**Metabolic engineering for improved microbial pentose fermentation**

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Global concern over the depletion of fossil fuel reserves, and the detrimental impact that combustion of these materials has on the environment, is focusing attention on initiatives to create sustainable approaches for the production and use of biofuels from various biomass substrates. The development of a low-cost, safe and eco-friendly process for the utilisation of renewable resources to generate value-added products with biotechnological potential as well as robust microorganisms capable of efficient fermentation of all types of sugars are essential to underpin the economic production of biofuels from biomass feedstocks. *Saccharomyces cerevisiae*, the most established fermentation yeast used in large scale bioconversion strategies, does not however metabolise the pentose sugars, xylose and arabinose, and it was not until the late 1970s that the first steps were taken to develop methods to engineer pentose metabolism in this yeast. The ability of *S. cerevisiae* in fermenting lignocellulose hydrolysates has been demonstrated repeatedly. *S. cerevisiae* produces ethanol with stoichiometric yields from hexose sugars and tolerates a wide spectrum of inhibitors and elevated osmotic pressure. For these reasons, it has been recognised that genetic engineering of naturally fermenting microorganisms such as *S. cerevisiae* is required for transport and efficient bioconversion of pentose sugars to bioethanol. Pathways for pentose sugar metabolism are essential for microorganisms living on decaying plant material and are of prime interest in biotechnology when low-cost plant hydrolysates are to be fermented to ethanol efficiently.

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**Pentose Metabolism**

A common step in the catabolism of both xylose and arabinose in all microorganisms is that both sugars are converted to D-xylulose-5-phosphate. However, the pathways to convert L-arabinose and D-xylose to D-xylulose-5-phosphate are distinctly different in bacteria and fungi (Fig. 1). In bacteria, D-xylose is converted to D-xylulose by an isomerase (EC 5.3.1.5) and then phosphorylated by xylulokinase (EC 2.7.1.53) while L-arabinose is first converted to L-ribulose by an isomerase (EC 5.3.1.3), and then phosphorylated by ribulokinase (EC 2.7.1.57). L-Ribulose-5-phosphate is then converted to D-xylulose-5-phosphate by an epimerase (EC 5.3.1.3).

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**Introduction**

The baker’s yeast, *Saccharomyces cerevisiae* is the most well established fermentation yeast for large scale ethanolic fermentation of the hexose sugars glucose, mannose and galactose. However, unlike some other yeast species such as Pachysolen sp. and Pichia sp., *S. cerevisiae* does not metabolise the pentose sugars, xylose and arabinose, and it was not until the late 1970s that the first steps were taken to develop methods to engineer pentose metabolism in this yeast. The ability of *S. cerevisiae* in fermenting lignocellulose hydrolysates has been demonstrated repeatedly. *S. cerevisiae* produces ethanol with stoichiometric yields from hexose sugars and tolerates a wide spectrum of inhibitors and elevated osmotic pressure. For these reasons, it has been recognised that genetic engineering of naturally fermenting microorganisms such as *S. cerevisiae* is required for transport and efficient bioconversion of pentose sugars to bioethanol. Pathways for pentose sugar metabolism are essential for microorganisms living on decaying plant material and are of prime interest in biotechnology when low-cost plant hydrolysates are to be fermented to ethanol efficiently.
In fungi, both pentose sugars go through oxidation and reduction reactions before they are phosphorylated by xylulokinase. D-xylose is reduced to xylitol by a reduced nicotinamide adenine dinucleotide phosphate (NADPH)-consuming reaction and xylitol is then oxidised by an NAD⁺-consuming reaction to form D-xylulose. L-Arabinose goes through four redox reactions; two are NAD⁺-dependent oxidation reactions and two reductions are linked to NADPH consumption. All of the enzymes in the fungal D-xylose pathway can also be used in the L-arabinose pathway. D-xylulose then enters the Pentose Phosphate Pathway (PPP) after phosphorylation to D-xylulose-5-phosphate. The conversion of L-arabinose and D-xylose to D-xylulose is redox neutral, but different redox cofactors are used, which affects cellular demands for oxygen. Fermentation of D-xylose and L-arabinose to equimolar amounts of ethanol and CO₂ under anaerobic conditions is possible in S. cerevisiae engineered for pentose metabolism but the fermentation requires careful aeration otherwise the fermentation product is mainly biomass or xylitol and CO₂. Pentoses are therefore not efficiently fermented to ethanol because of the imbalance of these redox cofactors. Since NADPH is regenerable mainly in the oxidative phase of the PPP, where the reduction of NADP⁺ is coupled to the generation of CO₂, it has an effect on the redox balance. When extra CO₂ is produced in this pathway, the pentose fermentation to ethanol and CO₂ is no longer redox neutral. To remove excess NADPH, either xylitol is produced or aeration is required, which leads to further unwanted CO₂ production or a combination of both processes.²

Metabolic Engineering and the Redox Metabolism

Several metabolic engineering strategies have been developed to generate organisms (yeasts or bacteria) that can produce ethanol efficiently from biomass-derived hydrolysates. To date, no studies have been conducted to engineer filamentous fungi for ethanolic fermentation even though some species of anaerobic filamentous fungi were shown to produce ethanol and also ferment pentose sugars.¹ For commercial fermentation, yeasts present a number of advantages over bacteria. Yeasts have superior resistance to hydrolysate inhibitors, better growth at low pH and less stringent nutritional requirements. Saccharomyces sp. have been traditionally used in industry for fermentation of sugar-based materials. However, the best hexose fermentor to produce ethanol, S. cerevisiae, is unable to metabolise the pentose sugar components of biomass. Metabolic engineering has been used to improve the fermentative capability of S. cerevisiae. Expression of a xylose reductase (XYL1) and a xylitol dehydrogenase (XYL2) from Pichia stipitis in S. cerevisiae resulted in growth on xylose but low levels of ethanol production.³ In addition, the overexpression of the endogenous xylulose kinase (XYL3) together with XYL1 and XYL2 was undertaken⁴ and the effect and optimization of expression levels of these genes has been studied.⁵ The use of NAD(P)H-dependent XR and NAD⁺-dependent XDH from P. stipitis creates a cofactor imbalance resulting in xylitol accumulation. The effect of replacing the native P. stipitis Xr with a K_{270}M-mutated Xr⁶ which has reduced affinity for NADPH was investigated, resulting in enhanced ethanol yields and decreased xylitol formation. However, when the K_{m} for NADPH was enhanced, the K_{m} for xylose also increased concomitantly.

NADH-specific xylose reductase enzymes would enable efficient recycling of the co-enzyme in the next step in xylose and arabinose metabolism, which involves conversion of xylitol to xylose and arabinol to L-xylulose by NAD⁺-specific dehydrogenases. Furthermore NADH is more stable and intracellular concentrations are naturally much higher than NADPH. Recombinant S. cerevisiae strain carrying a single copy of the Candida tenuis xylose reductase K²⁷⁰R - N²⁷⁰D double mutant which was shown to have undergone
almost complete reversal of co-enzyme preference from NADPH to NADH and displayed improved fermentative capabilities in terms of ethanol when compared to S. cerevisiae harbouring the wild type C. tenuis xylose reductase.\(^7\)

The gene encoding Xylose reductase (TeXR) was also isolated from the thermophilic fungus Talaromyces emersonii,\(^8\) a fungus known by its thermostable lignocellulolytic enzyme systems.\(^9\) Amino acid residues identified in C. tenuis xylose reductase critical in substrate recognition and co-factor preference were conserved in TeXR (Fig. 2). The coenzyme selectivity of TeXR was altered by site-directed mutagenesis and the ability of TeXR\(^{K271R,N273D}\) double mutant to use NADH preferentially to NADPH as a coenzyme in the first step of pentose metabolism could have dramatic effects on improving the xylose conversion process by reducing the redox imbalance showing that T. emersonii may be a novel and highly efficient ‘toolbox’ for biotechnological conversion of lignocellulose to bioethanol. Comparable changes in altered coenzyme preference from NADPH to NADH were obtained with a GiXr\(^{K274R,N276D}\) double mutant\(^{10}\), PkXr\(^{K270R,N272D}\) double mutant\(^{11,12}\) and a PkXr\(^{K270S,S271G,N272P,R276F}\) quadruple mutant.\(^{11}\) The next logical steps in our approach would be to perform a detailed investigation of TeXR\(^{K271R,N273D}\) double mutant effects in engineered strains in order to improve xylose fermentation rates and establish a comparison between other xylose reductase mutants. The S. cerevisiae strain engineered with the C. tenuis double mutant showed 42% enhanced ethanol yield and decreased xylitol and glycerol production compared to the reference strain harbouring wild-type XR.\(^{13}\) Similar tendencies were observed with S. cerevisiae strains engineered with the P. stipitis PkXr\(^{K271R,N273D}\) double mutant.\(^{12}\)

Other strategies to introduce xylose metabolic pathways into S. cerevisiae in an attempt to decrease xylitol formation included the expression of the Thermus thermophilus xylose (glucose) isomerase\(^{14}\) and the XYLA gene encoding the Piromyces sp. xylose isomerase.\(^{15}\) However, the latter enzyme is strongly inhibited by xylitol and because S. cerevisiae produces an aldose reductase (Gre3) capable of reducing xylose to xylitol, a major by-product in the process was xylitol. Therefore GRE3 deletion strains are essential for xylose fermentation when a xylose isomerase is introduced into S. cerevisiae.\(^{16}\) Comparison of xylose-fermenting ability by S. cerevisiae strains engineered with xylose reductase and xylitol dehydrogenase with strains engineered with xylose isomerase revealed that the Xr-Xdh xylose
utilization pathway is much better than the xylose isomerase pathway due to the insufficient in vivo activity of xylose isomerase. This low xylose isomerase activity limits xylose utilization in recombinant S. cerevisiae strains and therefore it would be useful to exploit further the Xr-Xdh pathway using T. emersonii XrK271R. N273D double mutant to potentially construct a new pathway to reduce the redox imbalance achieving more effective ethanol production from xylose by recombinant S. cerevisiae.

NADPH is mainly regenerated in the oxidative part of PPP and is coupled to the generation of CO₂. In this pathway, D-glucose-6-phosphate dehydrogenase (G6PDH), encoded by ZWF1 and 6-phosphogluconate dehydrogenase, encoded by GND1 and GND2 are responsible for the oxidation of D-glucose-6-phosphate and release of 2 moles of NADPH and 1 mole of CO₂ per mole of D-glucose-6-phosphate. Ethanol yield from xylose can be increased by lowering the oxidative PPP flux. Modifications in the pathway such as deletion of ZWF1 and GND1 were proven to block PPP giving an ethanol yield of 0.41 g g⁻¹ and a xyitol yield of only 0.05 g g⁻¹ and a reduced phosphogluconate isomerase activity, an enzyme that converts glucose-6-phosphate into fructose-6-phosphate, was shown to increase ethanol yield.

Insertion of a NADP⁺-dependent D-glyceraldehyde-3-phosphate dehydrogenase (NADP-GAPDH) from Kluyveromyces lactis facilitated NADPH regeneration with no production of CO₂ in S. cerevisiae strains with a ZWF1 deletion. In S. cerevisiae strains exhibiting a range of productions levels of G6PDH, overexpression of a transhydrogenase from Azotobacter vinelandii, reduced the xyitol yield but enhanced the yield of glycerol during xylose fermentation.

A different approach to modifying the redox metabolism would require modification of ammonia assimilation in recombinant S. cerevisiae. For ammonia incorporation, ATP-dependent synthesis of glutamine from glutamate and ammonia is catalysed by glutamine synthetase, while a NADPH-dependent glutamate dehydrogenase, encoded by GDH1 is responsible for 2-ketoglutarate amination. Another glutamate dehydrogenase, encoded by GDH2 is NADH-dependent and converts glutamate to 2-ketoglutarate and ammonium. Typically all microorganisms that fix atmospheric nitrogen assimilate ammonia through the GS-GOGAT complex which converts ammonia to glutamate using NADH and ATP and consists of two enzymes: glutamate synthetase encoded by GLT1 and glutamine synthetase encoded by GLN1. Deletion of NADPH-dependent GDH1 and overexpression of NADH-dependent GDH2 or GS-GOGAT complex was shown to improve cofactor utilisation in xylose-fermenting S. cerevisiae and improved the ethanol yield.

In further metabolic engineering of xylose metabolism, the xylose transport step should receive special attention. The rather high \(K_m\) values of xylose transporters and xylose isomerise enzymes implies that achieving high rates of xylose fermentation may require the introduction of heterologous high-affinity xylose transporters that catalyses xylose uptake. S. cerevisiae also lacks an efficient transport system for pentose sugars, although the transport of these sugars occurs through hexose transporters with very low affinity and competition with glucose restricts xylose assimilation. Simultaneous and effective transport of both hexose and pentose sugars by pentose utilising strains of S. cerevisiae would therefore be a significant improvement for bioconversion of biomass feedstocks to bioethanol (Fig. 3). A gene encoding a glucose/xylose facilitated diffusion transporter and a gene encoding a glucose/xylose symporter from C. intermedia were successfully expressed in S. cerevisiae. However the C. intermedia symporter was not able to support vigorous growth of the recombinant S. cerevisiae strain on xylose or glucose when used as sole carbon sources. Among filamentous fungi relatively few sugar transporters have been identified and characterised.

T. emersonii, the thermophilic fungus used in our studies, inhabits the soil, decaying masses of plant material piles of agricultural and forestry products, and other accumulations of organic matter wherein the warm, humid and aerobic environment with accessible carbon sources provides the basic conditions for survival. T. emersonii also grows rapidly on hexoses and pentoses as a sole carbon sources and thermostable enzyme systems required for hemicellulose degradation have been described and characterised previously. So we therefore anticipated the presence of high affinity sugar transporter genes in T. emersonii genome.

We searched the T. emersonii chromosomal DNA for genes with strong sequence and predicted structural similarities with transporter genes from the
microorganisms whose function has been demonstrated and therefore the isolation of two putative transporters from the filamentous fungus T. emersonii, THTX (GenBank FJ985745) and TeXYLT (GenBank Accession No.: FJ985746) has been achieved. The transporters were shown to have 12 transmembrane domains which is a characteristic feature of the Major Facilitator Superfamily. Detailed sequence analysis of both transporters suggests that THTX is potentially a hexose transporter similarly to Gal2 from S. cerevisiae while TeXYLT has the potential to be a glucose/xylose transporter (unpublished data).

Despite the high similarity of THTX to fungal high-affinity transporters and the similarity of TeXYLT to other transporters previously shown to transport xylose, Talaromyces genes did not restore growth on different sugars of an engineered S. cerevisiae strain, in which all hexose transporters were deleted (unpublished data). We hypothesized that T. emersonii transporter proteins may not have been correctly directed to the plasma membrane, were folded incorrectly or a different composition of phospholipid sterols in S. cerevisiae may have caused changes in T. emersonii transporter conformation. Further expansion of these results of our research can open an alternative route to the development of industrial xylose-utilizing strains of S. cerevisiae.

Conclusion

More than a decade of research has been devoted to the development of strains for efficient pentose fermentation. The majority of the studies conducted used metabolic engineering through a rational selection of genes to be manipulated for the development of novel pentose-fermenting strains of S. cerevisiae with varying levels of success. The increased knowledge about pentose metabolism, substrate binding and cofactor specificity of pentose assimilating enzymes and sugar transport has contributed to the improvement of S. cerevisiae strains for bioconversion of pentose sugars to bioethanol. However, the mechanisms by which pentose-fermenting yeasts can accomplish an increased rate of ethanol production are still not fully understood and the simultaneous co-fermentation of hexose and pentose sugars still constitutes a major strain engineering challenge.

There is an increasing need to identify new sources of more stable biomass converting enzymes and more efficient systems to utilise all carbohydrate components of lignocellulose. Also better understanding of the sugar and oxygen regulatory system, sugar transport and the development of further transformation and expression systems with genes capable to overcome the cofactor imbalance will enable the construction of robust industrial yeast strains for pentose fermentation. The thermophilic fungus T. emersonii proved to be a potential source organism for extracellular and intracellular proteins with applications in bio-mass bioconversion strategies and future experiments using T. emersonii genes may further improve the desired traits or fermentative properties of the industrial organisms leading to a more efficient biotechnological conversion of lignocellulose to bioethanol.

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