

Improvement of xylose utilisation for bioprocesses (XyloImpro) Annual report 2008 (15th of January 2009)

Dr. Laura Ruohonen, VTT Technical Research Centre of Finland

Dr. Laura Salusjärvi, VTT

M. Sc. Eija Rintala, VTT

Abstract

The project deals with biotechnological production of fuel bioethanol and other chemicals from renewable lignocellulosic raw materials. This is highly important for ecoefficient and economically feasible bioethanol production, as well as production of also other bulk chemicals from lignocellulosic biomass. The main aim of the project is to further develop and improve *Saccharomyces cerevisiae* for production of bioethanol (and other chemicals) from the xylose fraction of plant biomass. The yeast *S. cerevisiae* is the most desired process organism for industrial scale since it is robust, has high ethanol tolerance and has been extensively used in large scale processes. It also tolerates well the inhibitors found in lignocellulosic hydrolysates. We have hydrolysate tolerant proprietary strains available that have been engineered to use xylose. This research focuses on fundamental understanding of those cellular factors that we consider still the most essential in improvement of xylose utilisation by yeasts to the level required in commercial production of bioethanol and other chemicals from lignocellulosic raw materials. These are:

1. Improvement of xylose transport to the cell
2. Engineering of cellular redox metabolism for improved xylose fermentation
3. Understanding of cell responses and regulatory mechanisms involved in xylose utilisation

Results

During the second year of the project, we have focused on two of the aforementioned topics, *i.e.* improvement of xylose transport to the cell, and studying possibilities to improve the xylose fermentation of recombinant yeast *S. cerevisiae* by engineering the cellular redox balance.

Improvement of xylose transport to the cell

Interestingly, specific xylose transporters, *e.g.* transporters that would only transport xylose have been identified neither in prokaryotes nor in eukaryotes. Our earlier work (Saloheimo *et al.*, 2007) had indicated that several of the endogenous hexose transporters (HXT), acting by facilitated diffusion mode, are capable of xylose transport into the cells of *S. cerevisiae*. We chose one of these hexose transporters, HXT1 to be mutagenised and screened for improved xylose transport and preferably also for reduced competition by glucose. We have taken several approaches to generate mutant libraries of HXT1, altogether four different libraries. One library consists of clones in which HXT1 has been randomly mutagenised by error-prone PCR over its entire sequence. The three other libraries consist of clones in which semi-targeted mutagenesis of HXT1 has been carried out on sequences known or hypothesised to be important for recognition of the sugar to be transported. Two of these libraries target one of the 12 transmembrane domains (transmembrane domain 3 or 10 randomised). The transmembrane domain 3 has high homology to the xylose-recognising domain of bacterial ABC sugar transporters, whereas the transmembrane domain 10 is known to be important for glucose versus galactose recognition. Especially, two specific amino acids of transmembrane

domain 10 had been shown to be important in the glucose versus galactose recognition (Kasahara and Maeda, 1998) and the third library consists of clones in which only these amino acids were randomised.

Screening for mutants of HXT1 for improved xylose transport has been conducted in the two following ways. First, a screening strategy based on xylitol production was developed for high throughput screening by robotics. A host strain carrying the gene encoding xylose reductase (XR) was transformed with the HXT1 mutant library randomised at the two amino acids known to play a role in sugar recognition. The strain lacks the major hexose transporters and thus grows only on maltose. The clones were grown on maltose plates, picked by a colony picker to microtitre plates for further growth on maltose for 2-3 days. Subsequently, consumption of maltose was measured as indicative of growth, and xylose was added. Production of xylitol was measured after 3 h of incubation. Second, screening for improved xylose uptake was carried out on xylose plates for improved growth on this sugar with a strain harbouring the entire xylose consumption pathway, *i.e.* in addition to XR also xylitol dehydrogenase and xylulose kinase were present in the screening host, and its major hexose transporter encoding genes either intact or deleted.

Preliminary screenings have been carried out with the two afore described approaches. Interestingly, the approach with randomisation of the two amino acids known to play a role in sugar recognition resulted in no improved variants, but in a vast majority of deteriorated variants, indicating the importance of these amino acids to remain conserved in the hexose transporters. However, some clones have been identified from the randomised HXT1 mutant library as faster growers on xylose. These clones will be further characterised and the screening will be continued.

Only recently, a glucose/xylose symporter (GXS) was identified from *Candida intermedia* (Leandro *et al.*, 2006, 2008). We have initiated collaboration with the University of Bari, Italy (Luigi Palmieri) to study the corresponding homologue of *Pichia stipitis*. We have now expressed the PsGXS in *S. cerevisiae* on a multicopy plasmid, and been able to measure modest xylose uptake *in vitro* using radioactive labelled xylose. However, no clear improvement of growth on xylose has been observed. In *C. intermedia* an interesting regulatory phenomenon is observed; the mRNA of GXS is degraded in the presence of high glucose in a strain with the native transporter encoding genes present, acting by facilitated diffusion. It is possible that similar regulation also occurs with the *P. stipitis* homologue. We are planning random mutagenesis of the PsGXS encoding gene to assess the possibility to overcome such regulation.

Engineering of cellular redox metabolism for improved xylose fermentation

The so called oxidoreductive pathway of xylose conversion in the recombinant yeast *S. cerevisiae* consists of two enzymes, xylose reductase (XR) and xylitol dehydrogenase (XDH). Even though redox neutral as such; the former enzyme reducing xylose to xylitol and the latter oxidising xylitol to xylulose, which enters the pentose phosphate pathway after phosphorylation by xylulokinase, the pathway results in redox cofactor imbalance in the cell. This is due to the different cofactor specificities of the two enzymes, the reductase preferring NADPH, the dehydrogenase being strictly NAD⁺-dependent. This pathway has been the only route to xylose-metabolising yeast *S. cerevisiae* strains until recently, thus enhancement of the oxidoreductive conversion continues to be important, not least due to also the complex global IPR situation in the field.

To overcome the cellular redox cofactor imbalance in the xylose yeast we have constructed xylose yeast strains that express an endogenous NADH-kinase encoding gene *POS5* in a truncated form encoding a cytosolic enzyme, or a MMP1489 gene encoding a bifunctional NADP⁺ phosphatase/NAD⁺-kinase enzyme from Archaeal *Methanococcus maripaludis*. Based on homology to the

corresponding protein of *Methanococcus jannaschii*, this protein has a NADP⁺-phosphatase activity in its N-terminus and a NAD⁺-kinase activity in its C-terminus. We are also testing whether the NAD⁺-kinase activity of *M. maripaludis* MMP1489 could be decreased and the NADH-kinase activity increased by two different amino acid changes in the protein, or whether the N-terminal NADP⁺-phosphatase domain of MMP1489 could function alone in the xylose-fermenting yeast strain. The *POS5* and MMP1489 genes are expressed separately and together in order to create a transhydrogenase-like cycle in the xylose-fermenting *S. cerevisiae*. In an optimal situation, Pos5p would convert NADH to NADPH for the XR reaction, and MMP1489 phosphatase activity would convert NADP⁺ to NAD⁺ for the XDH reaction. It is, however, not known what the physiological activity of MMP1489 protein will be when expressed in *S. cerevisiae*. The genes are integrated into the *ADH2* and *UTR1* loci. *ADH2* encodes the alcohol dehydrogenase isoenzyme that is mainly involved in oxidation of ethanol to acetaldehyde while *UTR1* encodes a cytosolic NAD⁺-kinase. Consequently, disruption of these genes may further improve the xylose fermentation properties of the strain.

Shake flask batch cultures, both microaerobic and anaerobic, showed that there was increased xylitol production by the strains with *POS5* or MMP1489 variants. Additionally, increased intracellular NADH-kinase activity was measured in the cell lysates of the transformants, showing that the enzymes encoded by these genes had *in vivo* activity in the heterologous yeast host. However, no clear phosphatase activity was detected in the strains with either the NADP⁺-phosphatase/NAD⁺-kinase encoding gene, or with the NADP⁺-phosphatase encoding part of the gene from *M. maripaludis*. Increased xylitol accumulation, 30-40% higher levels of xylitol observed compared to the control strain, suggests that NADPH concentration in the cells was increased being available for the XR reaction. However, the lack of phosphatase activity leaves NAD⁺ availability still as a limiting factor for enhanced xylose fermentation in these strains. We thus continue to assess possibilities to increase NAD⁺ concentration in the xylose-fermenting yeast hosts. An option would be to screen metagenomic or cDNA libraries for redox enzymes.

An alternative way to overcome the redox cofactor imbalance created by the oxidoreductive pathway would be to convert the xylose directly by isomerisation to xylulose, by a xylose isomerase enzyme. Extensive work on this option has only recently resulted in efficient xylose-metabolising yeast strains converting xylose by isomerisation to xylulose by *Piromyces sp.* xylose isomerase. We have looked for alternative/novel xylose isomerase encoding genes. Such gene candidates from *Tetraodon nigroviridis*, *Strongylocentrotus purpuratus*, in addition to barley xylose isomerase encoding gene, have so far been expressed in *S. cerevisiae* yeast. Preliminary shake flask batch cultivations and enzyme activity measurements did not, however, show any xylose isomerase activity in these strains. We thus need to conclude that these enzymes are either not true homologues of xylose isomerase or they are not functional in the yeast heterologous host.

In summary, work during the second year of the project has focused on means to overcome the cellular redox cofactor imbalance of xylose yeast, and on the improvement of xylose transport to the cell. We will continue around these topics during 2009, and in addition, focus on the regulatory mechanisms involved in xylose utilisation. We have earlier shown that the phosphoproteome appears different in recombinant *S. cerevisiae* metabolising xylose compared to glucose, and additionally, that the sensing and signalling pathways differ in xylose yeast compared to cells metabolising glucose (Salusjärvi *et al.*, 2008). We will continue to address these questions during 2009.

References

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