Bioengineered Bugs 1:6, 424-428; November/December 2010; © 2010 Landes Bioscience

Metabolic engineering for improved microbial pentose fermentation

Sara Fernandes[1] and Patrick Murray[2,*]

[1]IBB-Institute for Biotechnology and Bioengineering; Centre of Biological Engineering; Universidade do Minho; Braga, Portugal;

[2]Shannon Applied Biotechnology Centre; Limerick Institute Technology; Limerick, Ireland

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 utilization 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 bioengineering is required for introduction of efficient pentose metabolic pathways and pentose sugar transport proteins for bioconversion of these substrates. Our approach provided a basis for future experiments that may ultimately lead to the development of industrial *S. cerevisiae* strains engineered to express pentose metabolising proteins from thermophilic fungi living on decaying plant material and here we expand our original article and discuss the strategies implemented to improve pentose fermentation.

Key words: pentose fermentation, cofactor imbalance, metabolic engineering

Submitted: 05/04/10

Revised: 05/24/10

Accepted: 05/26/10

Previously published online: www.landesbioscience.com/journals/biobugs/article/12724

DOI: 10.4161/bbug.1.6.12724

*Correspondence to: Patrick G. Murray; Email: patrick.murray@lit.ie

Addendum to: Fernandes S, Tuohy MG, Murray PG. Xylose reductase from the thermophilic fungus *Talaromyces emersonii*: cloning and heterologous expression of the native gene (*Texr*) and a double mutant (*Texr*$^{K271R+N273D}$) with altered coenzyme specificity. J Biosci 2009; 34:881–90; PMID: 20093741.

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.[1] *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 recognized 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.

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 2.1.7.53) while L-arabinose is first converted to L-ribulose by an isomerase (EC 5.3.1.3), and then phosphorylated by ribulokinase (EC 2.1.7.47). L-Ribulose-5-phosphate is then converted to D-xylulose-5-phosphate by an epimerase (EC 5.3.1.3).

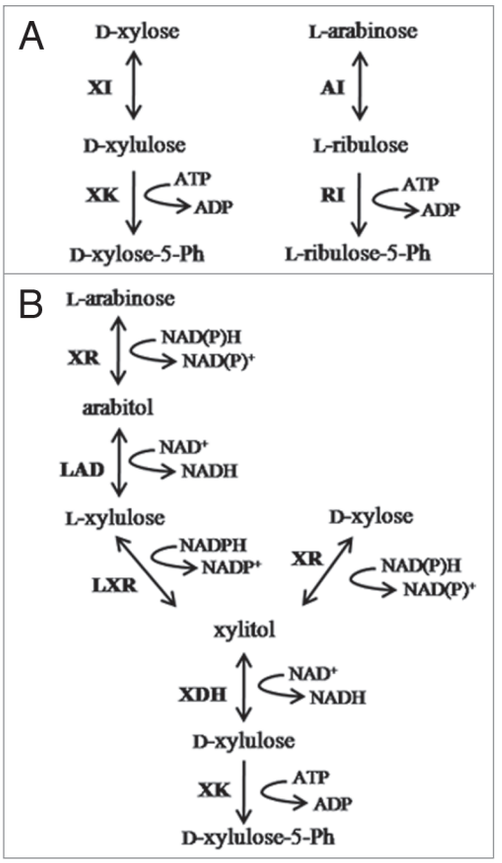

Figure 1. Bacterial (A) and fungal (B) pentose utilization pathways. Enzyme designations are: XI, xylose isomerase; XK, xylulose kinase; AI, arabinose isomerase; RI, ribulose kinase; XR, xylose reductase; LAD, L-arabitol dehydrogenase; LXR, L-xylulose reductase; XDH, xylitol dehydrogenase.

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 CO_2 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 CO_2. Pentoses are therefore not efficiently fermented to ethanol because of the imbalance of these redox cofactors. Since NADPH is regenerated mainly in the oxidative phase of the PPP, where the reduction of $NADP^+$ is coupled to the generation of CO_2, it has an effect on the redox balance. When extra CO_2 is produced in this pathway, the pentose fermentation to ethanol and CO_2 is no longer redox neutral. To remove excess NADPH, either xylitol is produced or aeration is required, which leads to further unwanted CO_2 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 undertaken[4] 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 Xr[6] 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 xylulose and arabitol 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 $K^{274}R$ - $N^{276}D$ double mutant which was shown to have undergone

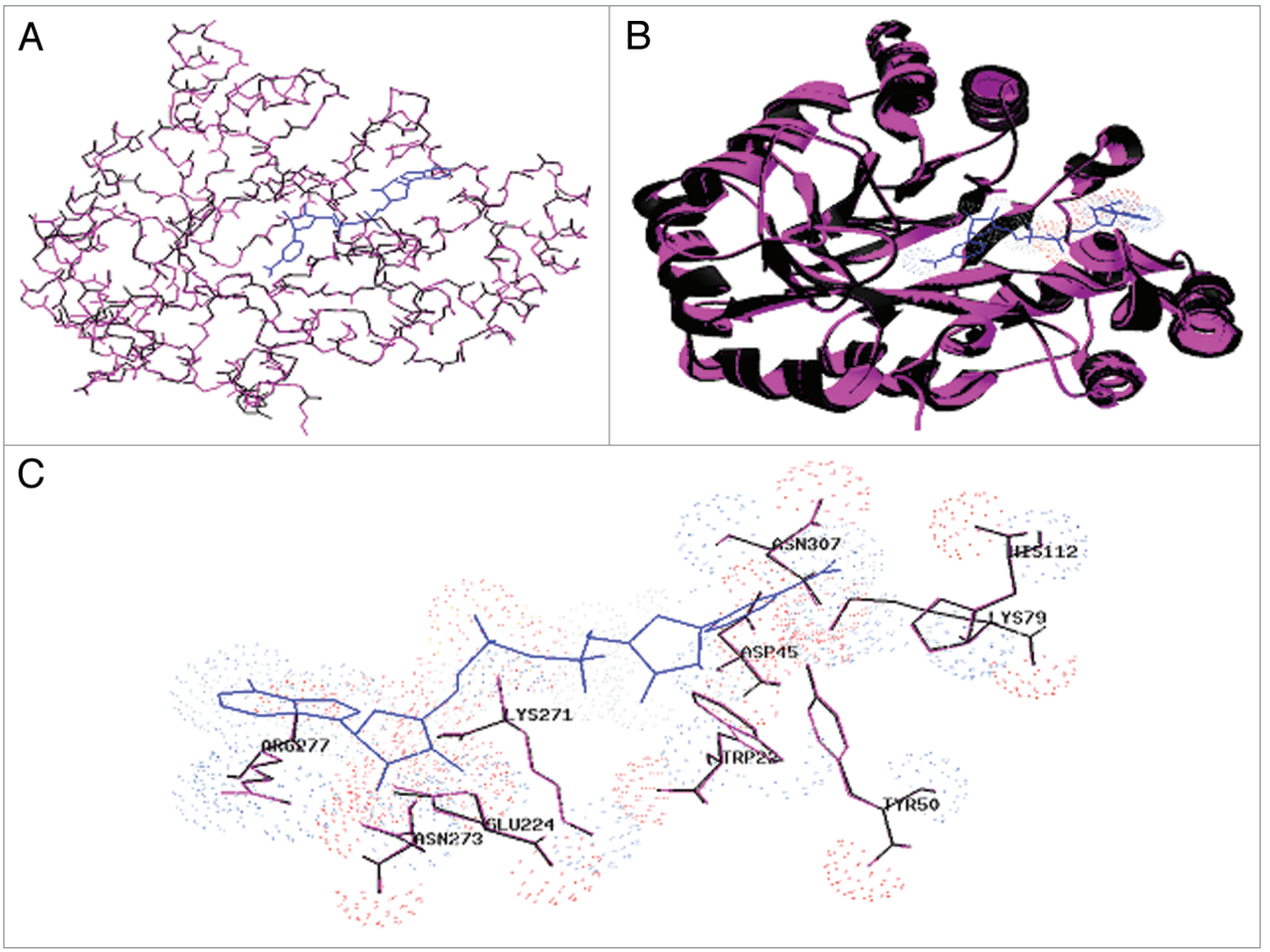

Figure 2. Superimposition using SWISS-MODEL of 2–319 amino acid backbone atoms (1,260) between the template structure 1mi3A (pink) *Te*XR (black) model (A) Cα trace, (B) ribbon trace and (C) amino acids relevant to catalysis, substrate binding and co-enzyme (blue) interaction.

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* harboring 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 *Te*Xr (**Fig. 2**). The coenzyme selectivity of *Te*Xr was altered by site-directed mutagenesis and the ability of *Te*Xr$^{K271R + N273D}$ 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 *Ct*Xr$^{K274R+N276D}$ double mutant[10] *Ps*Xr$^{K270R+N272D}$ double mutant[11,12] and a *Ps*Xr$^{K270S+S271G+N272P+R276F}$ quadruple mutant.[11] The next logical steps in our approach would be to perform a detailed investigation of *Te*Xr$^{K271R + N273D}$ 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* *Ps*Xr$^{K270R + N272D}$ 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* starins engineered with xylose reductase and xylitol dehydrogenase with strains engineered with xylose isomerase revealed that the Xr-Xdh xylose utilization pathway

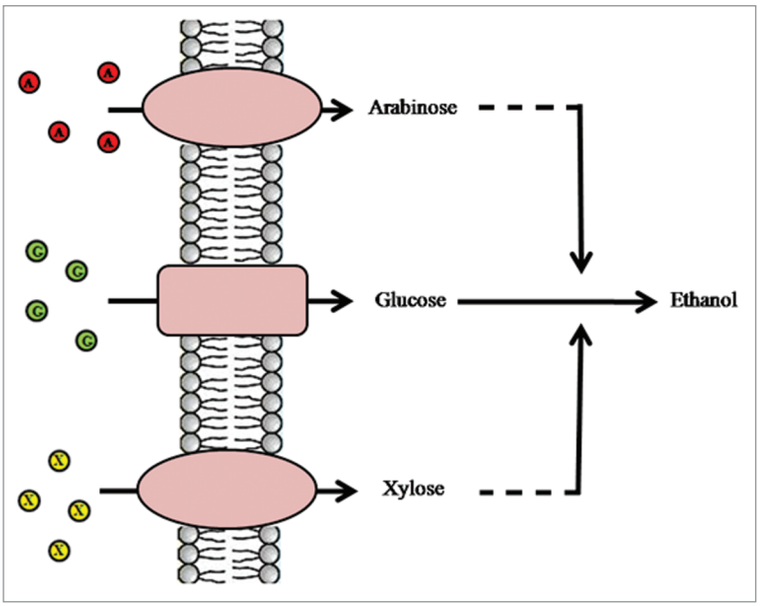

Figure 3. Ideal simultaneous transport and fermentation of hexose and pentose sugars.

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* $Xr^{K271R+N273D}$ 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 CO_2. 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 CO_2 per mole of D-glucose-6-phosphate.[17] 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 xylitol yield of only 0.05 g g^{-1},[18] and a reduced phosphoglucose isomerase activity, an enzyme that converts glucose-6-phosphate into fructose-6-phosphate, was shown to increase ethanol yield.[19] Insertion of a $NADP^+$-dependent D-glyceraldehyde-3-phosphate dehydrogenase (NADP-GAPDH) from *Kluyveromyces lactis* facilitated NADPH regeneration with no production of CO_2 in *S. cerevisiae* strains with a *ZWF1* deletion.[17] In *S. cerevisiae* strains exibiting a range of production levels of G6PDH, overexpression of a transhydrogenase from *Azotobacter vinelandii*, reduced the xylitol yield but enhanced the yield of glycerol during xylose fermentation.[20]

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. Virtually 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 utilization in xylose-fermenting *S. cerevisiae* and improved the ethanol yield.[21]

In further metabolic engineering of xylose metabolism, the xylose transport step should receive special attention. The rather high K_m values of xylose reductase 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 utilizing 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*[36] 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 characterized.

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 source provides the basic conditions for survival.[23] *T. emersonii* also grows rapidly on hexoses and pentoses as a sole carbon source and thermostable enzyme systems required for hemicellulose degradation have been described and characterized previously.[24,25] 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, Te*HTX (GenBank FJ985745) and *Te*XYLT (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 *Te*HXT is potentially a hexose transporter similarly to Gal2 from *S. cerevisiae* while *Te*XYLT has the potential to be a glucose/xylose transporter (unpublished data).

Despite the high similarity of *Te*HXT to fungal high-affinity transporters and the similarity of *Te*XYLT 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 utilize 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 biomass 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.

Acknowledgements

This work was supported by an Irish government Department of Agriculture, Food and Fisheries award (DAFF RSF-05-225) under the research stimulus fund program 2007–13.

References

1. Hahn-Hägerdal B, Jeppsson H, Olsson L, Mohagheghi A. An inter-laboratory comparison of the performance of ethanol-producing microorganisms in a xylose rich acid hydrolysate. Appl Environ Microbiol 1994; 41:62-72.
2. Hahn-Hägerdal B, Karhumaa K, Fonseca C, Spencer-Martins I, Gorwa-Grauslund MF. Towards industrial pentose-fermenting yeast strains. Appl Microbiol Biotechnol 2007; 74:937-53.
3. Kotter P, Ciriacy M. Xylose fermentation by *S. cerevisiae*. App Microb Biotechnol 1993; 38:776-83.
4. Ho NW, Chen Z, Brainard AP. Genetically engineered Saccharomyces yeast capable of effective cofermentation of glucose and xylose. Appl Environ Microbiol 1998; 64:1852-9.
5. Bao X, Gao D, Qu Y, Wang Z, Walfridssion M, Hahn-Hägerbal B. Effect on product formation in recombinant *S. cerevisiae* strains expressing different levels of xylose metabolic genes. Chin J Biotechnol 1997; 13:225-31.
6. Kostrzynska M, Sopher CR, Lee H. Mutational analysis of the role of the conserved lysine-270 in the *P. stipitis* xylose reductase. FEMS Microbiol Lett 1998; 159:107-12.
7. Petschacher B, Nidetzky B. Altering the coenzyme preference of xylose reductase to favor utilization of NADH enhances ethanol yield from xylose in a metabolically engineered strain of *Saccharomyces cerevisiae*. Microb Cell Fact 2008; 7:1-12.
8. Fernandes S, Tuohy MG, Murray PG. Xylose reductase from the thermophilic fungus *Talaromyces emersonii*: cloning and heterologous expression of the native gene (*Texr*) and a double mutant (*Texr*$^{K271R + N273D}$) with altered coenzyme specificity. J Biosci 2009; 34:881-90.
9. Moloney AP, McCrae SI, Wood TM, Coughlan MP. Isolation and characterization of the endoglucanases of *T. emersonii*. Biochem J 1985; 225:365-74.
10. Petschacher B, Leitgeb S, Kavanagh KL, Wilson DK, Nidetzky B. The coenzyme specificity of *Candida tenuis* xylose reductase (AKR2B5) explored by site-directed mutagenesis and X-ray crystallography. Biochem J 2005; 385:75-83.
11. Liang L, Zhang J, Lin Z. Altering coenzyme specificity of *Pichia stipitis* xylose reductase by the semi-rational approach CASTing. Microb Cell Fact 2007; 6:1-11.
12. Watanabe S, Saleh AA, Pack SP, Annaluru N, Kodaki T, Makino K. Ethanol production from xylose by recombinant *Saccharomyces cerevisiae* expressing protein-engineered NADH-preferring xylose reductase from *Pichia stipitis*. Microbiology 2007; 153:3044-54.
13. Petschacher B, Nidetzky B. Altering the coenzyme preference of xylose reductase to favor utilization of NADH enhances ethanol yield from xylose in a metabolically engineered strain of *Saccharomyces cerevisiae*. Microb Cell Fact 2008; 7:1-12.
14. Walfridsson M, Bao X, Anderlund M, Lilius G, Bulow L, Hahn-Hagerdal B. Ethanolic fermentation of xylose with *S. cerevisiae* harboring the *Thermus thermophilus xylA* gene, which expresses an active xylose (glucose) isomerase. Appl Environ Microbiol 1996; 62:4648-51.
15. Kuyper M, Harhangi HR, Stave AK, Winkler AA, Jetten MS, de Laat WT, et al. High-level functional expression of a fungal xylose isomerase: the key to efficient ethanolic fermentation of xylose by *S. cerevisiae*. FEMS Yeast Res 2003; 4:69-78.
16. Träff KL, Cordero Otero RR, van Zyl WH, Hahn-Hägerdal B. Deletion of the *GRE3* aldose reductase gene and its influence on xylose metabolism in recombinant strains of *S. cerevisiae* expressing the *xylA* and *XKS1* genes. Appl Environ Microbiol 2001; 67:5668-74.
17. Verho R, Londesborough J, Penttilä M, Richard P. Engineering redox cofactor regeneration for improved pentose fermentation in *S. cerevisiae*. Appl Environ Microbiol 2003; 69:5892-7.
18. Jeppsson M, Johansson B, Hahn-Hägerdal B, Gorwa-Grauslund MF. Reduced oxidative pentose phosphate pathway flux in recombinant xylose-utilizing *S. cerevisiae* strains improves the ethanol yield from xylose. Appl Environ Microbiol 2002; 68:1604-9.
19. Eliasson A, Boles E, Johansson B, Österberg M, Thevelein JM, Spencer-Martins I, et al. Xylulose fermentation by mutant and wild-type strains of *Zygosaccharomyces* and *Saccharomyces cerevisiae*. Appl. Microbiol. Biotechnol 2000; 53:376-82.
20. Jeppsson M, Johansson B, Jensen PR, Hahn-Hägerdal B, Gorwa-Grauslund MF. The level of glucose-6-phosphate dehydrogenase activity strongly influences xylose fermentation and inhibitor sensitivity in recombinant *S. cerevisiae* strains. Yeast 2003; 20:1263-72.
21. Roca C, Nielsen J, Olsson L. Metabolic engineering of ammonium assimilation in xylose-fermenting *S. cerevisiae* improves ethanol production. Appl Environ Microbiol 2003; 69:4732-6.
22. Stolk A. The genus Talaromyces. Studies in Mycology 1972; 2.
23. Tuohy MG, Coughlan MP. Production of thermostable xylan degrading enzymes by *T. emersonii* CBS 814.70. Bioresource Technol 1992; 39:131-7.
24. Tuohy MG, Puls J, Claeyssens M, Vrsanská M, Coughlan MP. The xylan-degrading enzyme system of *T. emersonii*: novel enzymes with activity against aryl beta-D-xylosides and unsubstituted xylans. Biochem J 1993; 290:515-23.