Engineering endogenous hexose transporters in Saccharomyces cerevisiae for efficient D-xylose transport
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SUMMARY

For 1st and 2nd generation bioethanol formation, the yeast *Saccharomyces cerevisiae* is the premier organism for fermentation. However, *S. cerevisiae* cannot naturally ferment pentose sugars like D-arabinose and D-xylose which are main products, next to D-glucose, of lignocellulosic biomass conversion in the 2nd generation bioethanol production process. Therefore, a xylose pathway was introduced via the expression of a fungal xylose isomerase in order for D-xylose to enter the pentose phosphate pathway (Figure 1). Although this resulted in the desired D-xylose fermentation, the consumption of D-xylose in the presence of high concentrations of D-glucose present in the lignocellulosic hydrolysate remains difficult. In general, xylose-fermenting *S. cerevisiae* strains first consume D-glucose, before D-xylose is metabolized. The figure shows the metabolic pathways involved.
The increased D-xylose affinity could be related to the replacement of the C-terminus of Hxt2, more specifically to a cysteine to proline mutation at position 505 in Hxt2. The Hxt2-Y353C mutation could provide a way to increase the D-xylose transport flux at low D-xylose concentration in e.g., at the end of an industrial fermentation when D-xylose concentration is nearly zero. However, these chimeric Hxt transporters are still capable of transporting D-glucose and thus the chimeras do not provide a solution for the D-glucose inhibition of D-xylose transport.

In chapter 3, therefore another approach was followed. Herein, a quadruple hexokinase deletion mutant of the D-xylose fermenting S. cerevisiae strain DS69473 was used in an in vivo engineering approach.
transporters for low affinity pentose uptake. To improve the specificity towards D-xylose the aforementioned DS71054 strain was used in an evolutionary engineering experiment to select for growth on D-xylose in the presence of high D-glucose concentrations. This resulted in D-glucose-tolerant growth of the yeast on D-xylose. This phenomenon, in DS71054-evoB, could be attributed to a mutation at N367 in the endogenous chimeric Hxt36 transporter causing a defect in D-glucose transport while still allowing uptake of D-xylose (Figure 1A). The Hxt36 N367I mutation created a specific D-xylose binding site, albeit with a slightly reduced affinity and lowered $V_{\text{max}}$. Specifically, the bulky isoleucine group prevents the aldehyde group of D-glucose to bind efficiently in the binding pocket, and hence the affinity for D-glucose is reduced significantly magnitude. Saturation mutagenesis of the N367 position yielded the variant Hxt36 N367A that transports D-xylose with a high rate and improved affinity. In fermentation trials, this mutant allowed the efficient co-consumption of D-glucose and D-xylose when provided in equimolar concentration. Therefore, this transporter can potentially be used for efficient lignocellulosic bioethanol production.

Although the endogenous hexose transporter Hxt36 was successfully engineered into a specific D-xylose transporter, Hxt36 is subjected to D-glucose-regulated protein degradation. Therefore, in chapter 5, protein degradation of hexose transporters was studied and optimized. In the absence of glucose or when the D-glucose is exhausted from the medium, some Hxt proteins with high D-xylose transport capacity are rapidly degraded and removed from the cytoplasmic membrane. Thus, turnover of such Hxt proteins may lead to poor growth on solely D-xylose. In contrast, Hxt11, which is normally not expressed in S. cerevisiae, is not subjected to protein degradation as not recognized by the quality control mechanisms. It remains on the cytoplasmic membrane both at high and low D-glucose concentrations. At low D-glucose concentration protein degradation is a major issue for the low affinity hexose transporters Hxt1, Hxt36 (Hxt3 variant) and Hxt5 which are also subjected to catabolite degradation. This is evidenced by a loss of the aforementioned hexose transporters from the membrane upon D-glucose depletion as monitored with GFP fusions. The catabolite degradation occurs through ubiquitination, which is a major signaling pathway for turnover. N-terminal lysine residues of the aforementioned Hxt proteins predicted to be the target of ubiquitination were replaced for arginine residues. The mutagenesis resulted in improved membrane localization when cells were grown on solely D-xylose concomitantly with significantly stimulated growth on D-xylose. The decreased ubiquitination also improved the late stages of sugar fermentation when cells are grown on both D-glucose and D-xylose.

Although the evolved hexokinase deletion DS71054-evoB strain bearing the Hxt36 N367I mutation from chapter 4 showed significantly improved growth on D-xylose in the presence of high concentrations of D-glucose, the mutant suffers from a reduced $V_{\text{max}}$ for xylose transport. This was partially solved by the aforementioned saturation mutagenesis of position N367, which yielded the Hxt36 N367A mutant with improved D-xylose transport properties, but this mutant also regained some D-glucose transport activity. Therefore, in chapter 6 a 2nd round of evolutionary engineering was performed in order to determine if the capacity of the Hxt36 N367I mutant to transport D-xylose can be improved by additional mutations while maintaining the strict specificity for D-xylose. The hexokinase deletion DS71054-evoB strain was evolved in a chemostat during three months on 1% D-xylose in the presence of 10% D-glucose. During the course of the experiment the dilution rate was gradually increased to select for faster growth. Whereas several intermediate evolved strains with improved growth rates were isolated, the final strain DS71054-evoG shows equal growth rates on D-xylose only as compared to D-xylose in presence of an excess D-glucose. The improved growth rate could be attributed to improved D-xylose uptake via increased expression levels of Hxt37 N367I, and an overall reduction in the expression of other Hxt transporters except for Hxt4. Re-introduction of the hexokinase Hxk2 (and other hexokinases) in the evolved DS71054-evoB mutant restored D-glucose consumption. However, the Hxk2 complemented evolutionary engineering lineage showed an increased D-xylose consumption that was paralleled by a decreased D-glucose consumption. Overall this led to a net reduction in sugar consumption and growth rate. On the other hand, consumption of D-glucose alone was restored to the levels of the parental and the non-evolved strain leading to the conclusion that glucose consumption per se was not altered in the evolved strains. When the Hxk2 complemented evolved strains were grown on a mixture of D-xylose and D-glucose, the progressive accumulation of trehalose-6-phosphate was observed. Trehalose-6-phosphate is an inhibitor of hexokinases and its