COMPARING INTRA AND EXTRACELLULAR HYDROLYSIS FOR CELLOBIOSE FERMENTATION BY *SACCHAROMYCES CEREVISIAE*

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ABSTRACT

Researchers have been constructing recombinant *Saccharomyces cerevisiae* strains capable of hydrolyzing cellobiose in the cytosol or extracellularly, however, few studies have focused on comparing the two strategies. Therefore, this study aimed to compare how cellobiose hydrolysis inside or outside the cell affects the ability of *S. cerevisiae* strains to ferment this sugar. For this, two cellobiose-fermenting recombinant *S. cerevisiae* strains were constructed: strain CEN.PK-X-Bgl1YL, expressing the periplasmic β-glucosidase BGL1 from *Yarrowia lipolytica*; and strain CEN.PK-X-B2-Mg, expressing the intracellular β-glucosidase SpBGL2 from *Spathaspora passalidarum* and the cellobiose transporter MgCBT2 from *Meyerozyma guilliermondii.* Both constructed strains showed cell growth and cellobiose fermentation in media containing the disaccharide as a carbon source; however, the strain CEN.PK-X-Bgl1YL, capable of hydrolyzing cellobiose extracellularly, showed faster growth and the best performance in batch fermentations. Furthermore, β-glucosidase activity and *p*NPβG transport activity revealed that transport across the plasma membrane was possibly the limiting factor in cellobiose fermentation by CEN.PK-X-B2-Mg. Thus, the results obtained in this study highlight that the heterologous expression of periplasmic β-glucosidases in *S. cerevisiae* could be an interesting strategy to overcome the problem of disaccharide transport, and thus allowing efficient cellobiose fermentation.

Keywords: Biorefineries 2G. Bioethanol. β-glucosidase. Heterologous expression. Yeast.

1 INTRODUCTION

Research groups worldwide have focused on optimizing the use of waste, with an emphasis on second-generation biorefineries (2G) that convert agro-industrial waste into various bioproducts, especially from lignocellulosic biomass.[1] However, enabling the use of these plant residues as substrates for bioproduct generation relies on the fermentation by microorganisms that metabolize the primary carbon sources present in lignocellulosic hydrolysates. The residual plant biomasses used as raw materials in these situations are rich in the polysaccharides cellulose and hemicellulose, which, when hydrolyzed, release as main products the monosaccharides glucose and xylose and the disaccharide cellobiose.[2] In this sense, it is essential that the choice of microorganisms for these processes takes into account the metabolism of these sugars.

As a fermenting microorganism, the yeast *Saccharomyces cerevisiae* stands out for its adaptation and robustness in the context of industrial fermentation conditions.[3] However, this yeast is unable to metabolize both xylose and the disaccharide cellobiose.[4] For *S. cerevisiae* to be able to metabolize cellobiose, the presence of a β-glucosidase capable of hydrolyzing the β-1,4 glycosidic bond between the two glucose subunits is indispensable. For this, two approaches have been explored: one involves the heterologous expression of genes encoding secreted or periplasmic β-glucosidases (extracellular hydrolysis), which only requires the yeast to transport glucose molecules to the cytoplasm for further metabolism; the other approach focuses on the heterologous expression of cytoplasmic β-glucosidases (intracellular hydrolysis) — in this last case, the yeast also depends on the heterologous expression of a permease capable of transporting cellobiose into the cell.[5,6]

In recent decades, through the heterologous expression approaches described above, researchers have been constructing modified *S. cerevisiae* strains capable of hydrolyzing cellobiose in the cytosol[7] or extracellularly[8]. However, few works have focused on comparing the two strategies, particularly using the same genetic background and similar cultivation conditions, factors that influence fermentation capacity and thus the comparison of fermentative performances.[7,8] Therefore, the present study aimed to compare the impact of intracellular and extracellular hydrolysis of the cellobiose disaccharide on the fermentation capacity of sugar by recombinant *S. cerevisiae* strains, in order to determine which of the two approaches allows the best fermentative performance of yeast with this abundant biomass carbohydrate.

2 MATERIAL & METHODS

The *S. cerevisiae* strain employed in this study was CEN.PK-X-RDK (isogenic to the CEN.PK2-1C strain, but containing the integrative plasmid pAUR-XKXDHXR[9]), described in detail by Kretzer (2023)[10]. Sequences encoding β-glucosidases SpBGL2 from *S. passalidarum* and BGL1 from *Y. lipolytica* UFMG-CM-Y6114 were amplified by PCR and cloned in the p424-GPD (*TRP1* GPDp-CYC1t - ATCC®87357) and p426-GPD (*URA3* GPDp-CYC1t - ATCC®87361) plasmids, respectively. The sequence encoding MgCBT2 cellobiose transporter from *M. guilliermondii* was amplified by PCR and cloned in the p426-GPD plasmid. 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.[11]

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 growth analysis, cells pre-cultured in synthetic medium were inoculated in YP medium containing 20 g L^{-1} of cellobiose in flasks filled to 1/5 of the volume with medium and maintained at 30°C with 180 rpm orbital shaking. For batch fermentations, cells were pre-grown in YP medium containing 20 g L^{-1} of cellobiose and inoculated with high cell density (~10 g L^{-1} dry yeast cells) in 20 mL of the same medium 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.[10]

Periplasmic and intracellular (permeabilized cells) β-glucosidase activity was determined in cells grown until the beginning of the exponential growth phase in YP medium containing 20 g L^{-1} of cellobiose. Periplasmic hydrolysis by β-glucosidases was determined *in vivo* with whole cells pre-incubated with sodium fluoride using 80 mM cellobiose or 2 mM *p*-nitrophenyl-β-d-glucopyranoside (*p*NPβG) as substrates.[12] Intracellular β-glucosidase activity was determined with the same substrates, but using permeabilized yeast cells.[13] For extracellular β-glucosidase activity, the culture supernatant was mixed with cellobiose or *p*NPβG. 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.[14] β-glucosidase activity and transport activity were expressed as U (g dry yeast cells)$^{-1}$, where one unit (U) corresponds to 1 µmol of glucose or *p*-nitrophenol produced per minute at 30°C. All data represent averages and standard error from two independent experiments.

3 RESULTS & DISCUSSION

A comparative study of the cellobiose fermentative performance by two different strains was conducted in this study. Firstly, the strain *S. cerevisiae* CEN.PK-X-Bgl1YL strain was constructed to represent the yeast with the capacity to hydrolyze cellobiose extracellularly, as it carries a plasmid containing the gene encoding the periplasmic β-glucosidase BGL1 from the yeast *Y. lipolytica*. Secondly, the *S. cerevisiae* CEN.PK-X-B2-Mg strain was constructed to represent the yeast that transports cellobiose into the cell and hydrolyzes it in the cytoplasm; as it carries a plasmid containing the gene encoding the intracellular β-glucosidase SpBGL2 from *S. passalidarum*, and another plasmid containing the gene encoding the cellobiose transporter MgCBT2 from *M. guilliermondii.*

In growth assays in YP medium containing cellobiose as a carbon source (Figure 1-A), the strain CEN.PK-X-Bgl1YL showed a higher rate of both cell growth and disaccharide consumption compared to the CEN.PK-X-B2-Mg strain. Similarly, in the batch fermentation assay with high cell density (Figure 1-B), a faster cellobiose consumption by the strain CEN.PK-X-Bgl1YL can be verified, with total consumption of the disaccharide in ~32 hours. Furthermore, this strain, which expresses the periplasmic enzyme, produced approximately 10 g L^{-1} of ethanol in the fermentation assay, whereas the strain CEN.PK-X-B2-Mg (expressing the transporter and the intracellular enzyme) was unable to consume all cellobiose during the total time of the experiment, and produced a smaller amount of ethanol at the end of fermentation (Figure 1-B).

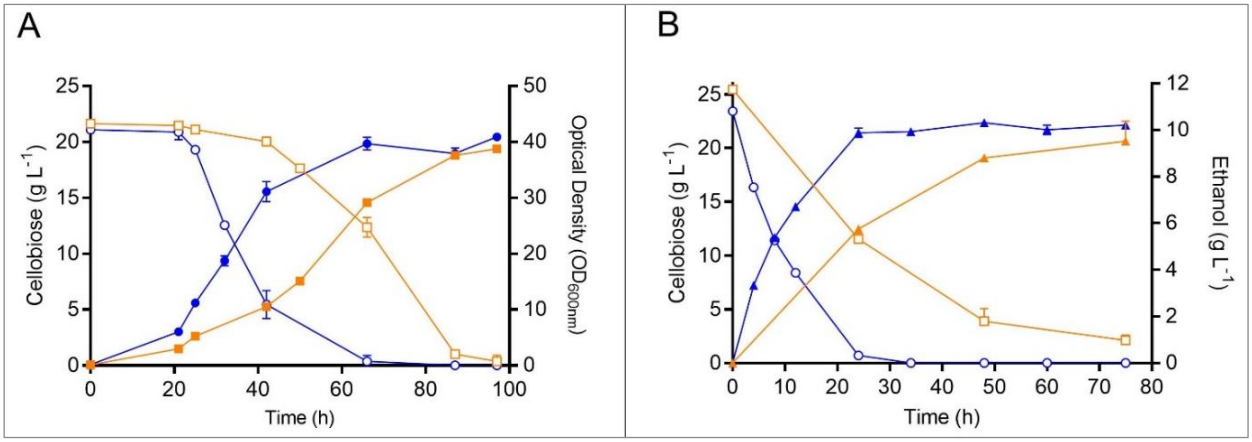

Figure 1: Cell growth, sugar consumption, and fermentation profiles by recombinant strains. In A: Cell growth (closed symbols) and cellobiose consumption (open symbols) during aerobic growth. In B: Cellobiose consumption (open symbols) and ethanol production (closed triangles) in batch fermentations with high cell density (10 g L^{-1} dry cells). Blue lines: strain CEN.PK-X-Bgl1YL. Orange lines: strain CEN.PK-X-B2-Mg.

In assays for β-glucosidase enzymatic activity, the strain CEN.PK-X-Bgl1YL (Figure 2-A) exhibited enzymatic activity with both substrates (cellobiose and its analog *p*NPβG) using either permeabilized cells or intact cells (periplasmic activity), while the activities measured in the culture medium were negligible. The β-glucosidase activity for both substrates was very similar when comparing values from permeabilized cells with those from the periplasmic assay, indicating that the BGL1 enzyme from *Y. lipolytica* is primarily present in the periplasm of cells when expressed in *S. cerevisiae*, as observed by Guo et al. (2016)[15] using this enzyme overexpressed in the yeast *Y. lipolytica*.

Since the SpBGL2 enzyme from *S. passalidarum* has already been characterized in *S. cerevisiae* previously, and was found to be intracellular[10], we analyzed β-glucosidase activity in strain CEN.PK-X-B2-Mg using only the permeabilized cells with both substrates. For this strain, we also evaluated the ability to transport the analog *p*NPβG into the cells, as it also expresses the MgCBT2 transporter from *M. guilliermondii*. Upon analyzing the enzymatic and transport activities (Figure 2-B), it can be observed that the strain exhibited β-glucosidase activity with both substrates. However, it is noticeable that the *p*NPβG transport activity by the strain was ~100 times lower, when compared to the enzymatic activity measured in the permeabilized cells with the same substrate.

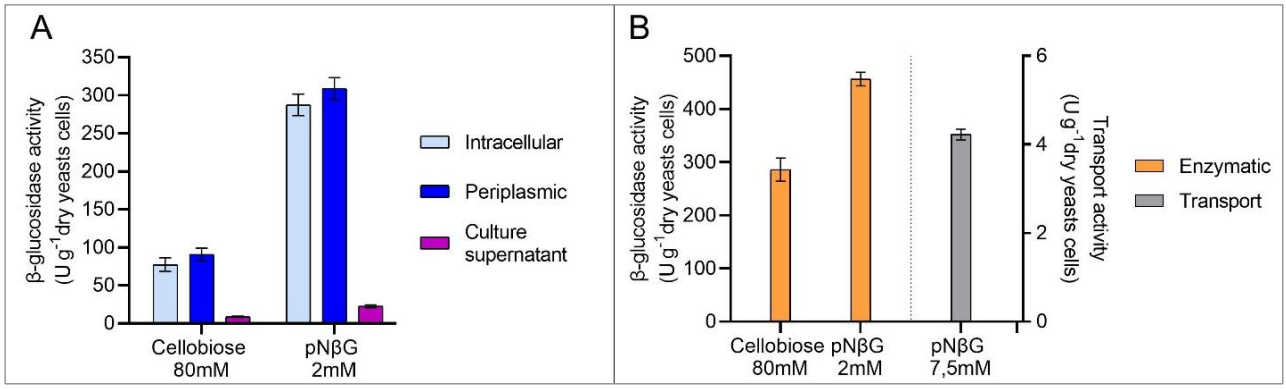

Figure 2: β-glucosidase activity and *p*NPβG transport activity. In A: Periplasmic, intracellular (permeabilized cells) and culture supernatant β-glucosidase activity by strain CEN.PK-X-Bgl1YL. In B: Intracellular β-glucosidase activity and transport activity by strain CEN.PK-X-B2-Mg.

Analyzing Figures 2-A and 2-B, it is noticeable that the hydrolysis activities presented by the strain CEN.PK-X-B2-Mg (carrying the intracellular enzyme) were higher for both substrates, when compared with the enzymatic activities obtained with the yeast CEN.PK-X-Bgl1YL (carrying the periplasmic enzyme). However, this didn't reflect better cellobiose consumption by the CEN.PK-X-B2-Mg strain, when compared to the CEN.PK-X-Bgl1YL strain (Figures 1-A and 1-B). The strain CEN.PK-X-B2-Mg, however, despite demonstrating high levels of intracellular enzymatic activity, showed a low capacity to transport the *p*NPβG analog into the cells, suggesting that the transport of cellobiose of this strain may have been the limiting factor for fermentation of the sugar. In fact, previous studies have shown that recombinant *S. cerevisiae* strains expressing heterologous β-glucosidases intracellularly had their cellobiose consumption capacities limited by the low rate of sugar uptake.[7,16] On the other hand, strain CEN.PK-X-Bgl1YL hydrolyzes cellobiose extracellularly, and thus only needs to transport the glucose generated in the periplasm to the cytoplasm, a mechanism that is highly efficient in *S. cerevisiae*.[17] Thus, these results suggest that extracellular hydrolysis by the CEN.PK-X-Bgl1YL strain appears more advantageous for cellobiose metabolism than the sugar transport and intracellular hydrolysis by strain CEN.PK-X-B2-Mg.

4 CONCLUSION

In the present work, two strains of *S. cerevisiae* were constructed — one of them (strain CEN.PK-X-Bgl1YL) expressing the periplasmic β-glucosidase BGL1 from the yeast *Y. lipolytica*, and the other (strain CEN.PK-X-B2-Mg) expressing the intracellular β-glucosidase SpBGL2 from the yeast *S. passalidarum* and the cellobiose transporter MgCBT2 from the yeast *M. guilliermondii*. Although both strains exhibited cellular growth and cellobiose consumption, strain CEN.PK-X-Bgl1YL, capable of hydrolyzing cellobiose extracellularly, demonstrated the best fermentative performance. Furthermore, analysis of β-glucosidase enzyme activity and *p*NPβG transport revealed that transport across the plasma membrane possibly was the limiting factor for efficient cellobiose fermentation by the yeast CEN.PK-X-B2-Mg. The results obtained in this study highlight that the heterologous expression of periplasmic β-glucosidases in *S. cerevisiae* may be an interesting strategy to overcome the cellobiose transport problem by recombinant strains, and thus enabling the efficient fermentation of this sugar.

REFERENCES

[1] PATEL, A., SHAH, A. R. 2021. J. Bioresour. Bioprod. 6 (2). 108-128.
[2] BOHN, L. R., DRESCH, A. P., CAVALI, M., VARGAS, A. C. G., FÜHR, J. F., TIRONI, S. P., FOGOLARI, O., MIBIELLI, G. M., ALVES JR., S. L., BENDER, J. P. 2021. Research, Society and Development. 10 (11). e149101118914.
[3] STAMBUK, B. U. 2019. Yeasts: The leading figures on Bioethanol production. *In:* Ethanol as a Green Alternative Fuel: Insight and Perspectives TREICHEL, H., ALVES JR, S.L., FONGARO, G., MÜLLER, C. (eds). 1ed. Nova science publishers, New York, 57-91.
[4] PATIÑO, M.G., ORTIZ, J.P., VELÁSQUEZ, M., STAMBUK, B.U. 2019. Yeast. 36 (9). 541-556.
[5] ALVES JR, S. L., SCAPINI, T., WARKEN, A., KLANOVICZ, N., PROCÓPIO, D. P., TADIOTO, V., STAMBUK, B. U., BASSO, T. O., TREICHEL, H. 2022. Engineered *Saccharomyces* or Prospected non- *Saccharomyces:* Is There Only One Good Choice for Biorefineries? *In*: Yeasts: From Nature to Bioprocesses. ALVES JR, S.L., TREICHEL, H., BASSO, T., STAMBUK, B.U. (eds.). Bentham Science Publisher, Singapore. 243–283.
[6] ELIODÓRIO, K. P., CUNHA, G. C. DE G. E., MÜLLER, C., LUCARONI, A. C., GIUDICI, R., WALKER, G. M., ALVES, S. L., BASSO, T. O. 2019. Adv. Appl. Microbiol. 109. 61-119.
[7] HA, S.-J., GALAZKA, J.M., KIM, S.R., CHOI, J.H., YANG, X., SEO, J.H., GLASS, N.L., CATE, J.H., JIN, Y.S. 2010. Proc. Natl. Acad. Sci. USA. 108. 504-509.
[8] ZHANG, L., GUO, Z. P., HONG, J. H., DING, Z. Y., GAO, Z. Q., HE, Z. M., SHI, G. Y. 2012. Ann. Microbiol. 62. 539-544.
[9] MATSUSHIKA, A., WATANABE, S., KODAKI, T., MAKINO, K., SAWAYAMA, S. 2008. J. Biosci. Bioeng.. 105 (3). 296-299.
[10] KRETZER, L. G. 2023. Development of recombinant *Saccharomyces cerevisiae* capable of fermenting cellobiose. Thesis. Federal University of Santa Catarina. UFSC.
[11] AUSUBEL, F.M., BRENT, R., KINGSTON, R.E., MOORE, D.D., SEIDMAN, J.G., SMITH, J.A., STRUHL, K. 2003. Current Protocols in Molecular Biology. John Wiley & Sons.
[12] SILVEIRA, M. C. F., CARVAJAL, E., BON, E. P. S. 1996. Anal. Biochem. 238. 26-28.
[13] STAMBUK, B. U. 1999. Biochem. Educ. 27. 177-180.
[14] HOLLATZ, H., STAMBUK, B. U. 2001. J. Microbiol. Methods. 146. 253-259.
[15] GUO, Z., DUQUESNE, S., BOZONNET, S., CIOCI, G., NICAUD, J. M., MARTY, A. 2015. Biotechnol. Biofuels. 8. 109-125.
[16] CASA-VILLEGAS, M., POLAINA, J., MARÍN-NAVARRO, J. 2018. Process Biochem, 75. 59-67.
[17] OZCAN, S., JOHNSTON, M. 1999. Microbiol. Mol. Biol. Rev. 63. 554-569.

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