Article

Not peer-reviewed version

Screening and Engineering Yeast Transporters to Improve Cellobiose Fermentation by Recombinant *Saccharomyces cerevisiae*

Leonardo G. Kretzer , Marilia M. Knychala , Lucca C. da Silva , Isadora C. C. da Fontoura , Maria José Leandro , César Fonseca , Kevin J. Verstrepen , Boris U. Stambuk *

Posted Date: 20 August 2024

doi: 10.20944/preprints202408.1444.v1

Keywords: cellobiose; transporter; β-glucosidase; Spathaspora passalidarum; *Meyerozyma guilliermondii*; recombinant *Saccharomyces cerevisiae*

Article

Screening and Engineering Yeast Transporters to Improve Cellobiose Fermentation by Recombinant *Saccharomyces cerevisiae*

Leonardo G. Kretzer [1,2], Marilia M. Knychala [1,3], Lucca C. da Silva [1], Isadora C. C. da Fontoura [1], Maria José Leandro [3,4], César Fonseca [3,5], Kevin J. Verstrepen [2] and Boris U. Stambuk [1,*]

[1] Department of Biochemistry, Federal University of Santa Catarina, Florianópolis 88040-900, SC, Brazil; leo_kretzer@hotmail.com (L.G.K.); marilia.mknychala@gmail.com (M.M.K.); luccacorreadasilva@gmail.com (L.C.S.); isaacernach1@gmail.com (I.C.C.F.)
[2] VIB-KU Leuven Center for Microbiology, and Department of Microbial and Molecular Systems, KU Leuven, Leuven, Belgium. kevin.verstrepen@kuleuven.be (K.J.V.)
[3] Laboratório Nacional de Energia e Geologia, I.P., Unidade de Bioenergia, Estrada do Paço do Lumiar 22, 1649-038 Lisboa, Portugal; mjose.leandro@itqb.unl.pt (M.J.L.); cesfonseca76@gmail.com (C.F.)
[4] ITQB NOVA, Instituto de Tecnologia Química e Biológica António Xavier, Av. da República, 2780-157 Oeiras, Portugal;
[5] Present address: Novonesis, R&D, 2970 Hørsholm, Denmark
* Correspondence: boris.stambuk@ufsc.br; Tel.: +55-48-3721-4449

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 (*SpBGL7*) from *Spathaspora passalidarum*, and co-expressed the cellobiose transporter *SiHXT2.4* from *Scheffersomyces illinoinensis*, and two putative 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 ubiquitinylation 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 lysine residues 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 *SpBGL7* 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: cellobiose; transporter; β-glucosidase; *Spathaspora passalidarum*; *Meyerozyma guilliermondii*; recombinant *Saccharomyces cerevisiae*

1. Introduction

The production and use of sustainable alternatives to fossil fuels has been growing for the last few decades, and it will certainly become a necessity in the near future due to the environmental impact of fossil fuels on the climate. Bioethanol is the primary liquid biofuel used in the USA and Brazil, responsible for approx. 85% of the global production [1–3]. This so-called first-generation (1G) bioethanol is made from food-based plant sugars from corn (starch) or sugarcane (sucrose). Although accounting for approx. 1% of the global fuel ethanol production, second-generation (2G) bioethanol is made from plant residues rich in lignocellulosic biomass [3–5]. This renewable biomass is

 doi:10.20944/preprints202408.1444.v1

composed of cellulose (a linear polymer of β-1,4 linked glucose molecules), hemicellulose (a branched and highly heterogeneous polymer containing both hexoses and pentoses), and lignin. The biochemical conversion of lignocellulosic biomass involves several steps, including pre-treatment, enzymatic hydrolysis and fermentation, being the efficient conversion of all the sugars available a major requirement for an economically feasible 2G bioethanol process [5–8].

An optimized enzyme blend (containing endoglucanases, exoglucanases, hemicellulases, pectinases, β-glucosidases, xylanases, β-xylosidases, laccases, etc.) is required for the efficient conversion of cellulose and hemicellulose polymers into sugar syrups to be fermented by yeasts [9,10]. To avoid cellobiose accumulation, a strong inhibitor of both cellobiohydrolases and endoglucanases [11–14], enzyme blends usually contain high levels of β-glucosidases, increasing 2G bioethanol production costs. Thus, a solution for efficient conversion of lignocellulose to ethanol is the use of microorganisms capable of efficient cellobiose fermentation. Many yeasts can secrete β-glucosidases, and cellobiose is hydrolyzed to glucose in the extracellular environment, followed by glucose uptake and fermentation [15–18]. Other yeasts species have intracellular β-glucosidases, and thus cellobiose needs to be transported into the cell by cellodextrin permeases to allow its fermentation [19–23]. Some few yeasts may even have both pathways, extracellular hydrolysis besides transport and intracellular hydrolysis of cellobiose [24,25].

S. cerevisiae is a favored platform for microbial engineering efforts to produce biofuels from cellulosic hydrolysates because of its industrial robustness and easy genetic manipulation, but it lacks the capacity to ferment cellobiose. The uptake and intracellular hydrolysis of cellobiose, an abundant mechanism for cellobiose utilization in filamentous fungi and also engineered in *S. cerevisiae* [26,27], is an interesting strategy to avoid the production of glucose in the medium and thus the competition between glucose and xylose for the *HXT* transporters of *S. cerevisiae* [28], which often considerable delays xylose consumption and the fermentation process [29–33]. Furthermore, complete enzymatic conversion of cellulose to glucose is problematic because high glucose concentrations inhibit both cellulases and β-glucosidases [10–12]. The first report showing the successful expression in *S. cerevisiae* of cellodextrin transporters and intracellular β-glucosidase was with genes from the cellulolytic fungi *Neurospora crassa* [26]. The authors expressed an intracellular β–glucosidase (encoded by the *gh1-1* gene) and two transporters (*CDT-1* and *CDT-2* genes) that transport cellobiose and higher cellodextrins, as well as playing a critical role in hemicellulose sensing and utilization by *N. crassa*, while *CDT-2* seems to also transport xylobiose and longer xylodextrins [26,34–36]. Another difference between these permeases is that *CDT-1* is an active transporter, while *CDT-2* seems to transport cellobiose by facilitated diffusion, although both transporters were reported to have very similar affinity to cellobiose [26,37].

There are dozens of other manuscripts reporting the use of the *gh1-1* β-glucosidase and *CDT-1* and *CDT-2* transporters for cellobiose fermentation by several recombinant yeast, and while other fungal cellodextrin/xylodextrin permeases have been expressed in *S. cerevisiae* [38–42], unfortunately these transporters were characterized just by growth assays, with little information regarding kinetics of cellobiose transporters and their contribution to ethanol production during growth [27,43]. Regarding cellobiose transporters from yeasts, only two permeases (*HXT2.4* from *Sc. stipitis* and *Ls120451* from *Lipomyces starkeyi*) have been shown to allow cellobiose fermentation by recombinant *S. cerevisiae* when also expressing the *gh1-1* β-glucosidase from *N. crassa* [44,45].

In the present work we have cloned an intracellular β-glucosidase (*SpBGL7*) from the cellobiose fermenting yeast *Sp. passalidarum* [46], and used a *S. cerevisiae* laboratory strain CEN.PK2-1C expressing this β-glucosidase to screen for cellobiose transporters from yeasts. The co-expression of the cellobiose transporter *SiHXT2.4* from *Sc. illinoinensis*, and two putative transporters from *C. tropicalis* (*CtCBT1*) and *M. guilliermondii* (*MgCBT2*), allowed growth on cellobiose by the recombinant *S. cerevisiae* strains. However, only the *MgCBT2* permease allowed cellobiose fermentation, although the consumption of cellobiose was stuck (incomplete). Since the incomplete consumption of cellobiose was the consequence of a drop in the transport activity by the cells, we analyzed this transporter in relation to the presence of lysine residues in the N- or C-terminal domains with potential for ubiquitinylation, and thus for being involved in downregulation of the transporter

 doi:10.20944/preprints202408.1444.v1

through endocytosis and vacuolar degradation [47,48]. The construction of a truncated version of the transporter lacking lysine residues at both the C- and N-terminal domain (*MgCBT2*ΔNΔC) allowed efficient fermentation of all the cellobiose present in the medium by an industrial fuel-ethanol yeast strain. The results obtained highlight the importance of removing lysine residues involved in endocytosis to allow efficient expression of heterologous sugar transporters in *S. cerevisiae*.

2. Materials and Methods

2.1. Strains, Media and Growth Conditions

The yeast strains and plasmids used in this study are listed in Table 1. The *Escherichia coli* DH5α strain (F′/ endA1hsdR17 (rK-mK+) glnV44 thi-1 recA1 gyrA (NaIr) relA1Δ (lacZYA-argF) U169 deoR Φ80dlac Δ(LacZ) M15) [49], was used for cloning and plasmid propagation, and was grown in Luria broth containing 1% tryptone, 0.5% yeast extract, 0.5% sodium chloride, pH 7.0, and 100 mg/L ampicillin when required (Sigma-Aldrich Brazil Ltda., São Paulo, SP, Brazil).

Table 1. Yeast strains and plasmids used in this study.

Strains and plasmids	Relevant features or genotype	Source
Yeast strains:		
C. tropicalis UFMG-HB-93a	Isolated from decaying sugarcane bagasse in São Paulo, Brazil	[50]
M. guilliermondii NRRL Y-27844	Clinical isolate	USDA-ARC Culture Collection
Sc. illinoinensis UFMG-CM-Y512	Isolated from rotting wood in Rio de Janeiro, Brazil	[51]
Sp. passalidarum UFMG-CM-Y474	Isolated from rotting wood in Roraima, Brazil	[52]
S. cerevisiae CEN.PK2-1C	*MAT*a *leu2-3,112 ura3-52 trp1-289 his3-Δ*1 *MAL2-8*^{c} *SUC2*	[53]
S. cerevisiae B7	CEN.PK2-1C + pGPD-424-SpBGL7	This work
S. cerevisiae B7-HXT2.4	CEN.PK2-1C + pGPD-424-SpBGL7 + pGPD-426-SsHXT2.4	This work
S. cerevisiae B7-CBT1	CEN.PK2-1C + pGPD-424-SpBGL7 + pGPD-426-CtCBT1	This work
S. cerevisiae B7-CBT2	CEN.PK2-1C + pGPD-424-SpBGL7 + pGPD-426-MgCBT2	This work
S. cerevisiae CAT-1	Industrial strain isolated from Usina VO Catanduva, São Paulo, Brazil	[54,55]
S. cerevisiae MP-C5H1	Isogenic to CAT-1, but *AUR1*::pAUR-XKXDHXR *loxP-KanMX-loxP*-P$_{ADH1}$::[4- 59Δ]*HXT1*	[56]
S. cerevisiae MP-B7	Isogenic to MP-C5H1, but ARS208::P$_{TEF1}$-*SpBGL7*-T$_{PGK1}$	This work
S. cerevisiae MP-B7-CBT2	Isogenic to MP-B7, but ARS1309::P$_{TDH3}$-*MgCBT2*-T$_{CYC1}$	This work
S. cerevisiae MP-B7-CBT2ΔC	Isogenic to MP-B7, but ARS1309::P$_{TDH3}$-*MgCBT2*ΔC-T$_{CYC1}$	This work
S. cerevisiae MP-B7-CBT2ΔNΔC	Isogenic to MP-B7, but ARS1309::P$_{TDH3}$-*MgCBT2*ΔNΔC-T$_{CYC1}$	This work
Plasmids:		
pGPD-424	*Amp*^{R} *ori* 2μ *TRP1* P$_{TDH3}$-T$_{CYC1}$	ATCC® 87357™ [57]
pGPD-426	*Amp*^{R} *ori* 2μ *URA3* P$_{TDH3}$-T$_{CYC1}$	ATCC® 87361™ [57]
pGPD-424-SpBGL7	*Amp*^{R} *ori* 2μ *TRP1* P$_{TDH3}$-*SpBGL7*-T$_{CYC1}$	This work

 doi:10.20944/preprints202408.1444.v1

pGPD-426-SsHXT2.4	*Amp*^{R} *ori* 2µ *URA3* P$_{TDH3}$-*SsHXT2.4*-T$_{CYC1}$	This work
pGPD-426-CtCBT1	*Amp*^{R} *ori* 2µ *URA3* P$_{TDH3}$-*CtCBT1*-T$_{CYC1}$	This work
pGPD-426-MgCBT2	*Amp*^{R} *ori* 2µ *URA3* P$_{TDH3}$-*MgCBT2*-T$_{CYC1}$	This work
pGPD-426-MgCBT2ΔC	*Amp*^{R} *ori* 2µ *URA3* P$_{TDH3}$-*MgCBT2*ΔC-T$_{CYC1}$	This work
pGPD-426-MgCBT2ΔNΔC	*Amp*^{R} *ori* 2µ *URA3* P$_{TDH3}$-*MgCBT2*ΔNΔC-T$_{CYC1}$	This work
pV1382	*Amp*^{R} *ori CEN ARS URA3 NAT*^{R} P$_{TEF1}$-*CaCas9*-T$_{CYC1}$ P$_{SNR52}$-sgRNA-T$_{SNR52}$	[58]
pV1382-ARS1309	pV1382 P$_{SNR52}$-sgRNA(ARS1309)-T$_{SNR52}$	This work
pV1382-ARS208	pV1382 P$_{SNR52}$-sgRNA(ARS208)-T$_{SNR52}$	This work
pMV	*Amp*^{R} *ori* (pBR322 derivative)	BGI Group
pMV-SpBGL7	*Amp*^{R} *ori* [5′ARS208-P$_{TEF1}$-*SpBGL2*-T$_{PGK1}$-3′ARS208]	This work

Yeasts were grown in rich YP medium (1% yeast extract, 2% Bacto peptone, Sigma-Aldrich), or in synthetic complete (YNB) medium (0.67% yeast nitrogen base without amino acids, supplemented with 1.92 g/L of yeast synthetic Drop-out media without uracil, or 1.82 g/L without uracil and tryptophan, Sigma-Aldrich), with 20 g/L glucose or cellobiose as carbon source. The pH of the medium was adjusted to pH 5.0 with HCl, and when required, 2% Bacto agar (Sigma-Aldrich) or 0.1 g/L nourseothricin (cloNAT, WERNER BioAgents GmbH, Jena, Germany) were added to the medium. The laboratory strains transformed with plasmids were pre-grown in YNB with glucose as carbon source, and used to inoculate new YNB medium containing 20 g/L cellobiose with an initial cell concentration of 0.1 optical density at 600 nm (A_{600nm}), measured with a Cary 60 UV-VIS spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). Growth was performed aerobically in cotton plugged Erlenmeyer flasks filled to 1/5 of the volume with medium at 28°C with 160 rpm orbital shaking. Cellular growth was followed by absorbance measurements at 600 nm (A_{600nm}). For batch fermentations, cells were collected in the exponential phase of growth, centrifuged at 6,000 *g* for 5 min at 4°C, and washed twice with sterile water, and inoculated at a high cell density (~10 g dry cell weight/L) into a 25 mL flask containing 20 mL of rich YP medium containing 20 g/L cellobiose at 30°C, with shaking at 100 rpm. For the industrial strains, fermentations were also performed in rich YP medium containing 20 g/L glucose or cellobiose, or 20 g/L cellobiose plus 20 g/L xylose. Culture samples were harvested regularly, centrifuged (5,000 *g*, 1 min at 4^{0}C), and supernatants used for the quantification of substrates and fermentation products.

2.2. Molecular Biology Techniques

Standard procedures for DNA manipulation and analysis, as well as bacterial and yeast transformation, were employed [59,60]. Purification of plasmids, PCR products or Gibson Assembly products was performed using the QIAquick PCR Purification Kit (QIAGEN Antwerp, Belgium). The genomic DNA from the yeast strains was purified using a YeaStar Genomic DNA kit (Zymo Research, Irvine, CA, USA). For plasmid extraction, we used the manual mini-prep method [60], or using the commercial QIAprep Spin Miniprep Kit (QIAGEN). DNA fragments for cloning, sequencing, or transformations were PCR-amplified using Phusion High-Fidelity (Thermo Fisher Scientific Inc., Waltham, MA, USA) or PrimeSTAR® GXL DNA polymerases (Takara Bio Europe SAS, Saint-Germain-en-Laye, France). Purified plasmids, products of Gibson Assembly, or cells lysed by incubation at 100°C in 20 mM NaOH for 10 min (colony PCR) served as DNA templates.

Based on the genome of *C. tropicalis* and *M. guilliermondii* [61], *Sc. stipitis* [62] and *Sp. passalidarum* [63], primers were designed (Table 2) to amplify genes encoding a β-glucosidase from *Sp. passalidarum* (*SpBGL7*, NCBI Gene ID: 18871961, primers SpBGL7-F and SpBGL7-R), the cellobiose transporters *HXT2.4* from *Sc. illinoinensis* (the *HXT2.4* gene from *Sc. stipitis* has NCBI Gene ID: 4850978, primers HXT2.4-F and HXT2.4-R), *CtCBT1* from *C. tropicalis* (NCBI Gene ID: 8298855, primers CtCBT1-F and CtCBT1-R), and *MgCBT2* from *M. guilliermondii* (NCBI Gene ID: 5129179, primers MgCBT2-F and MgCBT2-R), introducing restriction sites (for *Bam*HI, *Eco*RI, *Hind*III, *Sal*I or *Xho*I enzymes) for cloning

 doi:10.20944/preprints202408.1444.v1

into multicopy shuttle vectors containing strong and constitutive promoters and terminators (pGPD-424 and pGPD-426, Table 1) as well as the *TRP1* and *URA3* genes used as selective marker.

Table 2. Primers used in this study.

Primer [1]	Sequence [2]
Cloning:	
SpBGL7-F	**GAATTC**ATGACCGTGTCTGATTTCGATGTTG
SpBGL7-R	**CTCGAG**CTAATTACCTTTCCAGAAGAAACTTTGATC
HXT2.4-F	GGC**GGATCC**AAAATGTCTGACAAACTTCACAACATCAAG
HXT2.4-R	GGC**CTCGAG**GTCGACATAATCAGGTATAATTTATTGACTAAAGCTTAG
CtCBT1-F	GGC**GAATTC**AAAATGTCATCCAAAGATAATATCATCATCACTGAAG
CtCBT1-R	GGC**CTCGAG**GTCGACCTAGGCCAATTTTTCAACGTGATCAACC
MgCBT2-F	GGC**GGATCC**ATGGTTTCCAATTCGTCTTCATACTGG
MgCBT2-R	GGC**AAGCTT**TCATACTTTTTCAGCATGTTCAAGCG
MgCBT2ΔC-F	CAT**GGATCC**ATGGGTTTCCATTCGTCTTC
MgCBT2ΔC-R	TGA**AAGCTT**TCACGGAGTGGCAAGAATATGGA
MgCBT2ΔNΔC-F	CAT**GGATCC**ATGCACCAGGATATCGCTACTCA
CRISPR-Cas9:	
sgRNA.ARS1309-F	5P-GATCGCCTGTGGTGACTACGTATCCG
sgRNA.ARS1309-R	5P-AAAACGGATACGTAGTCACCACAGGC
sgRNA.ARS208-F	5P-GATCGGTCCGCTAAACAAAAGATCTG
sgRNA.ARS208-R	5P-AAAACAGATCTTTTGTTTAGCGGACC
ARS208-F	CCGCAGTGTCTTGCGTCTCTGATCTTACCTGGTGAATTGG
ARS208-R	TTGGCAGTGACTCCGTCTCTAGTAGGTGCCAGTTGAATAG
Sequencing:	
seq.p1382.sgRNA-F	GCTGTAGAAGTGAAAGTTGG
seq.p1382.sgRNA-R	CAAGTTGATAACGGACTAGC

[1] All primers were synthesised by Integrated DNA Technologies (IDT, Coralville, IA, USA). [2] Bold sequences indicate restriction enzyme sites (*Bam*HI, *Eco*RI, *Hind*III, *Sal*I or *Xho*I) used for cloning.

To clone and overexpress modified versions of the *MgCBT2* transporter, we amplified the *MgCBT2* gene from the pGPD-426-MgCBT2 plasmid (Table 1) using specific primer pairs: MgCBT2ΔC-F and MgCBT2ΔC-R (Table 2) for generating a version of the gene that encodes a truncated permease in the C-terminal region (pGPD-426-MgCBT2ΔC plasmid, Table 1), and MgCBT2ΔNΔC-F and MgCBT2ΔC-R (Table 2) for generating a version that encodes a truncated permease in both N- and C-terminal regions (pGPD-426-MgCBT2ΔNΔC plasmid, Table 1). These primers ensured the retention of the ATG codon for the initial methionine and the TGA stop codon for protein synthesis termination. The gene encoding the transporter truncated in the C-terminal lacked base pairs 4 to 60, resulting in a protein lacking the first 19 amino acid residues after the initial methionine. The gene encoding the transporter truncated in both N- and C-terminal regions, in addition to having the same modification described above, also lacked the last 36 coding base pairs, resulting in a protein lacking the last 12 amino acid residues in addition to the first 19 after the initial methionine.

The pV1382 plasmid (Table 1) served as the platform for expressing the CRISPR-Cas9 system in *S. cerevisiae* [58]. The ARS208 and ARS1309 loci were chosen for integrating the overexpression modules of the *SpBGL7* and *MgCBT2* genes, respectively, based on the research by Reider Apel and colleagues [64]. After sequencing both regions of interest in the MP-C5H1 strain genome (Table 1),

 doi:10.20944/preprints202408.1444.v1

we identified 20 bp segments to serve as the crRNA recognition sites. These segments were required to be followed by a protospacer adjacent motif (PAM) sequence recognized by the CRISPR-Cas9 system ("NGG" in this case). For the ARS208 site, the selected sequence was "GTCCGCTAAACAAAAGATCT", followed by the PAM sequence "TGG", located approximately 325 base pairs upstream of the ARS208 locus. For the ARS1309 site, the chosen sequence was "CCTGTGGTGACTACGTATCC", followed by the PAM sequence "AGG", situated approximately 180 base pairs upstream of the ARS1309 locus.

Each DNA fragment responsible for crRNA transcription, specific to the sequences mentioned above, was cloned into pV1382 as described [58]. The vector pV1382 was treated with enzyme *Bsm*BI (New England Biolabs, Leiden, The Netherlands), and the 5' ends of each DNA strand of the linearized plasmid were dephosphorylated using alkaline phosphatase (Quick CIP, New England Biolabs), followed by purification. The digested plasmid and the specific pair of oligonucleotides (sgRNA.ARS1309-F and sgRNA.ARS1309-R, Table 2) to target the CRISPR-Cas9 system to the ARS1309 locus, and sgRNA.ARS208-F and sgRNA.ARS208-R primers (Table 2) for the ARS208 locus, were incubated at 15°C for 16 hours in the presence of T4 DNA ligase (Thermo Fisher). The resulting plasmids were sequenced using the seq.p1382.sgRNA-F and seq.p1382.sgRNA-R primers (Table 2) to verify the correct insertion of the DNA fragments, yielding plasmids pV1382-ARS1309 and pV1382-ARS208 (Table 1).

To assemble the PCR-amplified DNA fragments for constructing the repair and gene overexpression modules, we utilized Gibson Assembly® with the NEBuilder® HiFi DNA Assembly kit (New England Biolabs). Each PCR reaction employed a pair of primers (primer sequences can be provided upon request) designed with at least 20 base pairs at the 3' end that anneal to the beginning or end of the intended amplification region, and at least 20 nucleotides at the 5' end identical to the adjacent end of the DNA portion in the other DNA molecule intended for joining. For constructing the repair fragment for inserting the gene encoding each version of the *MgCBT2* transporter, three initial fragments were joined: (I) a 515 bp DNA fragment identical to the region upstream of the cleavage site of the ARS1309 locus (5'ARS1309, obtained via colony PCR from the industrial strain MP-C5H1, Table 1); (II) a DNA fragment containing the P_{TDH3} promoter, the desired *MgCBT2* version, and the T_{CYC1} terminator (obtained via PCR using pGPD-426-MgCBT2, pGPD-426-MgCBT2ΔC, or pGPD-426-MgCBT2ΔNΔC plasmids as templates, Table 1); and (III) a 624 bp DNA fragment identical to the region downstream of the cleavage site of the ARS1309 locus (3'ARS1309, obtained via colony PCR from the industrial strain MP-C5H1).

For constructing the repair fragment containing the *SpBGL7* overexpression module, circular construction was chosen due to the low efficiency of Gibson Assembly in forming linear molecules from more than three distinct fragments. Circularization was achieved using the pMV vector (Table 1). The repair fragment was constructed by joining five distinct initial fragments: (I) a 695 bp DNA fragment identical to the region upstream of the cleavage site of the ARS208 locus (5'ARS208, obtained via colony PCR from strain MP-C5H1); (II) a DNA fragment containing 608 bp immediately upstream of the coding region of the *TEF1* gene, corresponding to its promoter region (P_{TEF1}, obtained via colony PCR from strain MP-C5H1); (III) a DNA fragment containing the *SpBGL7* gene (obtained via PCR using pGPD-424-SpBGL2 plasmid as template); (IV) a DNA fragment containing the 428 bp immediately downstream of the coding region of the *PGK1* gene, corresponding to its terminator region (T_{PGK1}, obtained via colony PCR from strain MP-C5H1); and (V) a 742 bp DNA fragment identical to the region downstream of the cleavage site of the ARS208 locus (3'ARS208, obtained via colony PCR from strain MP-C5H1). We changed the constitutive promoter (P_{TEF1}) and terminator (T_{PGK1}) controlling the *SpBGL7* gene to avoid any chromosomal instability with the promoter (P_{TDH3}) and terminator (T_{CYC1}) used for the *MgCBT2* permeases. Each purified PCR fragment was incubated at 50°C for 60 minutes in the presence of NEBuilder® HiFi DNA Assembly reagent. After incubation, the resulting plasmid (pMV-SpBGL7, Table 1) was transformed into *E. coli* DH5α.

For the insertion of the *SpBGL7* repair/overexpression module into the genome of the MP-C5H1 strain, transformations were performed using 300 ng of the purified pV1382-ARS208 plasmid and 10 µg of the repair/overexpression module amplified using primers ARS208-F and ARS208-R (Table 2)

 doi:10.20944/preprints202408.1444.v1

and the pMV-SpBGL7 plasmid as template (Table 1). Transformants were selected in YP-20 g/L glucose plates containing 0.1 g/L nourseothricin. Flipout of the pV1382-ARS208 plasmid was performed by overnight growth (twice) in nonselective liquid YP-20 g/L glucose medium. Drug-sensitive colonies, which had lost the plasmid, were identified by plating for single colonies on nonselective media and subsequent identification by replica plating to selective media. The correct insertion of the *SpBGL7* module at ARS208 in the MP-B7 strain (Table 1) was confirmed by sequencing. The same concentrations of the pV1382-ARS1309 plasmid and the repair/overexpression modules containing the different *MgCBT2* transporters (*MgCBT2*, *MgCBT2*ΔC or *MgCBT2*ΔNΔC), produced by Gibson Assembly, were used to transform strain MP-B7, yielding strains MP-B7-CBT2, MP-B7-CBT2ΔC and MP-B7-CBT2ΔNΔC, respectively (Table 1). All insertions at the ARS1309 locus were confirmed by sequencing.

2.3. Enzymatic and Transport Activity Assays

The hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside (*p*NPβG), cellobiose, or *p*-nitrophenyl-β-D-xylopyranoside (*p*NPβX) was determined using permeabilized yeast cells [65]. Approximately 50 µL of permeabilized cell suspension (at concentrations ranging from approximately 0.1 to 0.4 g/L) were added to 450 µL of 100 mM MOPS-NaOH, pH 6.8 buffer containing the desired amount of substrate, and incubated at 30°C for 10 minutes. The reaction was stopped by placing the tubes at 100°C for 3 minutes. Pre-boiled cells for 3 minutes were used as controls. We used final concentrations ranging from 0.05 to 10 mM of *p*NPβG and *p*NPβX, or 1 to 80 mM cellobiose as substrates. After the reaction, cells were centrifuged at 2600x *g* for 5 minutes, and the supernatant from assays using *p*NPβG and *p*NPβX were used to determine the enzymatic activity by measuring the concentration of *p*-nitrophenol released by substrate hydrolysis, at an absorbance of 400 nm. To determine cellobiose hydrolysis, the supernatant from the assays was used to measure the concentration of glucose formed using a commercial glucose oxidase-peroxidase kit (Glicose Pap Liquiform Labtest, Centerlab, Belo Horizonte, MG, Brazil). Activities are expressed as nmol of *p*-nitrophenol or glucose produced by (mg dry cell weight)$^{-1}$ min^{-1}. The values of K_m and V_{max} were determined through nonlinear regression applied to the Michaelis-Menten kinetic model using the GraphPad Prism v. 8.0 software (GraphPad Software, Boston, MA, USA).

The transport assays followed a colorimetric method originally developed for determination of α-glucoside (*p*-nitrophenyl-α-D-glucopyranoside) transport by yeast maltose permeases [66]. Cells were harvested from liquid culture, washed twice with chilled (4°C) sterile distilled water, and resuspended in 50 mM succinate-Tris pH 5.0 buffer to achieve a cell concentration of approximately 30 g/L. Aliquots of 50 µL of this cell suspension were transferred to Eppendorf tubes, and a volume of 50 µL of 10 mM *p*NPβG or *p*NPβX in the same buffer was added. The cells were incubated at 30°C for 10 minutes, during which the internalized substrate underwent hydrolysis due to the activity of the intracellular β-glucosidase. The reaction was stopped by incubating the tubes at 100°C for 3 minutes. Subsequently, 200 µL of 2 M $NaHCO_3$ was added. Pre-boiled cells for 3 minutes were used as negative controls. Cells were centrifuged at 2600x *g* for 5 minutes, and the supernatant was used to determine the concentration of *p*-nitrophenol produced, measured by absorbance at 400 nm. The transport activities are expressed as nmol of *p*NPβG or *p*NPβX transported (*p*-nitrophenol produced) by (mg dry cell weight)$^{-1}$ min^{-1}.

2.4. Analytical Methods

Cellobiose and xylose substrates, and ethanol, xylitol, and glycerol products in medium samples were quantified by high performance liquid chromatography (Prominence HPLC system) equipped with a RID-20A refractive index detector (Shimadzu Co., Tokyo, Japan) using an Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, CA, USA). The HPLC apparatus was operated at 50°C using 5 mM H_2SO_4 as mobile phase at a flow rate of 0.6 mL/min, and 10 µL injection volume.

2.5. Prediction of Lysine Residues in MgCBT2 Transporter with Ubiquitinylation Potential

 doi:10.20944/preprints202408.1444.v1

To predict potential transmembrane domains (TMs) of the *MgCBT2* transporter (NCBI GenBank ID: PP826654.1), we used PRALINE with the PSIPRED and HMMTOP methods [67]. The lysine residues with ubiquitinylation potential in the N- and C-terminal domains of the permease were determined with the BDM-PUB [68] and UbPred programs [69].

3. Results

3.1. Cloning and Expression of an Intracellular Yeast β-Glucosidase in S. cerevisiae

Initially we used the amino acid sequence of the intracellular β–glucosidase encoded by the *gh1-1* gene from *N. crassa* [26] to search for putative β–glucosidases from yeasts, but our analysis did not revealed a gene with significant identity. However, using the sequence of the β-glucosidase from the cellulolytic fungi *Thielavia terrestris* (*TtBG* gene, NCBI Gene ID: 11519932 [27]), revealed two genes with 33% (annotated as *SpBGL2*) and 29% (*SpBGL7*) of identity in the genome of the cellobiose-fermenting yeast *Sp. passalidarum* [46,63]. Attempts to clone the *SpBGL2* gene from the *Sp. passalidarum* UFMG-CM-Y474 strain (Table 1) failed, but we succeeded in cloning the *SpBGL7* β-glucosidase into the pGPD-424 plasmid (Table 1). When the pGPD-424-SpBGL7 plasmid was transformed into strain CEN.PK2-1C, the obtained strain (B7) could not use or grow on cellobiose (Figure 1), but we could clearly detect a intracellular β-glucosidase activity when we used permeabilized yeast cells, with a K_m of 18.2 ± 3.9 mM and V_{max} of 1,188 ± 85 nmol mg^{-1} min^{-1} with cellobiose, and higher affinity (K_m of 0.8 ± 0.2 mM and V_{max} of 5,368 ± 199 nmol mg^{-1} min^{-1}) with *p*NPβG. This enzyme had also hydrolytic activity with *p*NPβX (a synthetic analog of xylobiose), with a K_m of 1.0 ± 0.5 mM and V_{max} of 1,088 ± 175 nmol mg^{-1} min^{-1}.

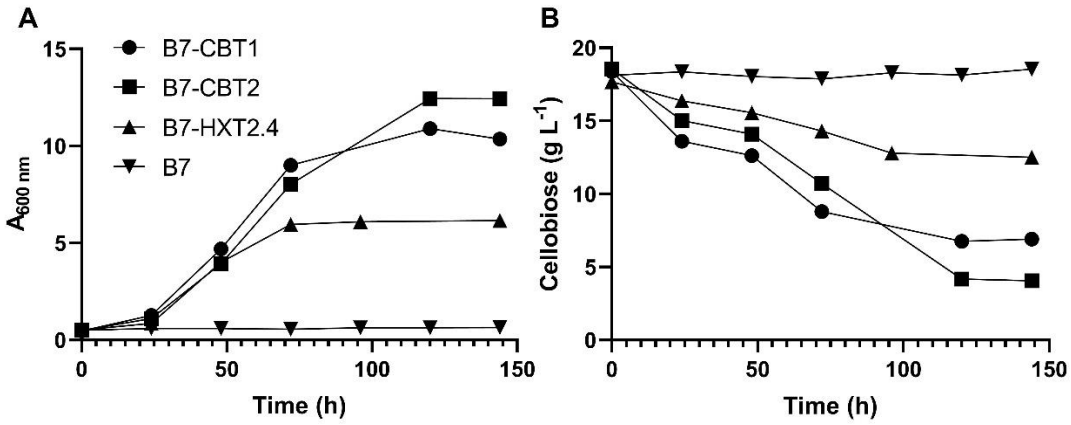

Figure 1. Cell growth (**A**) and cellobiose consumption (**B**) in YNB medium containing 20 g/L cellobiose by the indicated yeast strains harboring the intracellular *SpBGL7* β-glucosidase (strain B7), or this strain also transformed with the pGPD-426 plasmids containing the genes (*CtCBT1*, *MgCBT2* and *SiHXT2.4*) encoding sugar transporters.

3.2. Cloning and Expression of Yeast Sugar (Cellobiose) Transporters in S. cerevisiae

The *S. cerevisiae* strain B7, having high intracellular β-glucosidase activity but unable to grow on cellobiose, was used as platform to identifiy putative sugar transporters capable of mediating the uptake of cellobiose. First, and taking into account the close relationship between *Sc. stipitis* and *Sc. illinoinensis* [70], we used the genomic information of *Sc. stipitis* [63] to design primers and amplify the corresponding *HXT2.4* cellobiose transporter [44] from *Sc. illinoinensis.* Primers HXT2.4-F and HXT2.4-R (Table 2) allowed the amplification of a 1.7-1.8 Kb DNA fragment from the genome of *Sc. illinoinensis*, which was cloned into the pGPD-426 plasmid (Table 1). We also used the amino acid sequence of the *CDT-2* cellobiose transporter from *N. crassa* [26,34,36,37] to search for yeast putative cellobiose transporters, and found two sequences, one with 28% identity with *CDT-2* present in the genome of the yeast *C. tropicalis* (and thus named *CtCBT1*), and another sequence with 27% identity

 doi:10.20944/preprints202408.1444.v1

in the genome of *M. guilliermondii* (named as *MgCBT2*). Using specific primers for these genes (Table 2), these putative cellobiose transporters were cloned into the pGPD-426 plasmid (Table 1). As can be seen in Figure 1, the co-expression in *S. cerevisiae* of the intracellular *SpBGL7* β-glucosidase together with any of the three transporters (*SiHXT2.4*, *CtCBT1* and *MgCBT2*) allowed cell growth on cellobiose, although cellobiose consumption and cell growth was lower in the strain expressing the *SiHXT2.4* permease.

We next tested the capacity of these laboratory yeast strains to ferment cellobiose under microaerobic conditions (Figure 2). While the strain harboring the *SiHXT2.4* permease consumed small amounts of cellobiose, and could not produce any ethanol, the strain with the *CtCBT1* transporter also consumed very small amounts of cellobiose, and produced less than 1.5 g/L of ethanol from the sugar. The best results regarding cellobiose fermentation were with the strain expressing the *SpBGL7* β-glucosidase together with the *MgCBT2* permease, which consumed more than 16 g/L of cellobiose and produced almost 8 g/L of ethanol, although the fermentation was stuck (incomplete).

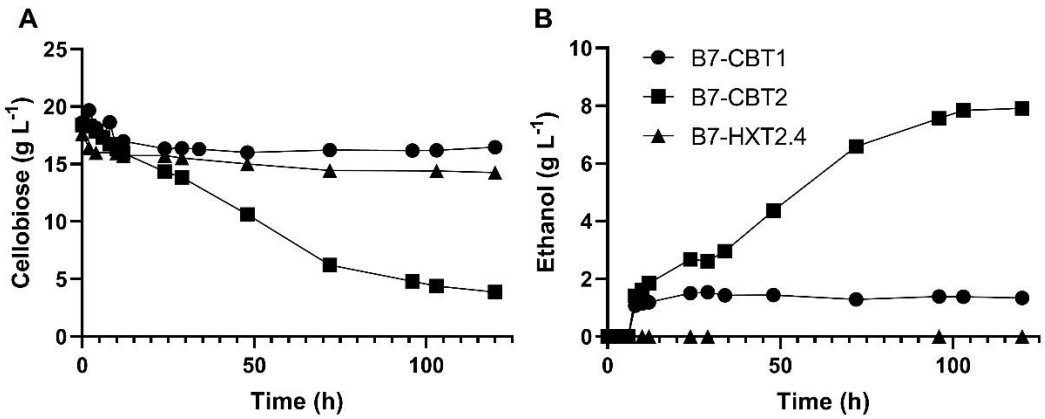

Figure 2. Cellobiose consumption (**A**) and ethanol production (**B**) during fermentation of 20 g/L cellobiose in rich YP medium by 10 g dry cell weight/L of the indicated yeast strains harboring the *SpBGL7* β-glucosidase and the genes *CtCBT1*, *MgCBT2* and *SiHXT2.4* encoding sugar transporters.

In order to better understand the reason for the cellobiose stuck fermentation by strain B7-CBT2 (pGPD-424-SpBGL7 + pGPD-426-MgCBT2, Table 1), we initially used *p*NPβG, a synthetic analog of cellobiose, in a colorimetric assay to determine the transport activity of the *MgCBT2* permease, as already described for the maltose permeases of *S. cerevisiae* [66]. After growth on cellobiose the B7-CBT2 strain was capable to transport 7.4 ± 1.6 nmol of *p*NPβG mg^{-1} min^{-1}. The B7-CBT2 cells were also capable to transport *p*NPβX (3.3 ± 0.1 nmol mg^{-1} min^{-1}), indicating that the *MgCBT2* permease probably also transports xylobiose. Figure 3 shows the intracellular β-glucosidase activity (determined with *p*NPβG), as well as the *p*NPβG transport activity (both activities were determined with 5 mM of substrate), during the stuck cellobiose fermentation by strain B7-CBT2.

Preprints.org (www.preprints.org) | NOT PEER-REVIEWED | Posted: 20 August 2024 doi:10.20944/preprints202408.1444.v1

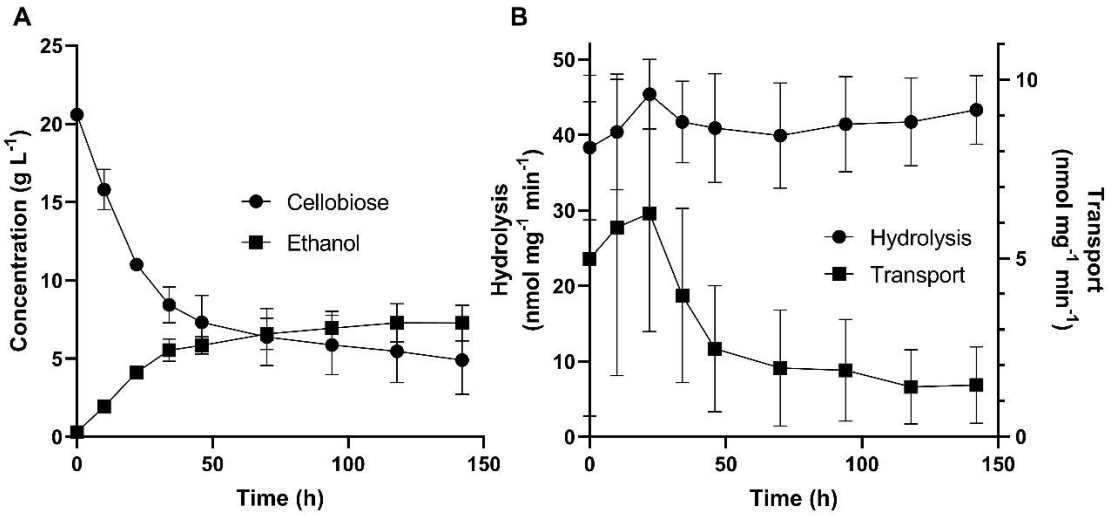

Figure 3. Cellobiose consumption and ethanol production (**A**), and pNPβG intracellular hydrolysis and transport activities (**B**) by strain B7-CBT2 during fermentation of 20 g/L cellobiose in rich YP medium.

It is evident in Figure 3B that the intracellular β–glucosidase activity remained high along all the fermentation period, while the transport activity was higher only in the first 36 h of the fermentation (at the time in which cellobiose was rapidly consumed and fermented, Figure 3A), and from 48 h on the transport activity dropped, and was accompanied by a significant drop in cellobiose consumption by the cells. Thus, our results indicate that the stuck cellobiose consumption is a consequence of a drop in the transport activity, and not in the intracellular hydrolysis of the disaccharide. Since a possible explanation for the drop in the transport activity is the well-known post-transcriptional mechanism of down-regulation of yeast sugar permeases, involving ubiquitinylation, endocytosis and vacuolar degradation [71], we next identified lysine residues (the site of ubiquitinylation) in the N- and C-terminal domains of the *MgCBT2* transporter, in order to develop strategies to avoid this drop in transport activity causing cellobiose stuck fermentations.

3.3. Identification of Possible Lysine Residues Involved in Ubiquitinylation and Down-Regulation of the MgCBT2 Cellobiose Transporter Expressed in S. cerevisiae

Our data indicated that the best cellobiose transporter from yeasts that we cloned was *MgCBT2* from *M. guilliermondii*, but it still could not allow the use and fermentation of all cellobiose (stuck fermentation) present in the medium. The analysis of the presence of lysine residues (K) with ubiquitinylation potential at the cytoplasmic C-terminal domain of the *MgCBT2* transporter revealed 3 lysine residues (K534, K536 and K544) with high ubiquitinylation potential present in the last 12 amino acids of the transporter (Figure 4), while at the cytoplasmic N-terminal domain only 2 lysine residues (K10 and K20) were found with medium ubiquitinylation potential (Figure 4).

A

```
MgCBT2        MVSNSSSYWKMTDSNSSIEKQQVITTQESGIDVTNHLEIDDHS..
MgCBT2ΔC      MVSNSSSYWKMTDSNSSIEKQQVITTQESGIDVTNHLEIDDHS..
MgCBT2ΔNΔC                      MQQVITTQESGIDVTNHLEIDDHS..
```

B

```
MgCBT2        ..FFPETSGYTLEEVAKVFGDDPDTTIHILATPKEKLSLEHAEKV
MgCBT2ΔC      ..FFPETSGYTLEEVAKVFGDDPDTTIHILATP
MgCBT2ΔNΔC    ..FFPETSGYTLEEVAKVFGDDPDTTIHILATP
```

 doi:10.20944/preprints202408.1444.v1

Figure 4. Sequence alignment of the cytoplasmic N-terminal first 43 amino acids (**A**) and the cytoplasmic C-terminal last 43 amino acids of the protein, after the last transmembrane domain (**B**), deduced from the *MgCBT2* gene, and the truncated versions of *MgCBT2* at the C-terminal domain (*MgCBT2*ΔC), or at both the N- and C-terminal domains (*MgCBT2*ΔNΔC). The lysine residues with medium (blue) or high (red) ubiquitinylation potential were determined with the BDM-PUB [68] and UbPred programs [69].

Considering that lysine residues with ubiquitinylation potential are involved in removing the transporters from the plasma membrane through endocytosis [71,72], we decided to remove these terminal lysine residues by simply truncating the *MgCBT2* permease. We removed the last 12 amino acid residues of the protein (with 3 lysine residues with high ubiquitinylation potential) by introducing a premature stop codon during cloning as described in Materials and Methods, and this modified transporter was denominated *MgCBT2*ΔC (Figure 4). Another truncated version of *MgCBT2* was produced were we not only removed the last 12 amino acid residues, but also the first 19 amino acid residues of the protein (after the initial methionine), producing the *MgCBT2*ΔNΔC transporter lacking all lysine residues with ubiquitinylation potential from both the N- and C-terminal domains (Figure 4).

These cellobiose transporters (*MgCBT2*, *MgCBT2*ΔC, and *MgCBT2*ΔNΔC), as well as the intracellular *SpBGL7* β-glucosidase, were introduced into the genome of an industrial *S. cerevisiae* strain (MP-C5H1) capable of fermenting xylose, using CRISPR-Cas9 as described in Materials and Methods. As can be seen in Figure 5, while the strain harboring the *MgCBT2*ΔC transporter continued to produce stuck cellobiose fermentations as the strain with 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, producing higher levels of ethanol. However, is important to note that although this yeast strain with *MgCBT2*ΔNΔC consumes all the sugar, the ethanol yield ($Y_{p/s}$ = 0.38 ± 0.01 g of ethanol/g of consumed cellobiose) is very similar to the one obtained with the strain harboring the truncated *MgCBT2*ΔC transporter ($Y_{p/s}$ = 0.39 ± 0.01 g of ethanol/g of consumed cellobiose), and lower when compared with the wild-type *MgCBT2* permease ($Y_{p/s}$ = 0.47 ± 0.02 g of ethanol/g of consumed cellobiose).

Finally, since direct cellobiose transport and intracellular hydrolysis by recombinant *S. cerevisiae* is interesting in the context of cellobiose-xylose co-fermentations [29–31], we performed such fermentations with the industrial xylose-fermenting strain MP-C5H1 harboring the *MgCBT2*ΔNΔC transporter and *SpBGL7* β-glucosidase (strain MP-B7-CBT2ΔNΔC, Table 1). As can be seen in Figure 6, xylose is indeed totally consumed and fermented in the first 24 h, and although cellobiose is also consumed during that period, it is consumed very slowly after that time point (thus a stuck cellobiose fermentation), even considering that the strain harbors the *MgCBT2*ΔNΔC transporter lacking lysine residues in both the N- and C-terminal domains. While cellobiose is consumed slowly, also the xylitol produced from xylose fermentation also drops, thus both are probably contributing to the slight increase in ethanol produced (from 7.5 to 8.9 g/L) by the cells during the slow fermentation.

Preprints.org (www.preprints.org) | NOT PEER-REVIEWED | Posted: 20 August 2024 doi:10.20944/preprints202408.1444.v1

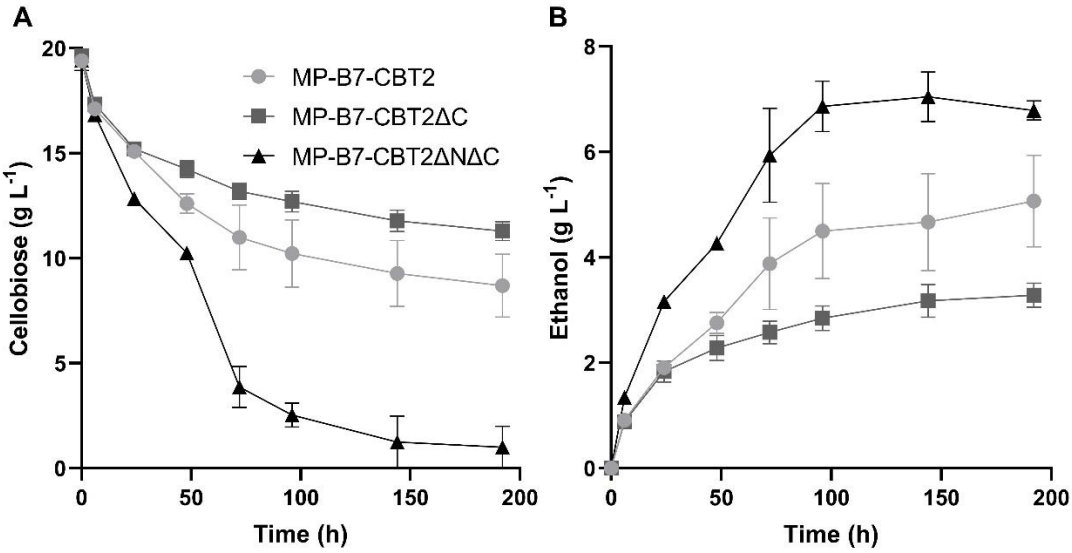

Figure 5. Cellobiose consumption (**A**) and ethanol production (**B**) during fermentation of 20 g/L cellobiose in rich YP medium by the indicated industrial yeast strains harboring the intracellular *SpBGL7* β-glucosidase and the *MgCBT2*, *MgCBT2*ΔC, and *MgCBT2*ΔNΔC cellobiose transporters.

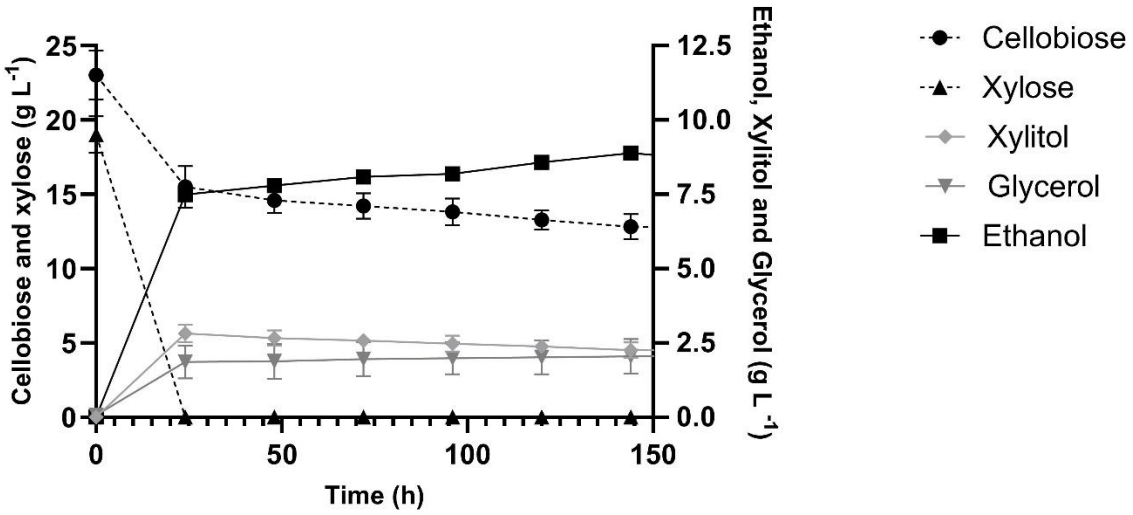

Figure 6. Co-fermentation of 20 g/L cellobiose plus 20 g/L xylose in rich YP medium by the recombinant industrial yeast strain MP-B7-CBT2ΔNΔC harboring the intracellular *SpBGL7* β-glucosidase and the *MgCBT2*ΔNΔC cellobiose transporter.

4. Discussion

We have cloned an intracellular β-glucosidase gene (*SpBGL7*) from the cellobiose fermenting yeast *Sp. passalidarum* [46], and expressed it in *S. cerevisiae*. While the β-glucosidase *gh1-1* from *N. crassa* belongs to the GH1 family of glycosyl hydrolases, the cloned enzyme belongs to the GH3 family, where are found not only β-glucosidases, but also β-xylosidases and other enzymes. The *SpBGL7* enzyme had higher affinity with *p*NPβG and *p*NPβX, and lower affinity with cellobiose, as already reported for other β-glucosidases characterized biochemically from yeasts [73,74]. Using the *S. cerevisiae* strain expressing the *SpBGL7* enzyme allowed the identification and cloning of 3 yeast cellobiose transporters that permitted growth of the strain in the presence of cellobiose. Figure 7 shows that the 3 transporters (we included the *HXT2.4* permease from *Sc. stipitis* [44], although the transporter we cloned was from *Sc. illinoinensis*) it is close to the known yeast cellobiose transporters,

 doi:10.20944/preprints202408.1444.v1

including the one cloned from *L. starkeyi* [45] and a cellobiose transporter *CEL1* from *Kluyveromyces dobzhanskii (that allows growth of S. cerevisiae on cellobiose, but no fermentation data is available) [75]. Note that in general Kluyveromyces yeasts can utilize cellobiose through the lactose permease encoded by LAC12. The cloned transporters are also closer to CDT-2 from N. crassa, and other transporters known to transport cellodextrins and xylobiose.*

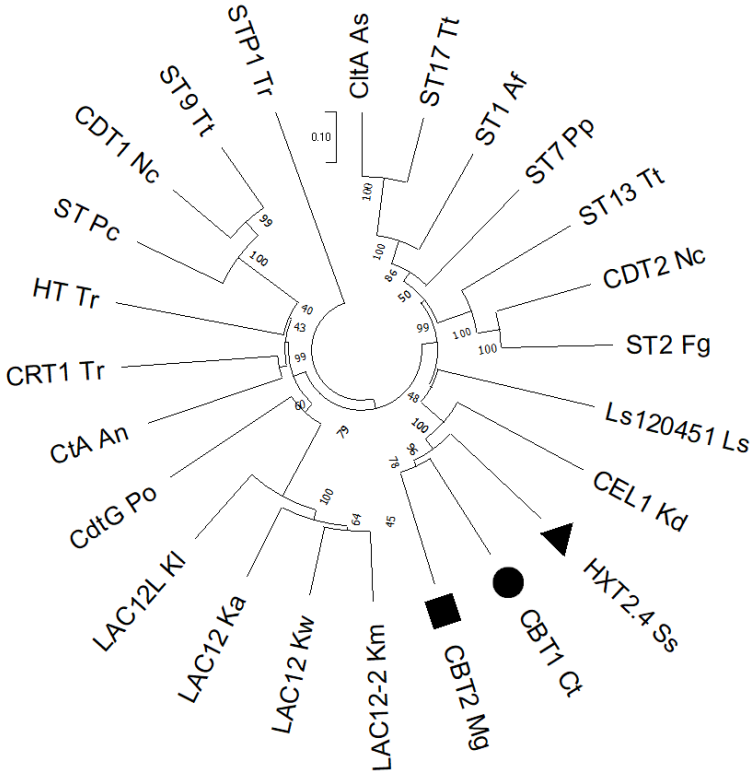

Figure 7. Phylogenetic classification of cellobiose transporters from various yeast and fungal hosts. The phylogenetic tree contains 24 transporter sequences, and the numbers at the nodes represent percentage bootstrap values based on 1500 samplings. The abbreviation of each species is added after the name of the transporter genes: As = *Aspergillus nidulans*, Tt = *Thielavia terrestris*, Af = *Aspergillus flavus*, Pp = *Postia placenta*, Nc = *Neurospora crassa*, Fg = *Fusarium graminearum*, Ls = *Lipomyces starkeyi*, Kd = *Kluyveromyces dobzhanskii*, Ss = *Scheffersomyces stipitis*, Ct = *Candida tropicalis*, Mg = *Meyerozyma guilliermondii*, Km = *Kluyveromyces marxianus*, Kw = *Kluyveromyces wickerhamii*, Ka = *Kluyveromyces aestuarii*, Kl = *Kluyveromyces lactis*, Po = *Penicillium oxalicum*, An = *Aspergillus niger*, Tr = *Trichoderma reesei*, and Pc = *Penicillium chrysogenum*.

Although the 3 transporters allowed growth on cellobiose by the recombinant *S. cerevisiae* strain, only the *MgCBT2* permease from *M. guilliermondii* allowed cellobiose fermentation, but the consumption of the sugar stopped after 50-75 h, leading to a stuck (incomplete) fermentation. Our analysis revealed that the reduced transport activity over time was responsible for this stuck fermentation, and that prompted us to look for lysine residues in the N- or C-terminal cytoplasmic domains of the *MgCBT2* transporter that could be ubiquitinylated, a signal that triggers the endocytosis and vacuolar degradation of plasma membrane transporters. Indeed, some years ago Sen and co-workers [76] showed that *S. cerevisiae* cells expressing the *CDT-2* cellobiose transporter also produced stuck cellobiose fermentations, and that cellobiose transport triggers the internalization of the permease, and they were able to identify that four α-arrestins (*ROD1*, *ROG3*, *ALY1*, and *ALY2*) are primarily responsible for this ubiquitinylation and internalization of the *CDT-2* transporter. We have also shown that other heterologous sugar transporters expressed in *S. cerevisiae* are removed

 doi:10.20944/preprints202408.1444.v1

from the plasma membrane through action of the *ROD1* and *ROG3* α-arrestins [77]. In another approach, mutated versions of the *CDT-2* permease were engineered changing cytoplasmic lysine residues into arginine, and revealed that lysine residues in the C-terminal domain were responsible for the α–arrestin mediated internalization of the transporter. A truncated C-terminal *CDT-2* transporter (losing the last lysine, K522) was also shown to remain stable at the plasma membrane, and either the mutant *CDT-2* permease lacking lysine residues at the C-terminal domain, or the same transporter truncated at this C-terminal domain, showed improved cellobiose consumption and fermentation [76]. Truncation at the C-terminal domain of other sugar transporters is an interesting strategy that allows stable expression of the permeases at the plasma membrane, allowing better sugar consumption and fermentation [77–79]. However, for other permeases (e.g. *HXT1*, *GAL2*) the lysine residues involved in ubiquitinylation and endocytosis can be present at the N-terminal domain [80,81]. For example, the *HXT1* hexose transporter from *S. cerevisiae* lacking practically all the N-terminal domain is stable and functional at the plasma membrane, allowing efficient xylose fermentation by recombinant *S. cerevisiae* cells [56,81–83].

In the case of the *MgCBT2* transporter, we found 3 lysine residues at the C-terminal domain with high ubiquitinylation potential, and two others (with medium ubiquitinylation potential) at the N-terminal domain. Truncation of the *MgCBT2* permease at the C-terminal domain did not improved cellobiose consumption, when the transporter was expressed in an industrial *S. cerevisiae* strain (Figure 5), but when both cytoplasmic terminal domains were truncated (removing the first 19 amino acids, and the last 12 amino acids), the *MgCBT2*ΔNΔC transporter allowed total cellobiose consumption and fermentation by the recombinant yeast cells. However, during co-fermentation of cellobiose with xylose, the cells expressing the *MgCBT2*ΔNΔC transporter could not consume all the cellobiose present in the medium after the total consumption and fermentation of xylose, indicating that probably other factors are impairing the activity of the *MgCBT2*ΔNΔC permease, or the intracellular metabolism of cellobiose. One such factor could be the ethanol produced during fermentation, as many nutrient transporters are sensitive to high ethanol concentrations [84,85]. Indeed, when we performed a co-fermentation of 20 g/L sucrose plus 20 g/L cellobiose by the industrial yeast strain harboring the *MgCBT2*ΔNΔC transporter, sucrose was efficiently fermented in 24 h, producing 10 g/L ethanol, and although some cellobiose was consumed initially, from that time point on, cellobiose was consumed very slowly, producing a stuck fermentation.

In general cellobiose fermentation by recombinant *S. cerevisiae* yeasts is much more slower than the fermentation of other sugar (e.g. glucose), with also lower ethanol yields [26,27]. Thus, several other strategies have been employed to increase cellobiose consumption and fermentation, including mutagenesis, direct evolution/selection (also called evolutionary engineering) [44,86–90], as well as increasing the copy number of transporter or β–glucosidase genes [91,92]. Our results show that the identification of cytoplasmatic lysine residues in the N- or C-terminal domain capable of ubiquitinylation, and removing such residues by truncating the transporter, is another interesting strategy for enhancing cellobiose consumption and fermentation. Another issue that needs to be considered is the fact that cellobiose is not recognized by *S. cerevisiae* as a sugar to be fermented [93], and thus it is also required to introduce/engineer transcriptional factors to increase cellobiose fermentation by this industrial yeast [94,95]. Another strategy recently described to increase cellobiose fermentation is the deletion of the mitochondrial adenylate kinase encoded by the *ADK2* gene, or the deletion of the *MRX1* gene involved in the response to oxidative stress that was shown to increase cellobiose (and xylose) fermentation by recombinant *S. cerevisiae* [96,97]. It would be interesting to verify if some of these strategies would enhance cellobiose fermentation by an industrial *S. cerevisiae* expressing the intracellular β-glucosidase encoded by the *SpBGL7* gene and the *MgCBT2*ΔNΔC cellobiose transporter.

5. Conclusions

In the present work we have cloned an intracellular β-glucosidase encoded by the *SpBGL7* gene from the xylose-fermenting yeasts *Sp. passalidarum*, and three cellobiose transporters from yeasts (*HXT2.4* from *Sc. illinoinensis*, *CBT1* from *C. tropicalis* and *CBT2* from *M. guilliermondii*) that allowed

 doi:10.20944/preprints202408.1444.v1

growth of recombinant *S. cerevisiae* cells on this carbon source. While the *CBT2* transporter allowed incomplete (stuck) fermentation of cellobiose, the truncation of the N- and C-terminal domains, removing lysine residues with ubiquitinylation potential, allowed the complete consumption and fermentation of cellobiose by an industrial yeast strain. This work therefore highlights the importance of post-translational modifications in the correct expression of novel sugar transporters in recombinant *S. cerevisiae* strains.

Author Contributions: Conceptualization, L.G.K., M.M.K., and B.U.S.; investigation, methodology, and formal analysis, L.G.K., M.M.K., L.C.S., I.C.C.F., and M.J.L.; resources, C.F., M.J.L., K.J.V. and B.U.S.; writing—review and editing, C.F., M.J.L. and B.U.S.; project administration and funding acquisition, C.F., K.J.V., and B.U.S. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported in part by grants and fellowships from the Brazilian agencies CAPES (process nº 7207-15-8 and 88887.575075/2020-00), CNPq (process nº 307290/2012-3, 478841/2013-2, 30862/2015-6, 308389/2019-0 and 309047/2023-4), and FINEP (process nº 01.09.0566.00/1421-08), and also by the Fundação para a Ciência e a Tecnologia (FCT), Portugal (CAPES/FCT process nº 359/14 and postdoctoral fellowship SFRH/BPD/102803/2014 to M.J.L.) and I.P., Project MOSTMICRO-ITQB. K.J.V. acknowledges funding from VIB, VLAIO, KU Leuven, FWO and project FWO SBO S004624N LAPLACE. This work is also part of the project "INCT Yeasts: Biodiversity, preservation and biotechnological innovation", supported by grants and fellowships from CNPq (process 406564/2022-1).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: All data used in this study are included in the manuscript, and further inquiries can be directed to the corresponding author.

Acknowledgments: We would like to thank Dr. Carlos A. Rosa (Federal University of Minas Gerais, Brazil) for providing some of the yeast strains used in the present work.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

References

1. Jaiswal, D.; de Souza, A.; Larsen, S.; Lebauer, D.S.; Miguez, F.E.; Sparovek, G.; Bollero, G.; Buckeridge, M.S.; Long, S.P. Brazilian sugarcane ethanol as an expandable green alternative to crude oil use. *Nat. Clim. Chang.* **2017**, *7*, 788–792. https://doi.org/10.1038/nclimate3410
2. Liu, Y.; Cruz-Morales, P.; Zargar, A.; Belcher, M.S.; Pang, B.; Englund, E.; Dan, Q.; Yin, K.; Keasling, J.D. Biofuels for a sustainable future. *Cell* **2021**, *184*, 1636–1647. https://doi.org/10.1016/j.cell.2021.01.052
3. Jacobus, A.P.; Gross, J.; Evans, J.H.; Ceccato-Antonini, S.R.; Gombert, A.K. *Saccharomyces cerevisiae* strains used industrially for bioethanol production. *Essays Biochem.* **2021**, *65*, 147–161. https://doi.org/10.1042/EBC20200160
4. dos Santos, L.V.; de Barros Grassi, M.C.; Gallardo, J.C.M.; Pirolla, R.A.S.; Calderón, L.L.; de Carvalho-Netto, O.V.; et al. Second-generation ethanol: The need is becoming a reality. *Ind. Biotechnol.* **2016**, *12*, 40–57. https://doi.org/10.1089/ind.2015.0017
5. Igwebuike, C.M.; Awad, S.; Andrès, Y. Renewable energy potential: Second-generation biomass as feedstock for bioethanol production. *Molecules* **2024**, *29*, 1619. https://doi.org/10.3390/molecules29071619
6. Gao, M.; Ploessl, D.; Shao, Z. Enhancing the co-utilization of biomass-derived mixed sugars by yeasts. *Front. Microbiol.* **2019**, *9*, 3264. https://doi.org/10.3389/fmicb.2018.03264
7. Broda, M.; Yelle, D.J.; Serwańska, K. Bioethanol production from lignocellulosic biomass-Challenges and solutions. *Molecules* **2022**, *27*, 8717. https://doi.org/10.3390/molecules27248717
8. Yadav, A.; Sharma, V.; Tsai, M.L.; Chen, C.W.; Sun, P.P.; Nargotra, P.; Wang, J.X.; Dong, C.D. Development of lignocellulosic biorefineries for the sustainable production of biofuels: Towards circular bioeconomy. *Bioresour. Technol.* **2023**, *381*, 129145. https://doi.org/10.1016/j.biortech.2023.129145
9. da Silva, A.S.; Espinheira, R.P.; Teixeira, R.S.S.; de Souza, M.F.; Ferreira-Leitão, V.; Bon, E.P.S. Constraints and advances in high-solids enzymatic hydrolysis of lignocellulosic biomass: a critical review. *Biotechnol. Biofuels.* **2020**, *13*, 58. https://doi.org/10.1186/s13068-020-01697-w
10. Østby, H.; Hansen, L.D.; Horn, S.J.; Eijsink, V.G.H.; Várnai, A. Enzymatic processing of lignocellulosic biomass: principles, recent advances and perspectives. *J. Ind. Microbiol. Biotechnol.* **2020**, *47*, 623-657. https://doi.org/10.1007/s10295-020-02301-8

11. Sørensen, A.; Lübeck, M.; Lübeck, P.S.; Ahring, B.K. Fungal β-glucosidases: a bottleneck in industrial use of lignocellulosic materials. *Biomolecules* **2013**, *3*, 612-631. https://doi.org/10.3390/biom3030612
12. Singhania, R.R.; Patel, A.K.; Sukumaran, R.K.; Larroche, C.; Pandey, A. Role and significance of β-glucosidases in the hydrolysis of cellulose for bioethanol production. *Bioresour. Technol*. **2013**, *127*, 500-507. https://doi.org/10.1016/j.biortech.2012.09.012
13. Pryor, S.W.; Nahar, N. β-glucosidase supplementation during biomass hydrolysis: How low can we go? *Biomass Bioenergy* **2015**, *80*, 298–302. https://doi.org/10.1016/j.biombioe.2015.06.005
14. Ferreira, R.G.; Azzoni, A.R.; Freitas, S. On the production cost of lignocellulose-degrading enzymes. *Biofuels Bioprod. Bioref*. **2021**, *15*, 85–99. https://doi.org/10.1002/bbb.2142
15. Barbosa, A.M.; Giese, E.C.; Dekker, R.F.; Borsato, D.; Briones Pérez, A.I.; Ubeda Iranzo, J.F. Extracellular β-glucosidase production by the yeast *Debaryomyces pseudopolymorphus* UCLM-NS7A: optimization using response surface methodology. *N. Biotechnol*. **2010**, *27*, 374-381. https://doi.org/10.1016/j.nbt.2010.05.013
16. Wang, X.; Liu, Z.L.; Weber, S.A.; Zhang, X. Two new native β-glucosidases from *Clavispora* NRRL Y-50464 confer its dual function as cellobiose fermenting ethanologenic yeast. *PLoS One* **2016**, *11*, e0151293. https://doi.org/10.1371/journal.pone.0151293
17. Vaz, J.E.; Rabelo, L.; Zaiter, M.A.; Pereira, W.E.S.; Metzker, G.; Boscolo, M.; da Silva, R.; Gomes, E.; da Silva, R.R. Functional properties and potential application of ethanol tolerant β-glucosidases from *Pichia ofunaensis* and *Trichosporon multisporum* yeasts. *3 Biotech*. **2021**, *11*, 467. https://doi.org/10.1007/s13205-021-03027-9
18. Albarello, M.L.; Giehl, A.; Tadioto, V.; dos Santos, A.A.; Milani, L.M.; Bristot, J.C.; Tramontin, M.A.; Treichel, H.; Bernardi, O.; et al. Analysis of the holocellulolytic and fermentative potentials of yeasts isolated from the gut of *Spodoptera frugiperda* larvae. *Bioener. Res.* **2023**, *16*, 2046-2057. https://doi.org/10.1007/s12155-023-10616-4
19. Jeffries, T.W.; Van Vleet, J.R. *Pichia stipitis* genomics, transcriptomics, and gene clusters. *FEMS Yeast Res*. **2009**, *9*, 793-807. https://doi.org/10.1111/j.1567-1364.2009.00525.x
20. Santos, R.O.; Cadete, R.M.; Badotti, F.; Mouro, A.; Wallheim, D.O.; Gomes, F.C.; Stambuk, B.U.; Lachance, M.A.; Rosa, C.A. *Candida queiroziae* sp. nov., a cellobiose-fermenting yeast species isolated from rotting wood in Atlantic Rain Forest. *Antonie Van Leeuwenhoek* **2011**, *99*, 635-642. https://doi.org/10.1007/s10482-010-9536-z
21. Lopes, M.R.; Lara, C.A.; Moura, M.E.F.; Uetanabaro, A.P.T.; Morais, P.B.; Vital, M.J.S.; Rosa, C.A. Characterization of the diversity and physiology of cellobiose-fermenting yeasts isolated from rotting wood in Brazilian ecosystems. *Fungal Biol*. **2018**, *122*, 668-676. https://doi.org/10.1016/j.funbio.2018.03.008
22. Barrilli, É.T.; Tadioto, V.; Milani, L.M.; Deoti. J.R.; Fogolari, O.; Müller, C.; Barros, K.O.; Rosa, C.A.; dos Santos, A.A.; et al. Biochemical analysis of cellobiose catabolism in *Candida pseudointermedia* strains isolated from rotten wood. *Arch. Microbiol*. **2020**, *202*, 1729-1739. https://doi.org/10.1007/s00203-020-01884-1
23. Kim, D.H.; Choi, H.J.; Lee, Y.R.; Kim, S.J.; Lee, S.; Lee, W.H. Comprehensive characterization of mutant *Pichia stipitis* co-fermenting cellobiose and xylose through genomic and transcriptomic analyses. *J. Microbiol. Biotechnol*. **2022**, *32*, 1485-1495. https://doi.org/10.4014/jmb.2209.09004
24. Ryu, S.; Hipp, J.; Trinh, C.T. Activating and elucidating metabolism of complex sugars in *Yarrowia lipolytica*. *Appl. Environ. Microbiol*. **2015**, *82*, 1334-1345. https://doi.org/10.1128/AEM.03582-15
25. Guo, Z.; Duquesne, S.; Bozonnet, S.; Cioci, G.; Nicaud, J,M.; Marty, A.; O'Donohue, M.J. Development of cellobiose-degrading ability in *Yarrowia lipolytica* strain by overexpression of endogenous genes. *Biotechnol. Biofuels* **2015**, *8*, 109. https://doi.org/10.1186/s13068-015-0289-9
26. Galazka, J.M.; Tian, C.; Beeson, W.T.; Martinez, B.; Glass, N.L.; Cate, J.H. Cellodextrin transport in yeast for improved biofuel production. *Science* **2010**, *330*, 84-86. https://doi.org/10.1126/science.1192838
27. Bae, Y.H.; Kang, K.H.; Jin, Y.S.; Seo, J.H. Molecular cloning and expression of fungal cellobiose transporters and β-glucosidases conferring efficient cellobiose fermentation in *Saccharomyces cerevisiae*. *J. Biotechnol*. **2014**, *169*, 34–41. https://doi.org/10.1016/j.jbiotec.2013.10.030
28. Gonçalves, D.L.; Matsushika, A.; de Sales, B.B.; Goshima, T.; Bon, E.P.S.; Stambuk, B.U. Xylose and xylose/glucose co-fermentation by recombinant *Saccharomyces cerevisiae* strains expressing individual hexose transporters. *Enzyme Microb. Technol*. **2014**, *63*, 13–20. https://doi.org/10.1016/j.enzmictec.2014.05.003
29. Li, S.; Du, J.; Sun, J.; Galazka, J.M.; Glass, N.L.; Cate, J.H.; Yang, X.; Zhao, H. Overcoming glucose repression in mixed sugar fermentation by co-expressing a cellobiose transporter and a β-glucosidase in *Saccharomyces cerevisiae*. *Mol. Biosyst*. **2010**, *6*, 2129-2132. https://doi.org/10.1039/c0mb00063a
30. 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. Engineered *Saccharomyces cerevisiae* capable of simultaneous cellobiose and xylose fermentation. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 504-509. https://doi.org/10.1073/pnas.1010456108
31. Ha, S.J.; Kim, S.R.; Kim, H.; Du, J.; Cate, J.H.; Jin, Y.S. Continuous co-fermentation of cellobiose and xylose by engineered *Saccharomyces cerevisiae*. *Bioresour, Technol*. **2013**, *149*, 525-531. https://doi.org/10.1016/j.biortech.2013.09.082

32. Parisutham, V.; Chandran, S.P.; Mukhopadhyay, A.; Lee, S.K.; Keasling, J.D. Intracellular cellobiose metabolism and its applications in lignocellulose-based biorefineries. *Bioresour. Technol*. **2017**, *239*, 496-506. https://doi.org/10.1016/j.biortech.2017.05.001
33. Oh, E.J.; Jin, Y.S. Engineering of *Saccharomyces cerevisiae* for efficient fermentation of cellulose. *FEMS Yeast Res*. **2020**, *20*, foz089. https://doi.org/10.1093/femsyr/foz089
34. Cai, P.; Gu, R.; Wang, B.; Li, J.; Wan, L.; Tian, C.; Ma, Y. Evidence of a critical role for cellodextrin transporter 2 (CDT-2) in both cellulose and hemicellulose degradation and utilization in *Neurospora crassa*. *PLoS One* **2014**, *9*, e89330. https://doi.org/10.1371/journal.pone.0089330
35. Znameroski, E.A.; Li, X.; Tsai, J.C.; Galazka, J.M.; Glass, N.L.; Cate, J.H. Evidence for transceptor function of cellodextrin transporters in *Neurospora crassa*. *J. Biol. Chem.* **2014**, *289*, 2610-2619. https://doi.org/10.1074/jbc.M113.533273
36. Li, X.; Yu, V.Y.; Lin, Y.; Chomvong, K.; Estrela, R.; Park, A.; Liang, J.M.; Znameroski, E.A.; Feehan, J.; Kim, S.R.; Jin, Y.S.; Glass, N.L.; Cate, J.H. Expanding xylose metabolism in yeast for plant cell wall conversion to biofuels. *Elife* **2015**, *4*, e05896. https://doi.org/10.7554/eLife.05896
37. Kim, H.; Lee, W.H.; Galazka, J.M.; Cate, J.H.; Jin, Y.S. Analysis of cellodextrin transporters from *Neurospora crassa* in *Saccharomyces cerevisiae* for cellobiose fermentation. *Appl. Microbiol. Biotechnol*. **2014**, *98*, 1087-1094. https://doi.org/10.1007/s00253-013-5339-2
38. Zhang, W.; Kou, Y.; Xu, J.; Cao, Y.; Zhao, G.; Shao, J.; Wang, H.; Wang, Z.; Bao, X.; Chen, G.; Liu, W. Two major facilitator superfamily sugar transporters from *Trichoderma reesei* and their roles in induction of cellulase biosynthesis. *J. Biol. Chem*. **2013**, *288*, 32861-72. https://doi.org/10.1074/jbc.M113.505826
39. dos Reis, T.F.; de Lima, P.B.; Parachin, N.S.; Mingossi, F.B.; de Castro Oliveira, J.V.; Ries, L.N.; Goldman, G.H. Identification and characterization of putative xylose and cellobiose transporters in *Aspergillus nidulans*. *Biotechnol. Biofuels* **2016**, *9*, 204. https://doi.org/10.1186/s13068-016-0611-1
40. Zhang, C.; Acosta-Sampson, L.; Yu, V.Y.; Cate, J.H.D. Screening of transporters to improve xylodextrin utilization in the yeast *Saccharomyces cerevisiae*. *PLoS One* **2017**, *12*, e0184730. https://doi.org/10.1371/journal.pone.0184730
41. Nogueira, K.M.V.; de Paula, R.G.; Antoniêto, A.C.C.; dos Reis, T.F.; Carraro, C.B.; Silva, A.C.; Almeida, F.; Rechia, C.G.V. et al. Characterization of a novel sugar transporter involved in sugarcane bagasse degradation in *Trichoderma reesei*. *Biotechnol. Biofuels* **2018**, *11*, 84. https://doi.org/10.1186/s13068-018-1084-1
42. Havukainen, S.; Valkonen, M.; Koivuranta, K.; Landowski, C.P. Studies on sugar transporter CRT1 reveal new characteristics that are critical for cellulase induction in *Trichoderma reesei*. *Biotechnol. Biofuels* **2020**, 13, 158. https://doi.org/10.1186/s13068-020-01797-7
43. Lin, H.; Zhao, J.; Zhang, Q.; Cui, S.; Fan, Z.; Chen, H.; Tian, C. Identification and characterization of a cellodextrin transporter in *Aspergillus niger*. *Front. Microbiol*. **2020**, *11*, 145. https://doi.org/10.3389/fmicb.2020.00145
44. Ha, S.J.; Kim, H.; Lin, Y.; Jang, M.U.; Galazka, J.M.; Kim, T.J.; Cate, J.H.; Jin, Y.S. Single amino acid substitutions in *HXT2.4* from *Scheffersomyces stipitis* lead to improved cellobiose fermentation by engineered *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol*. **2013**, *79*, 1500-1507. https://doi.org/10.1128/AEM.03253-12
45. de Ruijter, J.C.; Igarashi, K.; Penttilä, M. The *Lipomyces starkeyi* gene *Ls120451* encodes a cellobiose transporter that enables cellobiose fermentation in *Saccharomyces cerevisiae*. *FEMS Yeast Res*. **2020**, *20*, foaa019. https://doi.org/10.1093/femsyr/foaa019.
46. Long, T.M.; Su, Y.K.; Headman, J.; Higbee, A.; Willis, L.B.; Jeffries, T.W. Cofermentation of glucose, xylose, and cellobiose by the beetle-associated yeast *Spathaspora passalidarum*. *Appl. Environ. Microbiol*. **2012**, *78*, 5492-500. https://doi.org/10.1128/AEM.00374-12
47. Lauwers, E.; Erpapazoglou, Z.; Haguenauer-Tsapis, R.; André, B. The ubiquitin code of yeast permease trafficking. *Trends Cell Biol*. **2010**, *20*, 196-204. https://doi.org/10.1016/j.tcb.2010.01.004
48. Barata-Antunes, C.; Alves, R.; Talaia, G.; Casal, M.; Gerós, H.; Mans, R.; Paiva, S. Endocytosis of nutrient transporters in fungi: The ART of connecting signaling and trafficking. *Comput. Struct. Biotechnol. J*. **2021**, *19*, 1713-1737. https://doi.org/10.1016/j.csbj.2021.03.013
49. Meselson, M.; Yuan, R. DNA restriction enzyme from *E. coli*. *Nature* **1968**, *217*, 1110-1114. https://doi.org/10.1038/2171110a0
50. Lara, C.A.; Santos, R.O.; Cadete, R.M.; Ferreira, C.; Marques, S.; Gírio, F.; Oliveira, E.S.; Rosa, C.A.; Fonseca, C. Identification and characterisation of xylanolytic yeasts isolated from decaying wood and sugarcane bagasse in Brazil. Antonie Van Leeuwenhoek. 2014, 105, 1107-1119. https://doi.org/10.1007/s10482-014-0172-x
51. Cadete, R.M.; Melo-Cheab, M.A.; Dussán, K.J.; Rodrigues, R.C.L.B.; da Silva, S.S.; Gomes, F.C.O.; Rosa, C.A. Production of bioethanol in sugarcane bagasse hemicellulosic hydrolysate by *Scheffersomyces parashehatae*, *Scheffersomyces illinoinensis* and *Spathaspora arborariae* isolated from Brazilian ecosystems. *J. Appl. Microbiol*. **2017**, *123*, 1203-1213. https://doi.org/10.1111/jam.13559

52. Mouro, A.; dos Santos, A.A.; Agnolo, D.D.; Gubert, G.F.; Bon, E.P.S.; Rosa, C.A.; Fonseca, C.; Stambuk, B.U. Combining xylose reductase from *Spathaspora arborariae* with xylitol dehydrogenase from *Spathaspora passalidarum* to promote xylose consumption and fermentation into xylitol by *Saccharomyces cerevisiae*. *Fermentation* **2020**, *6*, 72. https://doi.org/10.3390/fermentation6030072
53. Entian, K.; Kotter, P. Yeast genetic strain and plasmid collections. *Methods Microbiol*. **2007**, *36*, 629-666. https://doi.org/10.1016/S0580-9517(06)36025-4
54. Babrzadeh, F.; Jalili, R.; Wang, C.; Shokralla, S.; Pierce, S.; Robinson-Mosher, A.; Nyren, P.; Shafer, R.W.; Basso, L.C.; et al.. Whole-genome sequencing of the efficient industrial fuel-ethanol fermentative *Saccharomyces cerevisiae* strain CAT-1. *Mol. Genet. Genomics* **2012**, *287*, 485-494. https://doi.org/10.1007/s00438-012-0695-7
55. Muller, G.; de Godoy, V.R.; Dário, M.G.; Duval, E.H.; Alves-Jr, S.L.; Bücker, A.; Rosa, C.A.; Dunn, B.; Sherlock, G.; Stambuk, B.U. Improved sugarcane-based fermentation processes by an industrial fuel-ethanol yeast strain. *J. Fungi* **2023**, *9*, 803. https://doi.org/10.3390/jof9080803
56. Pereira, I.O.; dos Santos, Â.A.; Gonçalves, D.L.; Purificação, M.; Guimarães, N.C.; Tramontina, R.; Coutouné, N.; Zanella, E.; et al. Comparison of *Spathaspora passalidarum* and recombinant *Saccharomyces cerevisiae* for integration of first- and second-generation ethanol production. *FEMS Yeast Res*. **2021**, *21*, foab048. https://doi.org/10.1093/femsyr/foab048
57. Mumberg, D.; Müller, R.; Funk, M. Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. *Gene* **1995**, *156*, 119-122. https://doi.org/10.1016/0378-1119(95)00037-7
58. Vyas, V.K.; Bushkin, G.G.; Bernstein, D.A.; Getz, M.A.; Sewastianik, M.; Barrasa, M.I.; Bartel, D.P.; Fink, G.R. New CRISPR mutagenesis strategies reveal variation in repair mechanisms among fungi. *mSphere* **2018**, *3*, e00154-18. https://doi.org/10.1128/mSphere.00154-18
59. Gietz, D.; St Jean, A.; Woods, R.A.; Schiestl, R.H. Improved method for high efficiency transformation of intact yeast cells. *Nucleic Acids Res*. **1992**, *20*, 1425. https://doi.org/10.1093/nar/20.6.1425
60. Ausubel, F.M.; Brent, R.; Kingston, R.E.; Moore, D.D.; Seidman, J.G.; Smith, J.A.; Struhl, K. *Short Protocols in Molecular Biology*, 3rd ed.; John Wiley & Sons: New York, USA, 1995.
61. Butler, G.; Rasmussen, M.D.; Lin, M.F.; Santos, M.A.; Sakthikumar, S.; Munro, C.A.; Rheinbay, E.; Grabherr, M.; Forche, A.; et al. Evolution of pathogenicity and sexual reproduction in eight *Candida* genomes. *Nature* **2009**, *459*, 657-662. https://doi.org/10.1038/nature08064
62. Jeffries, T.W.; Grigoriev, I.V.; Grimwood, J.; Laplaza, J.M.; Aerts, A.; Salamov, A.; Schmutz, J.; Lindquist, E.; Dehal, P.; Shapiro, H.; et al. Genome sequence of the lignocellulose-bioconverting and xylose-fermenting yeast *Pichia stipitis*. *Nat. Biotechnol*. 2007, 25, 319-326. https://doi.org/10.1038/nbt1290
63. Wohlbach, D.J.; Kuo, A.; Sato, T.K.; Potts, K.M.; Salamov, A.A.; Labutti, K.M.; Sun, H.; Clum, A.; Pangilinan, J.L.; Lindquist, et al. Comparative genomics of xylose-fermenting fungi for enhanced biofuel production. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 13212-13217. https://doi.org/10.1073/pnas.1103039108
64. Reider Apel, A.; d'Espaux, L.; Wehrs, M.; Sachs, D.; Li, R.A.; Tong, G.J.; Garber, M.; Nnadi, O.; Zhuang, W.; Hillson, N.J.; Keasling, J.D.; Mukhopadhyay, A. A Cas9-based toolkit to program gene expression in *Saccharomyces cerevisiae*. *Nucleic Acids Res*. **2017**, *45*, 496-508. https://doi.org/10.1093/nar/gkw1023
65. Stambuk, B.U. A simple experiment illustrating metabolic regulation: Induction versus repression of yeast α-glucosidase. *Biochem. Educ.* **1999**, *27*, 177–180. https://doi.org/10.1016/S0307-4412(98)00302-1
66. Hollatz, C.; Stambuk, B.U. Colorimetric determination of active α-glucoside transport in *Saccharomyces cerevisiae*. *J. Microbiol. Methods*. **2001**, *46*, 253–259. https://doi.org/10.1016/s0167-7012(01)00281-0
67. Pirovano W, Feenstra KA, Heringa J. PRALINE™: a strategy for improved multiple alignment of transmembrane proteins. *Bioinformatics* **2008**, *24*, 492-497. https://doi.org/10.1093/bioinformatics/btm636
68. Li, A.; Gao, X.; Ren, J.; Jin, C.; Xue, Y. BDM-PUB: Computational prediction of protein ubiquitination sites with a bayesian discriminant method, 2009. BDM-PUB: Prediction of Ubiquitination Website. Available online: http://bdmpub.biocuckoo.org/prediction.php (accessed on 30 November 2021)
69. Radivojac, P.; Vacic; V.; Haynes, C.; Cocklin, R.R.; Mohan, A.; Heyen, J.W.; Goebl, M.G.; Iakoucheva, L.M. Identification, analysis, and prediction of protein ubiquitination sites. *Proteins* **2010**, *78*, 365-380. https://doi.org/10.1002/prot.22555
70. Urbina, H.; Blackwel,l M. Multilocus phylogenetic study of the *Scheffersomyces* yeast clade and characterization of the N-terminal region of xylose reductase gene. *PLoS One* **2012**, *7*, e39128. https://doi.org/10.1371/journal.pone.0039128
71. Stambuk, B.U. Transcriptional and posttranslational regulation of a membrane nutrient transporter. *Biochem. Mol. Biol. Educ*. **2002**, *30*, 388-393. https://doi.org/10.1002/bmb.2002.494030060136
72. Horák, J. The role of ubiquitin in down-regulation and intracellular sorting of membrane proteins: insights from yeast. *Biochim. Biophys. Acta* **2003**, *1614*, 139-155. https://doi.org/10.1016/s0005-2736(03)00195-0
73. Skory, C.D.; Freer, S.N.; Bothast, R.J. Properties of an intracellular β-glucosidase purified from the cellobiose-fermenting yeast *Candida wickerhamii*. *Appl. Microbiol. Biotechnol*. **1996**, *46*, 353–359. https://doi.org/10.1007/BF00166229

74. Gueguen, Y.; Chemardin, P.; Arnaud, A. Purification and characterization of an intracellular β-glucosidase from a *Candida sake* strain isolated from fruit juices. *Appl. Biochem. Biotechnol*. **2001**, *95*, 151-162. https://doi.org/10.1385/abab:95:3:151
75. Varela, J.A.; Puricelli, M.; Ortiz-Merino, R.A.; Giacomobono, R.; Braun-Galleani, S.; Wolfe, K.H.; Morrissey, J.P. Origin of lactose fermentation in *Kluyveromyces lactis* by interspecies transfer of a neo-functionalized gene cluster during domestication. *Curr. Biol*. **2019**, *29*: 4284-4290.e2. https://doi.org/10.1016/j.cub.2019.10.044
76. Sen, A.; Acosta-Sampson, L.; Alvaro, C.G.; Ahn, J.S.; Cate, J.H.; Thorner, J. Internalization of heterologous sugar transporters by endogenous α-arrestins in the yeast *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol*. **2016**, *82*, 7074-7085. https://doi.org/10.1128/AEM.02148-16
77. Knychala, M.M.; dos Santos, A.A.; Kretzer, L.G.; Gelsleichter, F.; Leandro, M.J.; Fonseca, C.; Stambuk, B.U. Strategies for efficient expression of heterologous monosaccharide transporters in *Saccharomyces cerevisiae*. *J. Fungi* **2022**, *8*, 84. https://doi.org/10.3390/jof8010084
78. Li, H.; Schmitz, O.; Alper, H.S. Enabling glucose/xylose co-transport in yeast through the directed evolution of a sugar transporter. *Appl. Microbiol. Biotechnol*. **2016**, *100*, 10215-10223. https://doi.org/10.1007/s00253-016-7879-8
79. Luo, X.; Tao, X.; Ran, G.; Deng, Y.; Wang, H.; Tan, L.; Pang, Z. Molecular modification enhances xylose uptake by the sugar transporter KM_SUT5 of *Kluyveromyces marxianus*. *Int. J. Mol. Sci*. **2024**, 25, 8322. https://doi.org/10.3390/ijms25158322
80. Tamayo Rojas, S.A.; Schmidl, S.; Boles, E.; Oreb, M. Glucose-induced internalization of the *S. cerevisiae* galactose permease Gal2 is dependent on phosphorylation and ubiquitination of its aminoterminal cytoplasmic tail. *FEMS Yeast Res*. **2021**, *21*, foab019. https://doi.org/10.1093/femsyr/foab019
81. Roy, A.; Kim, Y.B.; Cho, K.H.; Kim, J.H. Glucose starvation-induced turnover of the yeast glucose transporter Hxt1. *Biochim. Biophys. Acta* **2014**, *1840*, 2878-2885. https://doi.org/10.1016/j.bbagen.2014.05.004
82. Martínez-Jimenez, F.D.; Pereira, I.O.; Ribeiro, M.P.A.; Sargo, C.R.; dos Santos, A.A.; Zanella, E.; Stambuk, B.U.; et al. Integration of first- and second-generation ethanol production: Evaluation of a mathematical model to describe sucrose and xylose co-fermentation by recombinant *Saccharomyces cerevisiae*. *Renew. Energy* **2022**, *192*, 326-339. https://doi.org/10.1016/j.renene.2022.04.094
83. de Oliveira Pereira, I.; dos Santos, Â.A.; Guimarães, N.C.; Lima, C.S.; Zanella, E.; Matsushika, A.; Rabelo, S.C.; Stambuk, B.U.; Ienczak, J.L. First- and second-generation integrated process for bioethanol production: Fermentation of molasses diluted with hemicellulose hydrolysate by recombinant *Saccharomyces cerevisiae*. *Biotechnol. Bioeng*. **2024**, *121*, 1314-1324. https://doi.org/10.1002/bit.28648
84. Santos, J.; Sousa, M.J.; Cardoso, H.; Inácio, J.; Silva, S.; Spencer-Martins, I.; Leão, C. Ethanol tolerance of sugar transport, and the rectification of stuck wine fermentations. *Microbiology* **2008**, *154*, 422-430. https://doi.org/10.1099/mic.0.2007/011445-0
85. Swinnen, S.; Goovaerts, A.; Schaerlaekens, K.; Dumortier, F.; Verdyck, P.; Souvereyns, K.; Van Zeebroeck, G.; Foulquié-Moreno, M.R.; Thevelein, J.M. Auxotrophic mutations reduce tolerance of *Saccharomyces cerevisiae* to very high levels of ethanol stress. *Eukaryot. Cell* **2015**, *14*, 884-897. https://doi.org/10.1128/EC.00053-15
86. Eriksen, D.T.; Hsieh, P.C.; Lynn, P.; Zhao, H. Directed evolution of a cellobiose utilization pathway in *Saccharomyces cerevisiae* by simultaneously engineering multiple proteins. *Microb. Cell Fact*. **2013**, *12*, 61. https://doi.org/10.1186/1475-2859-12-61
87. Lian, J.; Li, Y.; HamediRad, M.; Zhao, H. Directed evolution of a cellodextrin transporter for improved biofuel production under anaerobic conditions in *Saccharomyces cerevisiae*. *Biotechnol. Bioeng*. **2014**, *111*, 1521-31. https://doi.org/10.1002/bit.25214
88. Hu, M.L.; Zha, J.; He, L.W.; Lv, Y.J.; Shen, M.H.; Zhong, C.; Li, B.Z.; Yuan, Y.J. Enhanced bioconversion of cellobiose by industrial *Saccharomyces cerevisiae* used for cellulose utilization. *Front. Microbiol*. **2016**, *7*, 241. https://doi.org/10.3389/fmicb.2016.00241
89. Oh, E.J.; Kwak, S.; Kim, H.; Jin, Y.S. Transporter engineering for cellobiose fermentation under lower pH conditions by engineered *Saccharomyces cerevisiae*. *Bioresour. Technol*. **2017**, *245*, 1469-1475. https://doi.org/10.1016/j.biortech.2017.05.138
90. Kim, H.; Oh, E.J.; Lane, S.T.; Lee, W.H.; Cate, J.H.D.; Jin, Y.S. Enhanced cellobiose fermentation by engineered *Saccharomyces cerevisiae* expressing a mutant cellodextrin facilitator and cellobiose phosphorylase. *J. Biotechnol*. **2018**, *275*, 53-59. https://doi.org/10.1016/j.jbiotec.2018.04.008
91. Oh, E.J.; Skerker, J.M.; Kim, S.R.; Wei, N.; Turner, T.L.; Maurer, M.J.; Arkin, A.P.; Jin, Y.S. Gene amplification on demand accelerates cellobiose utilization in engineered *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol*. **2016**, *82*, 3631-3639. https://doi.org/10.1128/AEM.00410-16
92. Kim, H.J.; Lee, WH.; Turner, T.L.; Kwak, S.; Jin, Y.S. An extra copy of the β-glucosidase gene improved the cellobiose fermentation capability of an engineered *Saccharomyces cerevisiae* strain. *3 Biotech*, **2019**, *9*, 367. https://doi.org/10.1007/s13205-019-1899-x

 doi:10.20944/preprints202408.1444.v1

93. Chomvong, K.; Benjamin, D.I.; Nomura, D.K.; Cate, J.H.D. Cellobiose consumption uncouples extracellular glucose sensing and glucose metabolism in *Saccharomyces cerevisiae*. *mBio*. **2017**, *8*, e00855-17. https://doi.org/10.1128/mBio.00855-17
94. Lin, Y.; Chomvong, K.; Acosta-Sampson, L.; Estrela, R.; Galazka, J.M.; Kim, S.R.; Jin, Y.S.; Cate, J.H. Leveraging transcription factors to speed cellobiose fermentation by *Saccharomyces cerevisiae*. *Biotechnol. Biofuels* **2014**, *7*, 126. https://doi.org/10.1186/s13068-014-0126-6
95. Sullivan, S.F.; Shetty, A.; Bharadwaj, T.; Krishna, N.; Trivedi, V.D.; Endalur Gopinarayanan, V.; Chappell, T.C.; Sellers, D.M.; et al. Towards universal synthetic heterotrophy using a metabolic coordinator. *Metab. Eng*. **2023**, *79*, 14-26. https://doi.org/10.1016/j.ymben.2023.07.001
96. Zhang, Y.; Xu, Z.; Lu, M.; Ding, B.; Chen, S.; Wen, Z.; Yu, Y.; Zhou, L.; Jin, M. Rapid evolution and mechanism elucidation for efficient cellobiose-utilizing *Saccharomyces cerevisiae* through synthetic chromosome rearrangement and modification by LoxPsym-mediated evolution. *Bioresour. Technol*. **2022**, *356*, 127268. https://doi.org/10.1016/j.biortech.2022.127268
97. Blount, B.A.; Gowers, G.F.; Ho, J.C.H.; Ledesma-Amaro, R.; Jovicevic, D.; McKiernan, R.M.; Xie, Z.X.; Li, B.Z.; Yuan, Y.J.; Ellis, T. Rapid host strain improvement by in vivo rearrangement of a synthetic yeast chromosome. *Nat. Commun*. **2018**, *9*, 1932. https://doi.org/10.1038/s41467-018-03143-w