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

Mislav Oreb,* Heiko Dietz, Alexander Farwick and Eckhard Boles
Institute of Molecular Biosciences; Goethe-University Frankfurt am Main; Frankfurt am Main, Germany

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

Submitted: 06/18/12
Revised: 07/10/12
Accepted: 07/11/12
http://dx.doi.org/10.4161/bioe.21444
*Correspondence to: Mislav Oreb; Email: m.oreb@bio.uni-frankfurt.de

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.

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, forest 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 D-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. The conversion of D-arabinose to Xul5P is more complex; the pathway engineered in our laboratory involves codon-optimized D-arabinose isomerase from Bacillus licheniformis (araA), D-ribulokinase (araB) and D-ribulose-5-P 4-epimerase (araD) from E. coli. Through a cascade of reactions in the PPP, Xul5P is converted into glycolytic intermediates D-fuctose-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 lower than that of D-glucose.
slower than the fermentation of \( \alpha \)-glucose-galactose when xylulokinase (XK) and all enzymes of the non-oxidative part of the PPP, \( \alpha \)-ribulose 5-phosphate isomerase (RKI), \( \alpha \)-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 both \( \alpha \)-galactose and \( \alpha \)-glucose being 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 inhibition 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 \( S. \) cerevisiae is medi -[**348**]

10 hexose transporters, which also alleviates the inhibition. Both strategies already led to significantly improved co-fermentation of \( \alpha \)-glucose and \( \alpha \)-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 significantly improved co-fermentation of \( \alpha \)-glucose and \( \alpha \)-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 significantly improved co-fermentation of \( \alpha \)-glucose and \( \alpha \)-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 significantly improved co-fermentation of \( \alpha \)-glucose and \( \alpha \)-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 significantly improved co-fermentation of \( \alpha \)-glucose and \( \alpha \)-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 significantly improved co-fermentation of \( \alpha \)-glucose and \( \alpha \)-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 significantly improved co-fermentation of \( \alpha \)-glucose and \( \alpha \)-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 significantly improved co-fermentation of \( \alpha \)-glucose and \( \alpha \)-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 significantly improved co-fermentation of \( \alpha \)-glucose and \( \alpha \)-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 significantly improved co-fermentation of \( \alpha \)-glucose and \( \alpha \)-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 significantly improved co-fermentation of \( \alpha \)-glucose and \( \alpha \)-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 significantly improved co-fermentation of \( \alpha \)-glucose and \( \alpha \)-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 significantly improved co-fermentation of \( \alpha \)-glucose and \( \alpha \)-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 significantly improved co-fermentation of \( \alpha \)-glucose and \( \alpha \)-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 significantly improved co-fermentation of \( \alpha \)-glucose and \( \alpha \)-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 significantly improved co-fermentation of \( \alpha \)-glucose and \( \alpha \)-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 significantly improved co-fermentation of \( \alpha \)-glucose and \( \alpha \)-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 significantly improved co-fermentation of \( \alpha \)-glucose and \( \alpha \)-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 significantly improved co-fermentation of \( \alpha \)-glucose and \( \alpha \)-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 significantly improved co-fermentation of \( \alpha \)-glucose and \( \alpha \)-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 significantly improved co-fermentation of \( \alpha \)-glucose and \( \alpha \)-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 significantly improved co-fermentation of \( \alpha \)-glucose and \( \alpha \)-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 significantly improved co-fermentation of \( \alpha \)-glucose and \( \alpha \)-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 significantly improved co-fermentation of \( \alpha \)-glucose and \( \alpha \)-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 significantly improved co-fermentation of \( \alpha \)-glucose and \( \alpha \)-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 significantly improved co-fermentation of \( \alpha \)-glucose and \( \alpha \)-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 significantly improved co-fermentation of \( \alpha \)-glucose and \( \alpha \)-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 significantly improved co-fermentation of \( \alpha \)-glucose and \( \alpha \)-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 significantly improved co-fermentation of \( \alpha \)-glucose and \( \alpha \)-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 significantly improved co-fermentation of \( \alpha \)-glucose and \( \alpha \)-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 significantly improved co-fermentation of \( \alpha \)-glucose and \( \alpha \)-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 significantly improved co-fermentation of \( \alpha \)-glucose and \( \alpha \)-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 significantly improved co-fermentation of \( \alpha \)-glucose and \( \alpha \)-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 significantly improved co-fermentation of \( \alpha \)-glucose and \( \alpha \)-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 significantly improved co-fermentation of \( \alpha \)-glucose and \( \alpha \)-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 significantly improved co-fermentation of \( \alpha \)-glucose and \( \alpha \)-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 significantly improved co-fermentation of \( \alpha \)-glucose and \( \alpha \)-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 significantly improved co-fermentation of \( \alpha \)-glucose and \( \alpha \)-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 significantly improved co-fermentation of \( \alpha \)-glucose and \( \alpha \)-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 significantly improved co-fermentation of \( \alpha \)-glucose and \( \alpha \)-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 significantly improved co-fermentation of \( \alpha \)-glucose and \( \alpha \)-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 significantly improved co-fermentation of \( \alpha \)-glucose and \( \alpha \)-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 significantly improved co-fermentation of \( \alpha \)-glucose and \( \alpha \)-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 significantly improved co-fermentation of \( \alpha \)-glucose and \( \alpha \)-xylose growth medium can be applied as an evolutionary growth pressure to force the culture to accumulate beneficial mutations in order to overc....
are easily genetically modified and the functionality of α-arabinose or δ-xylose utilization pathways has been shown by various laboratories. Moreover, it has been possible to increase the resistance to fermentation inhibitors like acetate, furfural or hydroxyethylfurfural. 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
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 \(\text{d-}\)xylose and \(\text{l-}\)arabinose utilization pathways were stably integrated into the genome of the Ethanol Red strain. After evolutionary engineering, we obtained a strain efficiently fermenting \(\text{d-}\)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 \(\text{d-}\)xylose fermentation takes only about twice as long as that for \(\text{d-}\)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 \(\text{d-}\)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.

Acknowledgments
Financial support by German Federal Ministry for Environment, Nature Conservation and Nuclear Safety (BMU), Federal Ministry of Education and Research (BMBF), Federal Ministry of Food, Agriculture and Consumer Protection (BMELV) and the European Commission-Seventh Framework Programme (NEMO project) is gratefully acknowledged.

References
1. Weber C, Farwick A, Benisch F, Brat D, Dietz H, Stub T, et al. Trends and challenges in the microbial production of lignocellulosic bioalcohol fuels. Appl Microbiol Biotechnol 2010; 87:1303-15; PMID:20535464; http://dx.doi.org/10.1007/s00253-010-2707-z.
2. Van Vleet JH, Jeffries TW. Yeast metabolic engineering for hemicellulosic ethanol production. Curr Opin Biotechnol 2009; 20:300-6; PMID:19545992; http://dx.doi.org/10.1016/j.copbio.2009.06.001.
3. Young E, Lee SM, Alper H. Optimizing pentose utilization in yeast: the need for novel tools and approaches. Biotechnol Biofuels 2010; 3:24; PMID:20489029; http://dx.doi.org/10.1186/1754-6834-3-24.
4. Kohfad BC, Gupta B, Khana VP, Singh A, Zhang YHP. Bioethanol production from pentose sugars: Current status and future prospects. Bioresour Sustain Energy Rev 2011; 5:495-62. http://dx.doi.org/10.1016/j.bser.2010.07.058.

5. Bru D, Boke E, Windrum B. Functional expression of a bacterial xylose isomerase in Saccharomyces cerevisiae. Appl Environ Microbiol 2009; 75:2384-11. PMID:19324803. http://dx.doi.org/10.1128/AEM.05224-08.

6. Boker J, Boke E. A modified Saccharomyces cerevisiae strain that consumes L-Arabinose and produces ethanol. Appl Environ Microbiol 2003; 69:4146-50. PMID:12839782. http://dx.doi.org/10.1128/AEM.69.7.4146-4150.2003.

7. Windrum B, Boke E. Codon-optimized bacterial genes improve L-Arabinose fermentation in recombinant Saccharomyces cerevisiae. Appl Environ Microbiol 2008; 74:2043-50. PMID:18263741; http://dx.doi.org/10.1128/AEM.02395-07.

8. Young E, Pouget A, Cester A, Bailey A, Alper H. Functional survey for homologous sugar transport proteins, using Saccharomyces cerevisiae as a host. Appl Environ Microbiol. 2011; 77:3811-9. http://dx.doi.org/10.1128/AEM.06535-10.

9. Sahlin T, Boke E. Improving L-arabinose utilization of pentose fermenting Saccharomyces cerevisiae cells by heterologous expression of L-arabinose transporting sugar transporters. Biochim Biophys Acta 2011; 1812:43-50. http://dx.doi.org/10.1016/j.bbamcr.2010.09.003.

10. Hanashiki T, Boker E, Gudmundur M, Hacks-Hagedal B, Boke E. Characterization of the xylose-transporting properties of yeast hexose transporters and their influence on xylose utilization. Microbiology 2002; 148:2783-8. PMID:12213924.

11. Leander MJ, Forsman C, Gengelén P. Human and protozoan transport in ascomycetous yeasts an overview. FEMS Yeast Res 2005; 5:399-81. PMID:16480983; http://dx.doi.org/10.1111/j.1567-1364.2005.00509.x.

12. Younis F, Poonawala H, Ege S, Haq EI, Bailey A, Alper H. The role of ALS in xylose fermentation by engineered Saccharomyces cerevisiae mutant with improved ability to utilize xylose. FEMS Yeast Res 2011; 11:299-306. PMID:2129209. http://dx.doi.org/10.1111/j.1567-1364.2011.00719.x.

13. Petersen A, Alfreda J, Medig K, Kathomas K, Haks-Hagedal B, Greve-Grundemann MF, et al. A β-hydroxyethyl furfural inducing enzyme encoded by the Saccharomyces cerevisiae ADH6 gene confers HMF tolerance. Yeast 2006; 23:371-82. PMID:16652391. http://dx.doi.org/10.1002/yea.1673.

14. Younis F, Poonawala H, Forsman C, Ege S, Leander M, Heijnen JJ, Alper H. H. Functional survey for heterologous sugar transport proteins, using Saccharomyces cerevisiae as a host. Appl Environ Microbiol. 2011; 77:3811-9. http://dx.doi.org/10.1128/AEM.06535-10.

15. Boker E, Wisedeck K, Glatz C, Orts R, Gehr R, Heijnen JJ, Poonawala H, et al. Metabolomics, transcriptome and metabolite flux analysis of arabinoxylose fermentation by engineered Saccharomyces cerevisiae. Metab Eng 2009; 11:57-70. PMID:19004860. http://dx.doi.org/10.1016/j.meb.2008.08.003.

16. Petersen A, Alfreda J, Medig K, Kathomas K, Haks-Hagedal B, Greve-Grundemann MF, et al. A β-hydroxyethyl furfural inducing enzyme encoded by the Saccharomyces cerevisiae ADH6 gene confers HMF tolerance. Yeast 2006; 23:371-82. PMID:16652391. http://dx.doi.org/10.1002/yea.1673.