Overcoming glucose repression in mixed sugar fermentation by co-expressing a cellobiose transporter and a β-glucosidase in *Saccharomyces cerevisiae*†

Sijin Li, a Jing Du, a Jie Sun, a,b Jonathan M. Galazka, c N. Louise Glass, d Jamie H. D. Cate, c Xiaomin Yang e and Huimin Zhao* a

Received 22nd June 2010, Accepted 19th August 2010
DOI: 10.1039/c0mb00063a

Glucose repression is one of the main limitations in mixed lignocellulosic sugar fermentation for cost-effective production of fuels and chemicals. Here we report a novel strategy to overcome glucose repression by co-expressing a cellobiose transporter and a β-glucosidase in an engineered D-xylose-utilizing *Saccharomyces cerevisiae* strain. The resulting strain can simultaneously utilize cellobiose and D-xylose for ethanol production.

Lignocellulosic biomass consisting of cellulose, hemicellulose, and lignin is a potential sustainable source of mixed sugars for fermentation to fuels and chemicals.1 Co-utilization of all the available sugars including hexoses such as glucose and pentoses such as D-xylose and L-arabinose present in the lignocellulosic hydrolysates is critical to the overall process economics. Recombinant *Saccharomyces cerevisiae* strains containing a heterologous pentose utilization pathway can utilize mixed sugars including glucose, D-xylose, and L-arabinose to produce ethanol.2–5 However, one of the main limitations in the fermentation of mixed sugars is glucose repression, i.e. utilization of pentose sugars is inhibited by the presence of glucose (Fig. 1).6,7 This results in delayed utilization of pentose sugars and significantly reduced overall ethanol productivity.

Glucose repression is a well-studied regulatory mechanism in *S. cerevisiae*.8–11 Various approaches have been attempted to overcome glucose repression, such as evolutionary engineering12 and deletion of key genes involved in glucose repression, such as *MIG1* and *MIG2*.13 However, these approaches have met with limited success. Here we report a new strategy in which a cellobiose transporter gene and a β-glucosidase gene are co-expressed in *S. cerevisiae* and instead of glucose, pentose sugars derived from lignocellulose are used. In contrast, a mixture of cellobiose and pentose sugars will be used in this new strategy. The cellobiose will be transported into yeast cells via the heterologous cellobiose transporters, while pentose sugars will be transported into yeast cells by endogenous hexose transporters, thus mitigating the direct competition between glucose and pentose sugars for the same transporters that partly causes glucose repression. Once inside yeast cells, cellobiose will be converted to glucose by β-glucosidases. Pentose sugars are the products of dilute acid hydrolysis of hemicellulose. In conventional methods for sugar fermentation in *S. cerevisiae*, a mixture of glucose and pentose sugars derived from lignocellulose are used. In this new strategy, a mixture of cellobiose and pentose sugars will be used in this new strategy. The cellobiose will be transported into yeast cells via the heterologous cellobiose transporters, while pentose sugars will be transported into yeast cells by endogenous hexose transporters, thus mitigating the direct competition between glucose and pentose sugars for the same transporters that partly causes glucose repression. Once inside yeast cells, cellobiose will be converted to glucose by β-glucosidase and consumed, which should result in a low intracellular glucose concentration, thereby further alleviating glucose repression.

As proof of concept, the mixed sugar fermentation consisting of D-xylose and cellobiose was used as a model system. Specifically, an engineered xylose-utilizing yeast strain HZ3001 was used as a host to co-express a cellobiose transporter gene and a β-glucosidase gene. In this strain, the D-xylose utilization pathway consisting of xylose reductase, xylitol dehydrogenase, and xylulokinase from *Pichia stipitis* was integrated into the chromosome (ESI†). Three recently discovered cellobiose transporter genes from *Neurospora crassa*, including *cdt-1*, *NCU00809*, and *cdt-2*,14 and two
β-glucosidase genes, one from *N. crassa* (*ghl-1*) and the other from *Aspergillus aculeatus* (*BGL1*), were evaluated.

A total of six different strains, referred to as SL01 through SL06, were constructed by introducing a pRS425 plasmid harboring one of the cellobiose transporter genes and one of the β-glucosidase genes into the HZ3001 strain. In each plasmid, the cellobiose transporter gene and the β-glucosidase gene were assembled into the multi-copy plasmid pRS425 by the DNA assembler method (Fig. S1, ESI†). The empty pRS425 plasmid was introduced to the HZ3001 strain to yield the SL00 strain, which was used as a negative control. All strains were cultivated in YPA medium supplemented with 40 g L\(^{-1}\) cellobiose and 50 g L\(^{-1}\) D-xylose in shake-flasks, and their sugar consumption rates, cell growth rates, and ethanol titers were determined (Fig. S2, ESI†). Among all strains, the SL01 strain containing the β-glucosidase gene *ghl-1* from *N. crassa* and the cellobiose transporter gene *cdt-1* showed the highest sugar consumption rate and ethanol productivity. Thus, this strain was selected for further characterization.

Both SL01 and SL00 were cultivated using YPA medium supplemented with 40 g L\(^{-1}\) cellobiose and 50 g L\(^{-1}\) D-xylose in both shake-flasks and bioreactors (Fig. 2). In the shake-flask cultivation (Fig. 2a and b), 83% of the cellobiose was consumed in 96 hours by SL01, with a 41.2% higher overall D-xylose consumption rate (from 0.33 g L\(^{-1}\) h\(^{-1}\) to 0.46 g L\(^{-1}\) h\(^{-1}\)) compared to SL00. Consistent with the enhanced sugar consumption rate, 2.3-fold increased overall dry cell weight growth rate was observed (from 0.031 g dry cell weight L\(^{-1}\) h\(^{-1}\) to 0.072 g dry cell weight L\(^{-1}\) h\(^{-1}\)). The ethanol productivity was increased by more than 3.1-fold, from 0.07 g L\(^{-1}\) h\(^{-1}\) to 0.23 g L\(^{-1}\) h\(^{-1}\). The highest ethanol yield of 0.31 g per g sugar was reached in 48 hours, and the overall ethanol yield was 0.28 g per g sugar, representing a 23% increase compared to the SL00 strain. In the SL01 cultivation, a faster D-xylose consumption rate was observed, without the lag phase that is the hallmark of glucose repression in co-fermentation of glucose and D-xylose. Moreover, improved cell growth and ethanol production were also observed.

In the bioreactor cultivation (Fig. 2c and d), almost all of the cellobiose and 66% of the D-xylose were consumed in 48 hours by SL01, representing 42% increased D-xylose consumption rate (from 0.48 g L\(^{-1}\) h\(^{-1}\) to 0.68 g L\(^{-1}\) h\(^{-1}\)) and 1.02-fold increased dry cell weight growth rate (from 0.08 g dry cell weight L\(^{-1}\) h\(^{-1}\) to 0.17 g dry cell weight L\(^{-1}\) h\(^{-1}\)) compared to SL00. The ethanol productivity was increased by more than 4.4-fold (from 0.09 g L\(^{-1}\) h\(^{-1}\) to 0.49 g L\(^{-1}\) h\(^{-1}\)) and the ethanol yield was 0.39 g per g sugar. Compared to shake-flask cultivations, sugar consumption rates in the first 24 hours were lower due to the low cell density used in the beginning of batch cultivation.

Unexpectedly, a small amount of glucose was detected in the SL01 strain even though there was no glucose added in the

---

**Fig. 2** Concentrations of cellobiose (■), glucose (●), D-xylose (▲), ethanol (▼) and dry cell weight (□) of strains SL01 (a, c) and SL00 (b, d) in YPA medium supplemented with 40 g L\(^{-1}\) cellobiose and 50 g L\(^{-1}\) D-xylose in shake-flasks (a, b) and bioreactors (c, d), plotted as a function of time. Error-bars indicate standard deviations of triplicate samples.
medium (Fig. 2a and c). The maximum glucose concentration was reached in approximately 24 hours in both shake-flasks (12.1 g L\(^{-1}\)) and bioreactors (17.2 g L\(^{-1}\)) and then dropped to a very low level. However, no obvious glucose repression was observed and this glucose was also consumed. The reason for the observed transient presence of glucose in the extracellular medium will require future analysis.

A small amount of glucose (less than 10% of total sugars) is typically present in lignocellulosic hydrolysates when cellulase cocktails that are deficient in \(\beta\)-glucosidase activities were used to catalyze the hydrolysis of lignocellulosic materials.\(^{17,18}\) Thus, the fermentation performance of the engineered SL01 strain was also investigated using a mixture of cellobiose, \(\alpha\)-xylose, and glucose. Two concentrations of glucose, 5 g L\(^{-1}\) or 10 g L\(^{-1}\), were combined with 40 g L\(^{-1}\) cellobiose and 50 g L\(^{-1}\) \(\alpha\)-xylose as a mixed carbon source in bioreactors. In the batch cultivation with 5 g L\(^{-1}\) glucose (Fig. 3a and b), 81.5% cellobiose and 69.3% \(\alpha\)-xylose were consumed, respectively, by SL01 at 48 hours. Compared to SL00, the \(\alpha\)-xylose consumption rate was increased by 89%, from 0.38 g L\(^{-1}\) h\(^{-1}\) to 0.73 g L\(^{-1}\) h\(^{-1}\). The ethanol productivity was increased by 2.2-fold (from 0.13 g L\(^{-1}\) h\(^{-1}\) to 0.30 g L\(^{-1}\) h\(^{-1}\)). The ethanol yield was increased from 0.24 g per g sugar to 0.30 g per g sugar at 72 hours. As a control, the fermentation performance of the engineered SL01 strain was investigated using 10 g L\(^{-1}\) glucose or a mixture of 10 g L\(^{-1}\) glucose and 40 g L\(^{-1}\) cellobiose (Fig. S3, ESI\(^{\text{w}}\)). It was found that the presence of cellobiose increased the overall ethanol productivity, but its effect on the maximal ethanol yield and productivity seems to be insignificant.

Taken together, the engineered SL01 strain showed both a higher efficiency of sugar consumption and a higher rate of ethanol production than the SL00 wild type strain. More importantly, there was no significant glucose repression in the co-fermentation of three sugars even with glucose up to 10% total sugars (Fig. 3c and d), suggesting that this approach may be viable for industrial applications.

Conclusions

By co-expressing a cellobiose transporter gene and a \(\beta\)-glucosidase gene in an engineered \(\alpha\)-xylose-utilizing \textit{S. cerevisiae} strain and using mixed sugars including \(\alpha\)-xylose and cellobiose or \(\alpha\)-xylose, cellobiose, and a small amount of glucose as carbon sources, we demonstrated that these mixed sugars can be
consumed simultaneously to produce ethanol with high yields. Overcoming glucose repression in mixed sugar fermentation in *S. cerevisiae* improved the overall sugar utilization efficiency and ethanol productivity, which is highly desirable in biofuels production. Further studies on the discovery and engineering of more efficient cellobiose transporters and β-glucosidases coupled with engineering of a more efficient xylose-utilizing pathway are in progress.

This work was supported by the BP Energy Biosciences Institute.

**Notes and references**