,[10] hexoses and pentoses slightly compete during their catabolism. A further bottleneck imposed by competition for metabolites by different enzymes seems to occur in the non-oxidative part of PPP, namely after the first transketolase reaction (see **Fig. 2**), which yields sedoheptulose-7-phosphate (S7P) and GAP. In the "desired" reaction scheme, these metabolites are converted to erythrose-4-phosphate (E4P) and F6P by transaldolase; however, the highly abundant glycolytic enzyme glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) sequesters GAP produced by transketolase, leading to a stoichiometric imbalance with S7P and, consequently, to a bottleneck at the transaldolase reaction. Consistent with this, accumulation of S7P has been observed in yeast strains grown both on D-xylulose[15] and L-arabinose.[16] This effect can be partly suppressed by overexpression (or upregulation) of transaldolase genes.[16,17] The downregulation of certain GAPDH isoforms—which is a recurring observation made in different strains evolved to grow on D-xylose (refs. 18 and 19; Oreb and Boles, unpublished result) or L-arabinose (ref. 16; Wiedemann and Boles, unpublished results)—supports the view that this bottleneck is caused by the competition of GAPDH and TAL for GAP. An elegant possibility to channel GAP produced by TKL directly to TAL would be a physical interaction between these enzymes. Guided by this idea, we co-expressed both proteins fused to coiled-coil domains which undergo strong dimeric interactions. Our preliminary results show that D-xylose utilizing laboratory strains transformed with the fusion constructs exhibit a significantly faster growth on D-xylose compared with the reference strain expressing native TAL and TKL (Oreb and Boles, unpublished results). These first promising results suggest that other bottlenecks could also be relieved by substrate channeling; for example, the formation of D-xylitol could be suppressed by tethering of XI and XK on D-xylose transporter proteins. At the same time, co-localization of pentose-modifying enzymes with transporters could accelerate the transport rate by perpetuating a steep concentration gradient of the pentoses across the plasma membrane.

Genetic Engineering of Robust Industrial Yeast Strains

For efficient industrial production of ethanol from mixed-sugar hydrolysates it is crucial to implement the pentose fermentation technology into robust, industrially used yeast strains. Laboratory strains like S288C or the CEN.PK-series (Euroscarf)

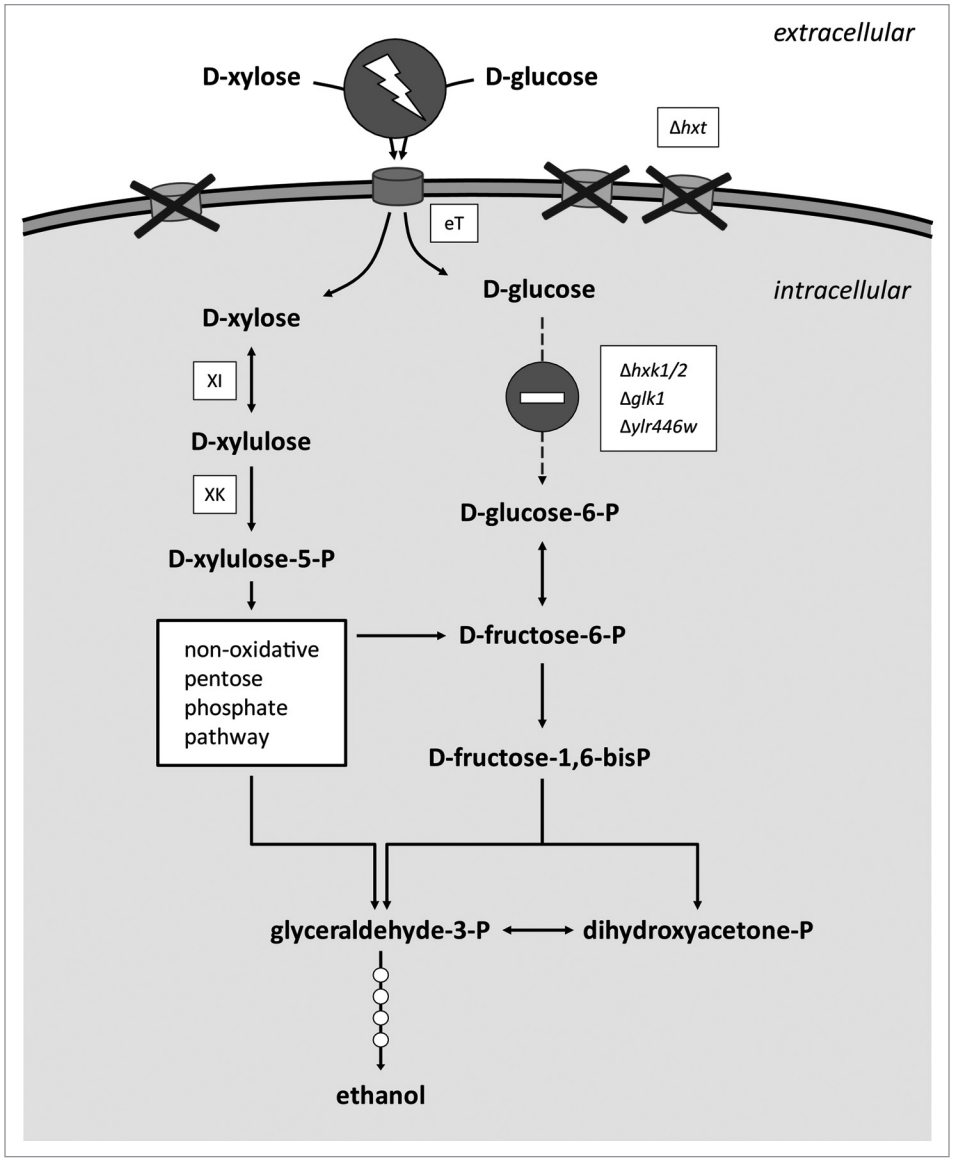

Figure 1. Schematic overview of the novel screening system. The strain has no hexose transporters (Δhxt) except the engineered one that is re-introduced (eT). Glycolysis is blocked at the first step by deletion of hexo-/gluco-kinases. Xylose metabolism is established by overexpression of the *Clostridium phytofermentans* xylose isomerase (XI) and endogenous xylulokinase *XKS1* (XK) and non-oxidative pentose phosphate pathway enzymes. The limitation of D-xylose utilization and the evolutionary pressure exerted by D-glucose concentrates on the re-introduced transporter.

are easily genetically modified and the functionality of L-arabinose or D-xylose utilization pathways has been shown by various laboratories.[6,8] Moreover, it has been possible to increase the resistance to fermentation inhibitors like acetate,[20] furfural or hydroxymethylfurfural.[21] Despite these advancements, most industrial yeast strains are still much more robust and resistant to the toxic inhibitor cocktail present in lignocellulosic hydrolysates. In addition, industrial yeast strains show higher specific ethanol productivities, ethanol yields and produce a lower amount of undesirable by-products like glycerol. Importantly, industrial yeast strains are extraordinarily stable under a variety of manufacturing conditions including drying and long-term storage.

On the other hand, genetic manipulation of diploid or even aneuploid industrial strains is challenging, especially if their exact genomic sequence is not known. Under large-scale fermentation conditions, the usage of plasmids is undesirable as their maintenance depends on selectable markers. All genetic manipulations should therefore be based on a stable integration into the genome. Consequently, gene deletions or insertions have to be performed for all alleles to obtain a stable genotype. At the same time, the number of transformation steps has to be kept small to avoid accumulation of negative mutations. Evolutionary engineering approaches should comply with industrial fermentation and propagation conditions in order to maintain all beneficial properties of a strain.

In our laboratory, the alcohol yeast strain Ethanol Red (Fermentis) that had been developed for ethanol industry has

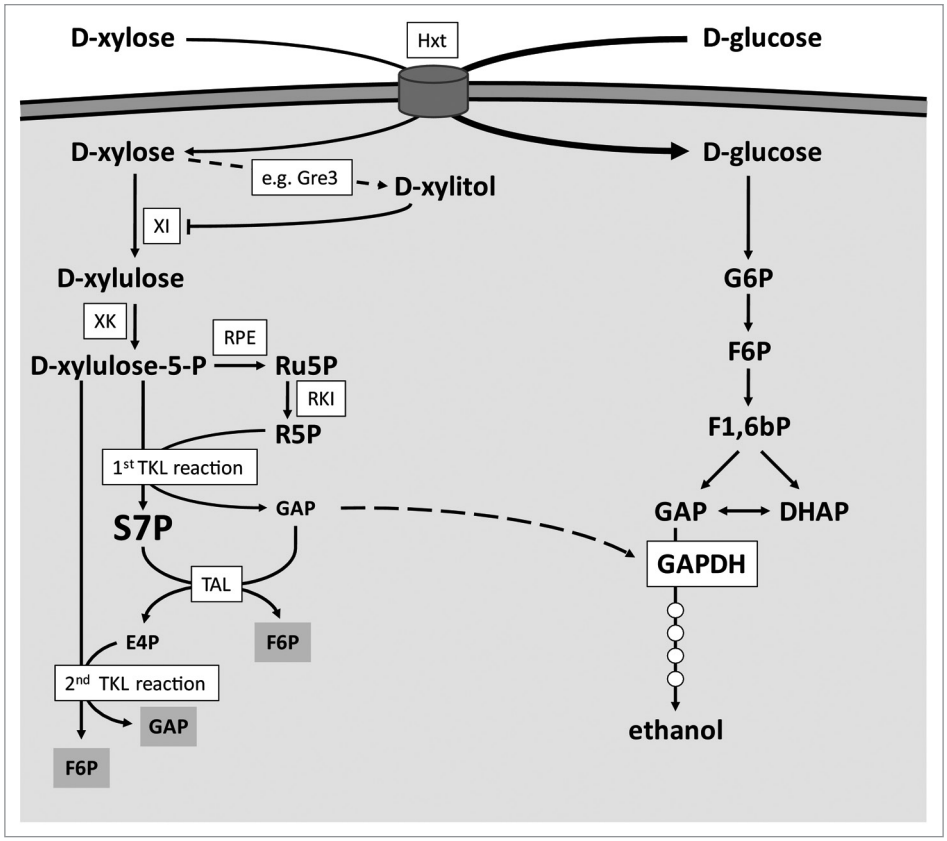

Figure 2. Bottlenecks in pentose fermentation by engineered yeast. Drain of pathway intermediates by competing pathways is depicted by dashed lines. The stoichiometric imbalance of reaction intermediates at the TAL reaction is depicted by the font size. For clarity, only those enzymes mentioned in the main text are shown and reverse reactions are not considered. The terminal metabolites of the PPP which can be further metabolized in glycolysis are shown in gray boxes. The glycolytic intermediates shown are D-glucose-6-phosphate (G6P), D-fructose-6-phosphate (F6P), D-fructose-1,6-bisphosphate (F1,6bP) and dihydroxyacetone-phosphate (DHAP). Other abbreviations used are the same as in the main text. The intermediates downstream of the GAPDH reaction are omitted for clarity.

proven to be a promising candidate for the fermentation of lignocellulosic hydrolysates. Moreover, we have successfully established protocols for the transfer of the developed pentose fermentation technologies into this strain. Elaborate genetic cassettes for D-xylose and L-arabinose utilization pathways were stably integrated into the genome of the Ethanol Red strain. After evolutionary engineering, we obtained a strain efficiently fermenting D-glucose and pentoses to ethanol with high production rates and nearly maximal yields (Dietz and Boles, unpublished results). With our currently best developed strain, e.g., complete D-xylose fermentation takes only about twice as long as that for D-glucose. The next steps will now be to introduce the newly developed specific pentose transporters and the substrate channeling modules into this strain to approach the main goal—efficient co-fermentation of pentoses and glucose.

Conclusions

For industrial ethanol production from lignocellulosic hydrolysates, yeast strains with high hexose and pentose fermentation rates and the ability to co-ferment D-glucose and pentoses are needed. Genetic engineering of industrial alcohol yeast strains for pentose utilization, along with the development of specific pentose transporters and substrate channeling complexes will pave the way to an economically feasible conversion of plant waste products into biofuels.

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