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OPTIMIZING THE DIRECT UPTAKE AND INTRACELLULAR CELLOBIOSE HYDROLYSIS BY RECOMBINANT *SACCHAROMYCES CEREVISIAE*

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ABSTRACT

Developing recombinant *Saccharomyces cerevisiae* strains capable of transporting and fermenting cellobiose directly is a promising strategy for second-generation ethanol production from lignocellulosic biomass. In this study we cloned and expressed in the CEN.PK2-1C strain an intracellular β-glucosidase (*SpBGL2*) from *Spathaspora passalidarum*, and co-expressed the known cellobiose transporter *SsHXT2.4* from *Scheffersomyces stipitis*, and two putative cellobiose transporters from *Candida tropicalis* (*CtCBT1*) and *Meyerozyma guilliermondii* (*MgCBT2*). While all three transporters allowed cell growth on cellobiose, only the *MgCBT2* permease allowed cellobiose fermentation, although cellobiose consumption was stuck (incomplete). The analysis of the β-glucosidase and transport activities revealed that the cells stopped to consume cellobiose due to a drop in the transport activity. Since ubiquitination of lysine residues at the N- or C-terminal domains of the permease are involved in the endocytosis and degradation of sugar transporters, we constructed truncated versions of the permease lacking lysines at the C-terminal domain (*MgCBT2*ΔC), and at both the C- and N-terminal domain (*MgCBT2*ΔNΔC), and co-expressed these permeases with the *SpBGL2* β-glucosidase in an industrial strain. While the strain harboring the *MgCBT2*ΔC transporter continued to produce stuck cellobiose fermentations as the wild-type *MgCBT2* permease, the strain with the *MgCBT2*ΔNΔC permease was able to consume and ferment all the cellobiose present in the medium. Thus, our results highlight the importance of expressing cellobiose transporters lacking lysine at the N- and C-terminal domains for efficient cellobiose fermentation by recombinant *S. cerevisiae*.

Keywords: Bioethanol. β-glucosidase. Cellobiose. Transporter. Lysine Ubiquitination. Yeast.

1 INTRODUCTION

Second-generation (2G) bioethanol from lignocellulosic biomass is a promising renewable fuel[1]. Residual plant biomasses used as raw materials for 2G bioethanol production are rich in the polysaccharides cellulose and hemicellulose, which need to be hydrolyzed to release the sugars that will be fermented by industrial *S. cerevisiae* yeasts. In the enzymatic cocktails used for biomass hydrolysis, the enzyme β–glucosidase (EC 3.2.1.21) is one of the most expensive enzymes used, contributing to the higher cost of 2G bioethanol production, when compared to first-generation bioethanol[2,3]. An interesting approach to overcome this limitation is the development of recombinant *S. cerevisiae* strains capable of fermenting cellobiose directly through the transport of the disaccharide by a permease and its intracellular hydrolysis, producing glucose that will be fermented into ethanol[4]. This intracellular hydrolysis of cellobiose has also the advantage of not producing glucose extracellularly, and consequently xylose (the second most abundant sugar in lignocellulosic biomass) will not compete with glucose for the *HXT* hexose transporters present in *S. cerevisiae*, which have higher affinity for glucose[5,6].

Therefore, the present study aimed to develop a *S. cerevisiae* strain capable of transporting cellobiose directly from the medium, and its intracellular hydrolysis mediated by a β–glucosidase (*SpBGL2*) from *Sp. passalidarum*. We co-expressed the known cellobiose transporter *SsHXT2.4* from *Sc. stipitis*, and two putative cellobiose transporters from *C. tropicalis* (*CtCBT1*) and *M. guilliermondii* (*MgCBT2*). While all three transporters allowed cell growth on cellobiose, only the *MgCBT2* permease allowed cellobiose fermentation, although cellobiose consumption was stuck (incomplete). Since ubiquitination of lysine residues at the N- or C-terminal domains of the permease are involved in the endocytosis and degradation of heterologous sugar transporters[7,8], we constructed truncated versions of the permease lacking lysines at the C-terminal domain (*MgCBT2*ΔC), and at both the C- and N-terminal domain (*MgCBT2*ΔNΔC), and co-expressed these permeases with the *SpBGL2* β-glucosidase in an industrial *S. cerevisiae* strain. Our results show that the strain with the truncated *MgCBT2*ΔNΔC permease was able to consume and ferment all the cellobiose present in the medium.

2 MATERIAL & METHODS

The *S. cerevisiae* strain used to characterize the β–glucosidase and cellobiose transporters was CEN.PK2-1C (*MAT*a *leu2-3,112 ura3-52 trp1-289 his3-Δ1 MAL2-8*^{c} *SUC2*). Sequences encoding the β-glucosidase *SpBGL2* from *Sp. passalidarum* and the cellobiose transporters *SsHXT2.4* from *Sc. stipitis*[9], *CtCBT1* from *C. tropicalis* and *MgCBT2* from *M. guilliermondii* were amplified by PCR and cloned into the p424-GPD (*TRP1* P_{GPD}-T_{CYC1} - ATCC®87357) and p426-GPD (*URA3* P_{GPD}-T_{CYC1} - ATCC®87361) plasmids, respectively. The *Escherichia coli* strain DH5α was used for cloning. Standard methods for DNA manipulation and analysis, as well as bacterial and yeast transformation, were employed[10]. Primers were designed for amplification of the *MgCBT2* gene from *M. guilliermondii* removing either the last 12 amino acids (*MgCBT2*ΔC), or the first 20 amino acids plus the last 12 amino acids (*MgCBT2*ΔNΔC) of the transporter, and cloned into the p426-GPD plasmid. These

transporters (*MgCBT2, MgCBT2*ΔC and *MgCBT2*ΔNΔC) together with the *SpBGL2* β-glucosidase were integrated into different ARS (autonomous replicating sequence) elements of the genome of the industrial xylose-fermenting strain MP-C5H1[11,12] by CRISPR-Cas9 technology[13,14]. The recombinant strain MP-C5H1 is derived from the Brazilian industrial strain CAT-1[15].

Yeasts were grown in synthetic medium (6.7 g L^{-1} of Yeast Nitrogen Base without amino acids, supplemented with 2 g L^{-1} of yeast synthetic drop-out media without uracil and/or tryptophan) or in rich YP medium (10 g L^{-1} of yeast extract, 20 g L^{-1} of peptone), containing 20 g L^{-1} of glucose or 20 g L^{-1} of cellobiose. For batch fermentations, cells were pre-grown in YP medium containing 20 g L^{-1} of glucose and inoculated with high cell density (~10 g L^{-1} dry yeast cells) in 20 mL of YP medium containing 20 g L^{-1} of cellobiose into closed 25 mL bottles (for microaerobic conditions) and maintained at 30°C. Cell growth was monitored using a spectrophotometer at 600 nm. Carbohydrate consumption and ethanol production were analyzed by high-performance liquid chromatography as previously described[14].

Intracellular β-glucosidase activity was determined using permeabilized yeast cells[16] with cellobiose or *p*NPβG as substrates. When cellobiose was used as substrate, the glucose released was measured using a commercial kit (Bioclin). The *p*-nitrophenol released by *p*NPβG hydrolysis was measured on a spectrophotometer at 400 nm. β-glucoside transport activity was determined using a colorimetric transport assay with 7.5 mM *p*NPβG, as previously described[17]. β-glucosidase activity and transport activity were expressed as nmol min^{-1} (g dry yeast cells)$^{-1}$ of glucose or *p*-nitrophenol produced 30°C. All data represent averages and standard error from two independent experiments.

3 RESULTS & DISCUSSION

The *S. cerevisiae* CEN.PK2-1C strain expressing the β-glucosidase *SpBGL2* from *Sp. passalidarum* showed an intracellular β-glucosidase activity with a K_m of 18.16 ± 3.87 mM for cellobiose, and higher affinity (K_m of 0.81 ± 0.16 mM) for *p*NPβG, while the V_{max} was 1,188 ± 85 nmol min^{-1} (mg dry cell)$^{-1}$ with cellobiose, and 5,368 ± 199 nmol min^{-1} (mg dry cell)$^{-1}$ with *p*NPβG. However, this strain was unable to grow on or consume cellobiose, and thus a cellobiose transporter was necessary to allow the recombinant yeast to consume the disaccharide. The expression of the known cellobiose transporter *SsHXT2.4* from *Sc. stipitis*[9], as well as two putative cellobiose transporters from *C. tropicalis* (*CtCBT1*) and *M. guilliermondii* (*MgCBT2*) allowed cell growth on cellobiose, but only the *MgCBT2* permease allowed cellobiose fermentation by the recombinant strain (data not shown). However, the CEN.PK2-1C strain expressing the *MgCBT2* transporter and the intracellular *SpBGL2* enzyme was unable to consume all the cellobiose present in the medium (Figure 1A), leaving approximately 25% of the initial cellobiose. The analysis of the β-glucosidase and transport activity of the cells during cellobiose consumption revealed a considerable loss of transport activity by the cells during fermentation, while the intracellular hydrolytic activity remained high during all the fermentation (Figure 1B), indicating that the stuck fermentation is due to drop in the cellobiose transport activity of the cells expressing the *MgCBT2* transporter.

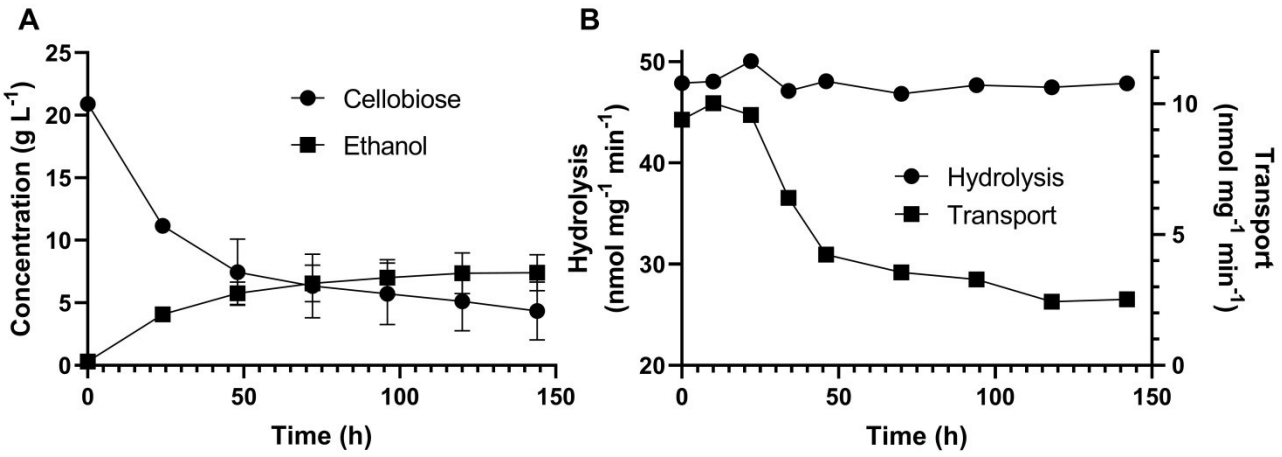

Figure 1: Cellobiose consumption and fermentation by recombinant strains expressing the *MgCBT2* transporter and the intracellular *SpBGL2* enzyme. In A: Cellobiose consumption (circles) and ethanol production (squares) during fermentation. In B: *p*NPβG hydrolysis (circles) or transport (squares) by the yeast cells during cellobiose fermentation (Figure 1A).

It is well known that ubiquitination of lysine residues at the N- or C-terminal domains of transporters promote the endocytosis and degradation of heterologous sugar transporters in yeasts[7,8], interfering with the stability of the permease at the plasma membrane. Using the BDM-PUB program[18] allowed us to identify that the *MgCBT2* transporter has indeed 3 lysine residues (K534, K536 and K544) at the C-terminal of the permease with high probability of ubiquitination, and a lysine residue (K20) at the N-terminal domain of the protein with low probability of ubiquitination. We thus removed part of the amino acid sequence of the C-terminal domain (last 12 amino acids) of the permease, producing the *MgCBT2*ΔC transporter, and we also removed the first 20 amino acids of the protein, producing the *MgCBT2*ΔNΔC transporter lacking lysine at both the C- and N-terminal domains. These transporters (*MgCBT2, MgCBT2*ΔC and *MgCBT2*ΔNΔC), together with the *SpBGL2* β-glucosidase (all four genes under control of strong and constitutive promoters) were integrated into different ARS elements of the genome of the industrial xylose-fermenting strain MP-C5H1 by CRISPR-Cas9 technology[14].

As it can be seen in Figure 2A, the industrial yeast strain expressing the *SpBGL2* β-glucosidase and the wild-type *MgCBT2* permease (strain B2-CTB2) also produced stuck cellobiose fermentations as the laboratory strain (Figure 1A), leaving approximately 50% of the initial cellobiose in the medium. On the other hand, the same industrial strain harboring the

*MgCBT2*ΔC transporter lacking the 3 lysine residues from the C-terminal domain (strain B2-CTB2ΔC) continued producing stuck fermentations (Figure 2A), even worse than the industrial strain expressing the wild-type *MgCBT2* permease. But a completely different profile was obtained with the industrial strain transformed with the transporter truncated in both the N- and C-terminal domain (strain B2-CTB2ΔNΔC), allowing the complete consumption of the disaccharide (Figure 2A). The profile of ethanol production during cellobiose fermentation followed the predicted trend: lower levels of ethanol were produced by strain B2-CTB2ΔC, while strain B2-CTB2ΔNΔC was the one that produced higher amounts of ethanol due to complete cellobiose consumption.

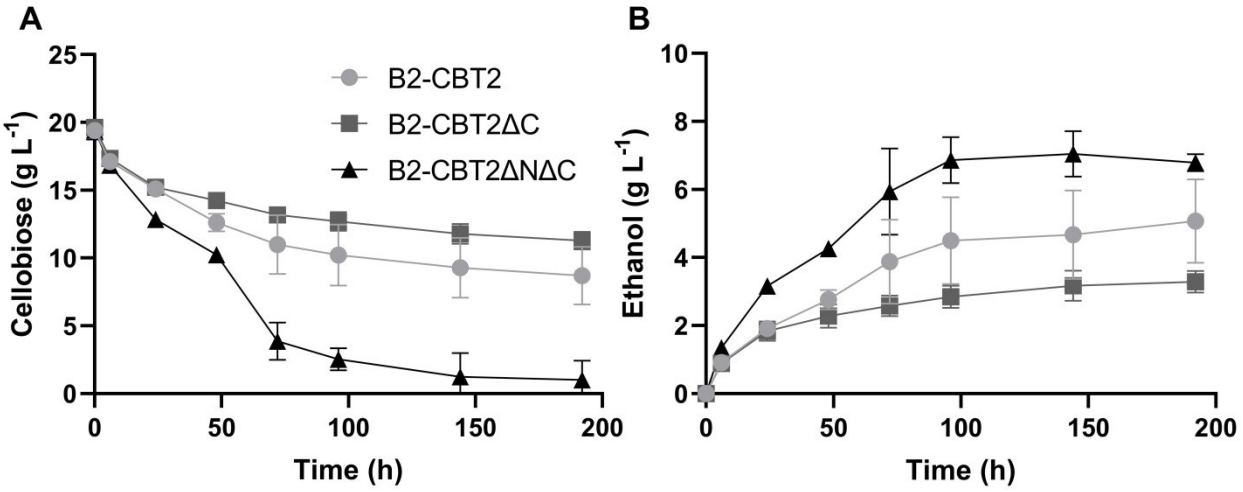

Figure 2: Cellobiose fermentation by industrial strains expressing the indicated *MgCTB2* genes. In A: cellobiose concentrations during fermentation. In B: ethanol concentrations during fermentation.

4 CONCLUSION

The expression of the intracellular β-glucosidase *SpBGL2* from the yeast *Sp. passalidarum* and the cellobiose transporter *MgCBT2* from the yeast *M. guilliermondii* allowed cellobiose fermentation by recombinant *S. cerevisiae*, although the fermentation was stuck. A truncated version in the N- and C-terminal domain of the *MgCBT2* transporter (*MgCBT2*ΔNΔC gene) removing lysine residues passible of ubiquitination, enabled the efficient consumption and fermentation of this sugar by an industrial strain. Our results highlight the importance of ubiquitination of lysine residues in the required correct expression of heterologous sugar transporters in *S. cerevisiae*.

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