

## **Improvement of xylose utilisation for bioprocesses (XyloImpro)**

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#### **Major achievements 2009**

- **Screening of mutant transporter libraries (*PsGXS*, *HXT1/TM3*, *HXT1/TM10*) for enhanced xylose uptake**
  - characterisation of previously found positive clones (cultivations, uptake measurements, sequencing, TRAC analysis of *HXT* expression in adapted H2219 strain)
  - second round of library screening (plates, Bioscreen cultivations, shake flask cultivations)
  - 12 clones with mutated *PsGXS* and improved growth on xylose compared with a control strain to be further characterized
- **Cloning of *bxl1* encoding  $\beta$ -xylosidase of *Trichoderma reesei* from cDNA bank and its expression in *S. cerevisiae* in order to construct a host strain for screening DNA libraries for xylose transporters**
- **Cloning and expression of fumarate reductase of *Trypanosoma brucei* in xylose-utilising *S. cerevisiae* (characterization of transformants going on)**
- **Screening of 424 strains from VTT Culture collection for aerobic and anaerobic growth on xylose and xylose reductase and xylitol dehydrogenase activity measurements of selected 47 strains**

#### **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 third year of the project, we have mainly 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), and the third library consists of clones in which two amino acids, known to be important for glucose versus galactose recognition 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 microtiter 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 carrying the entire xylose consumption pathway, *i.e.* in addition to XR also xylitol dehydrogenase (XDH) and xylulose kinase (XKS) were present in the screening host.

Screening of the *HXT1* mutant libraries has 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, two clones were identified from the randomised *HXT1* mutant library as faster growers on xylose. These clones were further characterised during 2009 in shake flask cultures on xylose and xylose uptake measurements showed that these clones had a somewhat increased xylose uptake. However, after re-transformation of the plasmids with mutagenised *HXT1* into the host strain the growth advantage and improved transport were lost suggesting that phenotype was rather due to strain specific adaptation to xylose than improved properties of mutagenised *HXT1*. Despite thorough screening on xylose plates, mutagenesis of transmembrane domains 3 or 10 did not result in clones and *HXT1* transporters with improved xylose uptake properties.

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*. When *PsGXS* was expressed in *S. cerevisiae* on a multicopy plasmid, modest xylose uptake *in vitro* was measured by using radioactive-labelled xylose. However, no clear improvement of growth on xylose was 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. In order to improve xylose transport properties of PsGXS when expressed in *S. cerevisiae*, random mutagenesis of the *PsGXS* encoding gene was carried out. Screening of the *PsGXS* mutant libraries was carried out on xylose plates and faster growing clones were further characterised by shake flask and Bioscreen cultivations. As a result six positive clones with mutated *PsGXS* that grew better on xylose compared with the control strain were found. Sequencing, further characterisation and verification of phenotype of these clones is ongoing.

Xylose uptake assays with radio-labelled xylose are tedious and not suited for large scale screening of transporter libraries while on the other hand screening for improved transport by growth on xylose plates leads generally to large amount of false positive clones. Thus, we have taken an approach to construct a host *S. cerevisiae* strain for more efficient screening of transport libraries based on the method published by Chen et al., 2009 for *E. coli*. Basically, as demonstrated with *E. coli*, xylose uptake could be measured with a xylose analogue *p*-nitrophenyl-D-xylopyranoside (pNPX) in a yeast strain expressing intracellular  $\beta$ -xylosidase.  $\beta$ -xylosidase releases *p*-nitrophenyl group from pNPX that gives a detectable colour the intensity of which is proportional to the amount of pNPX taken up by the cells. For this purpose we expressed  $\beta$ -xylosidase encoding *bx11* of *Trichoderma reesei* in *S. cerevisiae*. The signal sequence (first 20 amino acids) of *bx11* was removed in order to retain *bx11* intracellular. Unfortunately, *bx11* did not have any  $\beta$ -xylosidase activity in *S. cerevisiae* although expression of its gene was confirmed by Northern blot. Other options for  $\beta$ -xylosidases potentially more active in *S. cerevisiae* are being evaluated.

#### *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  $\text{NAD}^+$ -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, and a MMP1489 gene encoding a bifunctional  $\text{NADP}^+$  phosphatase/ $\text{NAD}^+$ -kinase enzyme from Archaean *Methanococcus maripaludis*. Based on homology to the corresponding protein of *Methanococcus jannaschii*, this protein has a  $\text{NADP}^+$ -phosphatase activity in its N-terminus and a  $\text{NAD}^+$ -kinase activity in its C-terminus. The *POS5* and MMP1489 genes were 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  $\text{NADP}^+$  to  $\text{NAD}^+$  for the XDH reaction.

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 the  $\text{NADP}^+$ -phosphatase/ $\text{NAD}^+$ -kinase encoding 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  $\text{NAD}^+$  availability still as a limiting factor for enhanced xylose fermentation in these strains.

We consequently continued to assess possibilities to increase  $\text{NAD}^+$  concentration in the xylose-fermenting yeast hosts. For this purpose we expressed in the xylose-utilising *S. cerevisiae* the fumarate reductase from *Trypanosoma brucei* alone and in combination with *POS5* or *MMP1489*. Unlike the endogenous  $\text{FAD}^+$ -linked fumarate reductase of *S. cerevisiae*, the enzyme from *T. brucei* is unique by reducing fumarate to succinate with  $\text{NADH}$ , thus producing  $\text{NAD}^+$  in the reaction. Preliminary results from aerobic shake flask cultures on xylose with the strains expressing the fumarate reductase encoding gene alone or together with *POS5* suggest that both strains grew slower compared with their controls. The strain with both fumarate reductase encoding gene and *POS5* had, however, considerably better growth compared with the strain with only fumarate reductase encoding gene, suggesting that fine-tuning of these reactions or further metabolic engineering on these pathways could result in closed redox balance and improved fermentation of xylose. Moreover, characterisation of these strains under anaerobic conditions is ongoing and will give information on the effect of these enzymes on xylose fermentation under conditions that are closer to the actual process, and also more challenging in respect to the redox status of the cell.

An alternative way to overcome the redox cofactor imbalance created by the oxidoreductive pathway would be to convert the xylose directly by a xylose isomerase, or to express in *S. cerevisiae* XR and XDH enzymes that would have more preference for  $\text{NADH}$  or  $\text{NADP}^+$ , respectively, than the enzymes of *P. stipitis*. We have looked for alternative/novel xylose isomerase, XR or XDH encoding genes by screening 427 yeast strains from VTT Culture Collection for growth on xylose. XR and XDH activities of selected 47 strains that had some micro-anaerobic growth on xylose were measured. Preliminary results are interesting. For example, some strains seemed to have very high XR and XDH activities, or very high XDH activity while some strains had more XDH activity with  $\text{NADP}^+$  than  $\text{NAD}^+$ . Further characterisation of these strains is ongoing.

In summary, work during the third 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 the ongoing work around these topics during 2010, and in addition, we are planning to screen metagenomic and cDNA libraries for xylose transporters and redox enzymes. Some focus will also be put on the regulatory mechanisms involved in xylose utilisation and how it differs between recombinant *S. cerevisiae* and naturally xylose-utilising yeast. 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 2010.

## References

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