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

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Abstract: Developing recombinant *Saccharomyces cerevisiae* strains capable of transporting and fermenting cellobiose directly is a promising strategy for second-generation ethanol production from lignocellulosic biomass. In this study, we cloned and expressed in the *S. cerevisiae* 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, one from *Candida tropicalis* (*CtCBT1* gene), and one from *Meyerozyma guilliermondii* (*MgCBT2* gene). While all three transporters allowed cell growth on cellobiose, only the *MgCBT2* permease allowed cellobiose fermentation, although cellobiose consumption was incomplete. The analysis of the β-glucosidase and transport activities revealed that the cells stopped consuming 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 incomplete 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*

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1. Introduction

The production and use of sustainable alternatives to fossil fuels have 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 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 is 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 the efficient conversion of lignocellulose to ethanol is the use of microorganisms capable of efficient cellobiose fermentation. Cellobiose can be found in small amounts in some fruits, honey, or sugar beet, but the main source of this disaccharide is cellulose degradation by microorganisms. 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 yeast 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 yeasts may even have both pathways, extracellular hydrolysis besides transport and intracellular hydrolysis of cellobiose [24,25].

S. cerevisiae is a 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 a 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 play 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 [26,36].

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 yeasts, and while other fungal cellodextrin/xylodextrin permeases have been expressed in *S. cerevisiae*, 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]. 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* [37,38].

In the present work, we have cloned an intracellular β-glucosidase (*SpBGL7*) from the cellobiose fermenting yeast *Spathaspora passalidarum* [39] 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 *Scheffersomyces illinoinensis* and two putative transporters, one from *Candida tropicalis* (*CtCBT1*) and one from *Meyerozyma 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 incomplete. We analyzed this transporter in relation to the presence of lysine residues in the N- or C-terminal domains with the potential for ubiquitinylation and, thus, for being involved in transporter downregulation through endocytosis and vacuolar degradation [40,41]. 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) [42], 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 Ltd.a., 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	[43]
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	[44]
Sp. passalidarum UFMG-CM-Y474	Isolated from rotting wood in Roraima, Brazil	[45]
S. cerevisiae CEN.PK2-1C	*MAT*a *leu2-3,112 ura3-52 trp1-289 his3*-Δ1 *MAL2-8*^{c} *SUC2*	[46]
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	[47,48]
S. cerevisiae MP-C5H1	Isogenic to CAT-1, but *AUR1*::pAUR-XKXDHXR *loxP-KanMX-loxP*-P_{ADH1}::[4- 59Δ]*HXT1*	[49]
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™ [50]
pGPD-426	*Amp*^{R} *ori* 2μ *URA3* P_{TDH3}-T_{CYC1}	ATCC® 87361™ [50]
pGPD-424-SpBGL7	*Amp*^{R} *ori* 2μ *TRP1* P_{TDH3}-*SpBGL7*-T_{CYC1}	This work
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

Table 1. *Cont.*

Strains and Plasmids	Relevant Features or Genotype	Source
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}	[51]
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 6000× *g* for 5 min at 4 °C, 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 (5000× *g*, 1 min at 4 °C), and supernatants were 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 [52,53]. Purification of plasmids, PCR products, or Gibson Assembly products was performed using the QIAquick PCR Purification Kit (QIAGEN Benelux B.V., 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 [53] or 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* [54], *Sc. stipitis* [55] and *Sp. passalidarum* [56], 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 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 synthesized 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* [51]. 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 [57]. After sequencing both regions of interest in the MP-C5H1 strain genome (Table 1), 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 [51]. 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 h 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 min 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) 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 subse-

quent 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 [58]. 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 min. The reaction was stopped by placing the tubes at 100 °C for 3 min. Pre-boiled cells for 3 min 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 2600× *g* for 5 min, and the supernatant from assays using *p*NPβG and *p*NPβX was 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 [59]. 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 min, 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 min. Subsequently, 200 µL of 2 M $NaHCO_3$ was added. Pre-boiled cells for 3 min were used as negative controls. Cells were centrifuged at 2600× *g* for 5 min, 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

To predict potential transmembrane domains (TMs) of the *MgCBT2* transporter (NCBI GenBank ID: PP826654.1), we used PRALINE with the PSIPRED and HMMTOP methods [60]. The lysine residues with ubiquitinylation potential in the N- and C-terminal

domains of the permease were determined with the BDM-PUB [61] and UbPred programs [62].

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 reveal 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* [39,56]. 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 intracellular β-glucosidase activity when we used permeabilized yeast cells, with a K_m of 18.2 ± 3.9 mM and V_{max} of 1188 ± 85 nmol mg^{-1} min^{-1} with cellobiose, and higher affinity (K_m of 0.8 ± 0.2 mM and V_{max} of 5368 ± 199 nmol mg^{-1} min^{-1}) with *p*NPβG. This enzyme also had hydrolytic activity with *p*NPβX (a synthetic analog of xylobiose), with a K_m of 1.0 ± 0.5 mM and V_{max} of 1088 ± 175 nmol mg^{-1} min^{-1}.

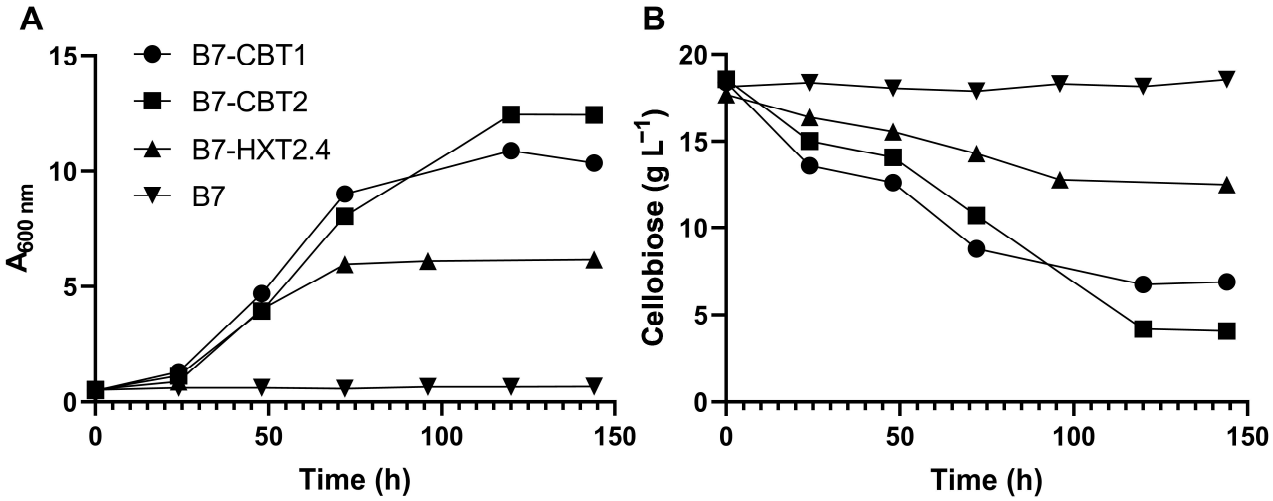

Figure 1. Cell growth (**A**) and cellobiose consumption (**B**) in YNB medium containing 20 g/L cellobiose by the indicated yeast strains harboring the intracellular *SpBGL7* β-glucosidase (strain B7), or this strain also transformed with the pGPD-426 plasmids containing the genes (*CtCBT1*, *MgCBT2*, 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 a platform to identify putative sugar transporters capable of mediating the uptake of cellobiose. First, and taking into account the close relationship between *Sc. stipitis* and *Sc. illinoinensis* [63], we used the genomic information of *Sc. stipitis* [56] to design primers and amplify the corresponding *HXT2.4* cellobiose transporter [37] 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] 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 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. Very low growth rates were obtained during cellobiose consumption, with $\mu = 0.0403\ h^{-1}$ for the strain expressing the *SiHXT2.4* permease, $\mu = 0.0408\ h^{-1}$ for the strain with the *CtCBT1* transporter, and $\mu = 0.0413\ h^{-1}$ for the strain expressing the *MgCBT2* 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 incomplete.

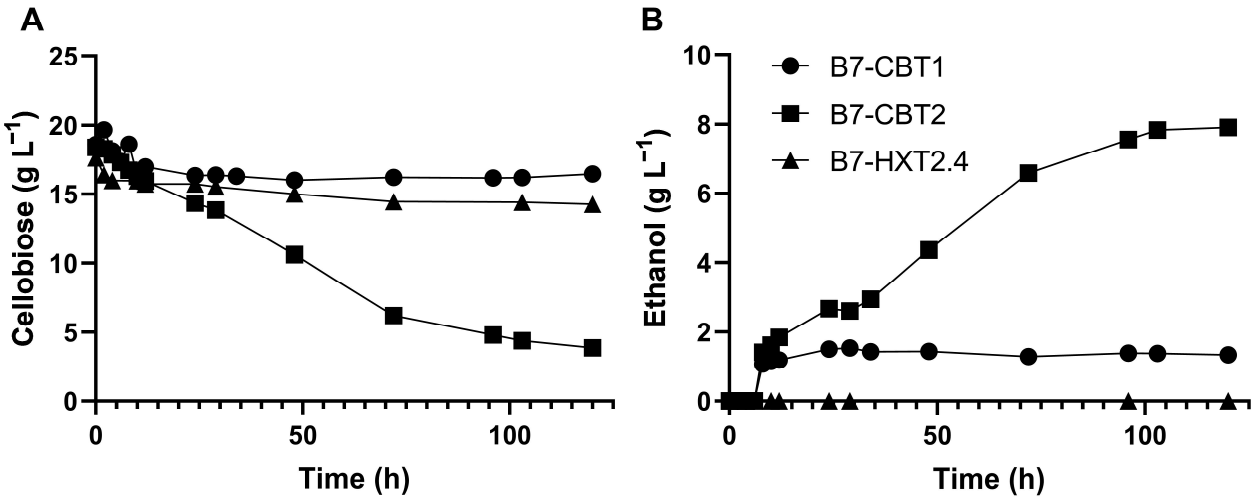

Figure 2. Cellobiose consumption (**A**) and ethanol production (**B**) during fermentation of 20 g/L cellobiose in rich YP medium by 10 g dry cell weight/L of the indicated yeast strains harboring the *SpBGL7* β-glucosidase and the genes *CtCBT1*, *MgCBT2*, and *SiHXT2.4* encoding sugar transporters.

In order to better understand the reason for the cellobiose incomplete 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* [59]. After growth on cellobiose, the B7-CBT2 strain was capable of transporting 7.4 ± 1.6 nmol of *p*NPβG $mg^{-1}\ min^{-1}$. The B7-CBT2 cells were also capable of transporting *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 incomplete cellobiose fermentation by strain B7-CBT2.

It is evident in Figure 3B that the intracellular β-glucosidase activity remained high throughout 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 incomplete 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 downregulation of yeast sugar permeases involving ubiquitinylation, endocytosis, and vacuolar degradation [64], 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 incomplete fermentations.

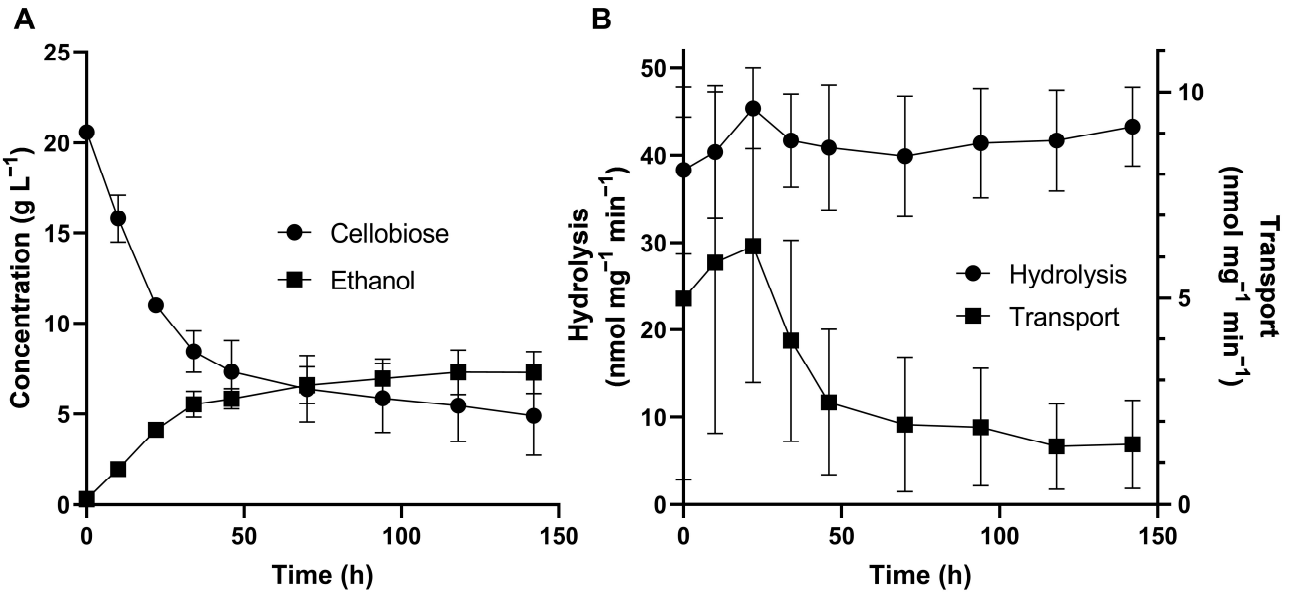

Figure 3. Cellobiose consumption and ethanol production (**A**), and pNPβG intracellular hydrolysis and transport activities (**B**) by strain B7-CBT2 during fermentation of 20 g/L cellobiose in rich YP medium.

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 (incomplete 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).

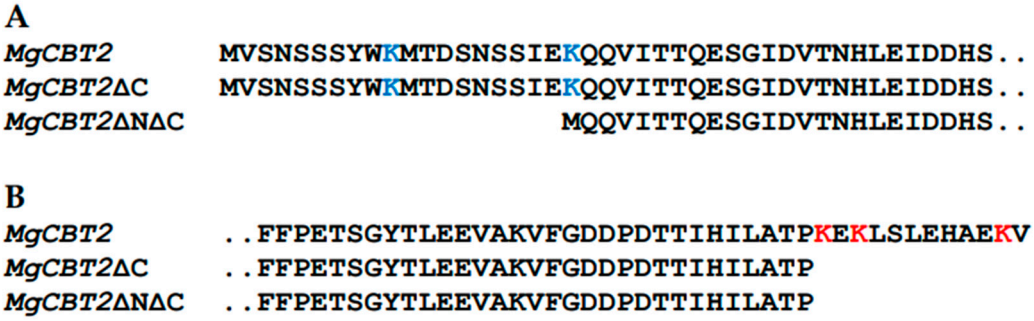

Figure 4. Sequence alignment of the cytoplasmic N-terminal first 43 amino acids (**A**) and the cytoplasmic C-terminal last 43 amino acids of the protein, after the last transmembrane domain (**B**), deduced from the *MgCBT2* gene, and the truncated versions of *MgCBT2* at the C-terminal domain (*MgCBT2*ΔC), 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 [61] and UbPred programs [62].

Considering that lysine residues with ubiquitinylation potential are involved in removing the transporters from the plasma membrane through endocytosis [64,65], 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 where 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 incomplete 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).

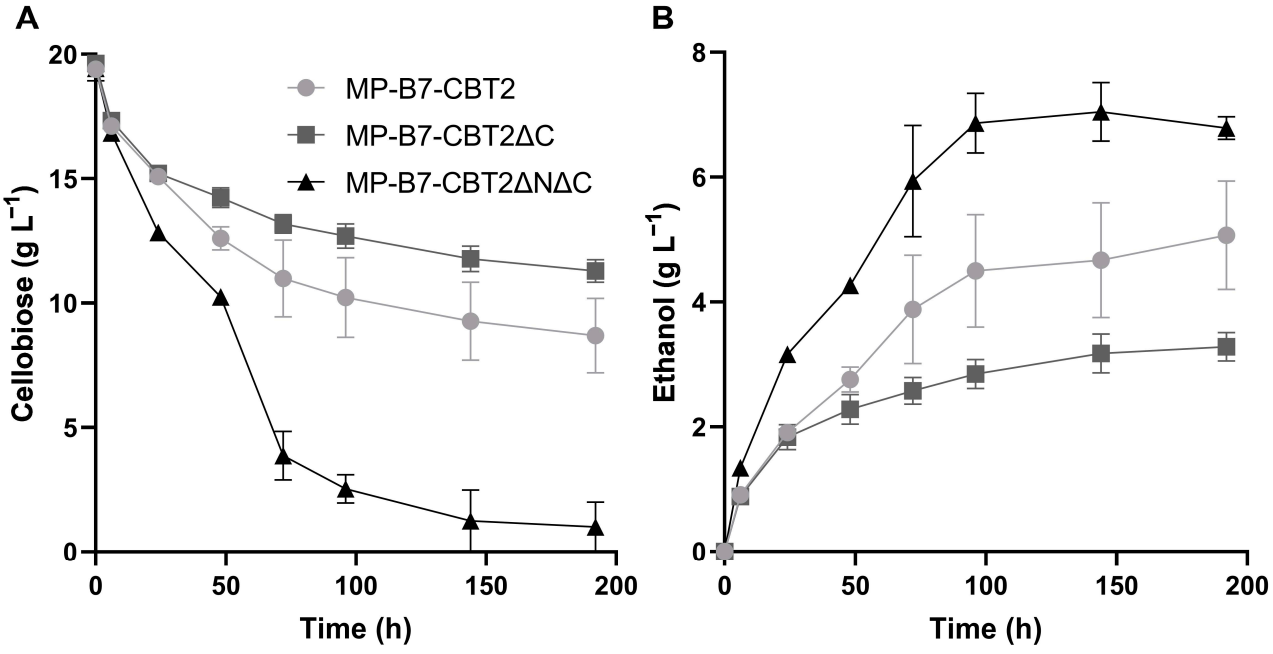

Figure 5. Cellobiose consumption (**A**) and ethanol production (**B**) during fermentation of 20 g/L cellobiose in rich YP medium by the indicated industrial yeast strains harboring the intracellular *SpBGL7* β-glucosidase and the *MgCBT2*, *MgCBT2*ΔC, and *MgCBT2*ΔNΔC cellobiose transporters.

Finally, since cellobiose transport and intracellular hydrolysis by recombinant *S. cerevisiae* may contribute to 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 an incomplete 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, 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.

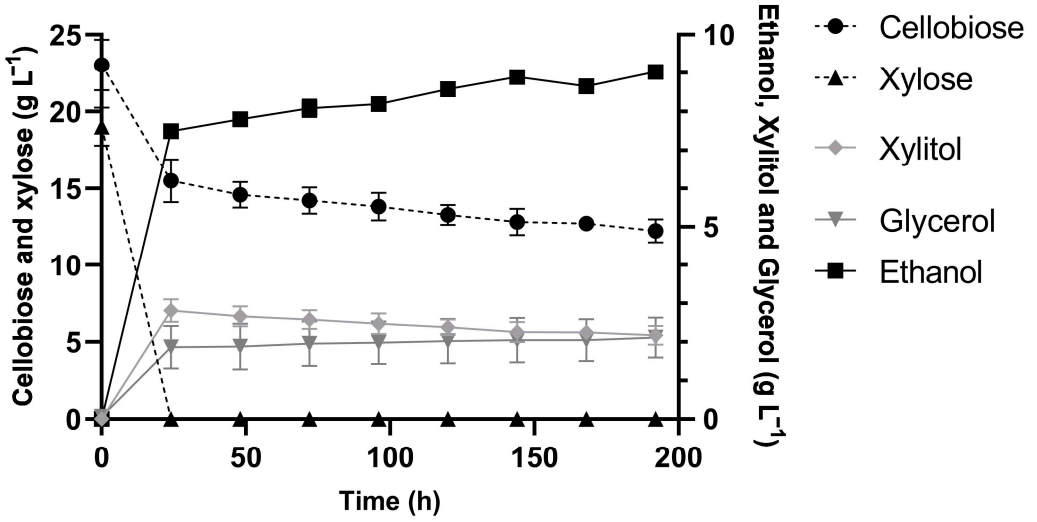

Figure 6. Co-fermentation of 20 g/L cellobiose plus 20 g/L xylose in rich YP medium by the recombinant industrial yeast strain MP-B7-CBT2ΔNΔC harboring the intracellular *SpBGL7* β-glucosidase and the *MgCBT2*ΔNΔC cellobiose transporter.

4. Discussion

We have cloned an intracellular β-glucosidase gene (*SpBGL7*) from the cellobiose fermenting yeast *Sp. passalidarum* [39] 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 [66,67]. Figure 7 shows a phylogenetic analysis of some β-glucosidases from yeasts and fungi, including the *BGL7* from *Sp. passalidarum* cloned in this work. Using the *S. cerevisiae* strain expressing the *SpBGL7* enzyme allowed the identification and cloning of three yeast cellobiose transporters that permitted the growth of the strain in the presence of cellobiose. Figure 8 shows that the three transporters (we included the *HXT2.4* permease from *Sc. stipitis* [37], although the transporter we cloned was from *Sc. illinoinensis*) are close to the known yeast cellobiose transporters, including the one cloned from *L. starkeyi* [38] and a cellobiose transporter *CEL1* from *Kluyveromyces dobzhanskii* (that allows growth of S. cerevisiae on cellobiose, but no fermentation data are available) [68]. 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.

Although the three transporters allowed the 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 an incomplete fermentation. Our analysis revealed that the reduced transport activity over time was responsible for this incomplete 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 [69] showed that *S. cerevisiae* cells expressing the *CDT-2* cellobiose transporter also produced incomplete 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 from the plasma membrane through the action of the *ROD1* and *ROG3* α-arrestins [70]. In another approach, mutated versions of the *CDT-2* permease were engineered changing cytoplasmic lysine residues into arginine and revealing 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 [69]. 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 [70–72]. However, for other permeases (e.g., *HXT1*, *GAL2*) the lysine residues involved in ubiquitinylation and endocytosis can be present in the N-terminal domain [73,74]. 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 [49,74–76].

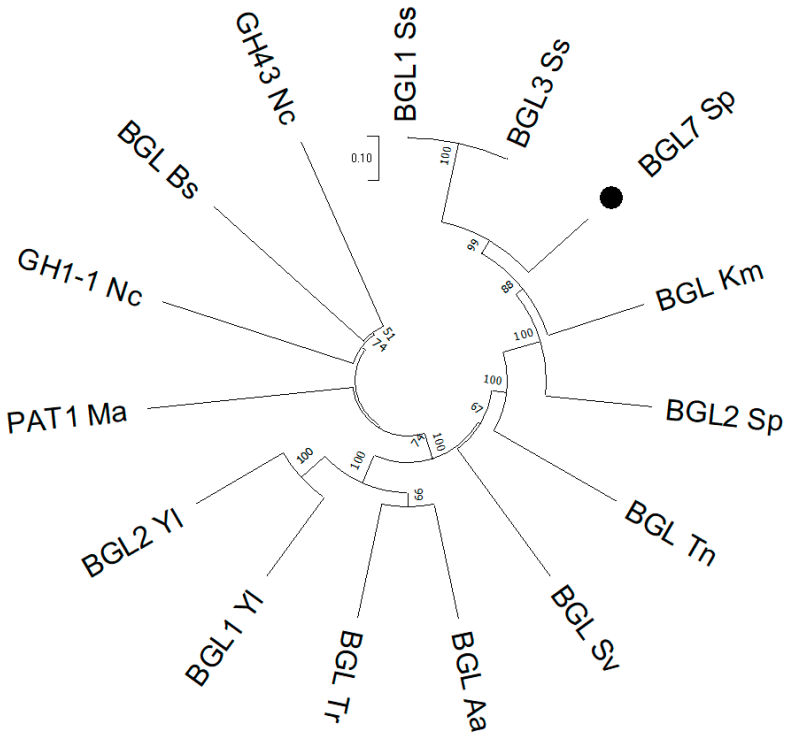

Figure 7. Phylogenetic classification of β-glucosidases from various yeast and fungal hosts. The phylogenetic tree contains 15 enzyme sequences, and the numbers at the nodes represent percentage bootstrap values based on 1500 samplings. The abbreviation of each species is added after the name of the β-glucosidase genes: Ss = *Scheffersomyces stipitis*, Sp = *Spathaspora passalidarum*, Km = *Kluyveromyces marxianus*, Tn = *Thermotoga neapolitana*, Sv = *Streptomyces venezuelae*, Aa = *Aspergillus aculeatus*, Tr = *Trichoderma reesei*, Yl = *Yarrowia lipolytica*, Ma = *Moesziomyces antarcticus*, Nc = *Neurospora crassa*, and Bs = *Bacillus subtilus*.

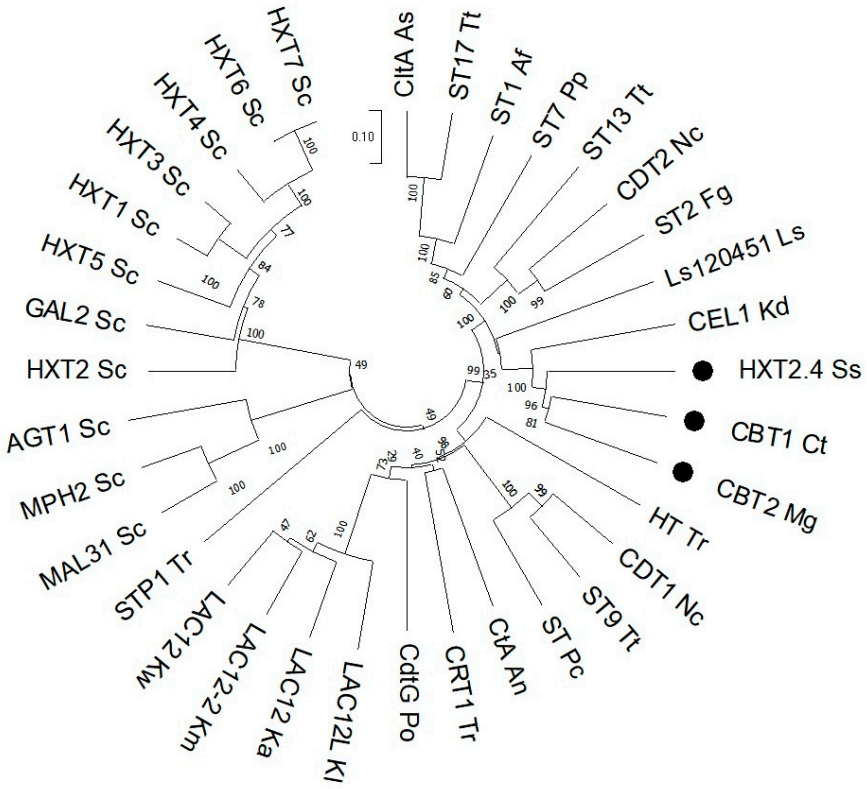

Figure 8. Phylogenetic classification of glucose, maltose, and cellobiose transporters from various yeast and fungal hosts. The phylogenetic tree contains 35 transporter sequences, and the numbers at the nodes represent percentage bootstrap values based on 1500 samplings. The abbreviation of each species 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*, Pc = *Penicillium chrysogenum*, and Sc = *Saccharomyces cerevisiae*.

In the case of the *MgCBT2* transporter, we found three 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 improve 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. Although the ethanol yield of the strain with the *MgCBT2*ΔNΔC transporter was lower than the ethanol yield of the unchanged *MgCBT2* permease, there was a clear advantage with the *MgCBT2*ΔNΔC transporter in terms of ethanol titer, as the transporter truncated in the N- and C-terminal domains produced 33.7% more ethanol than the *MgCBT2* permease, and more than doubled the amount of ethanol produced with the *MgCBT2*ΔC transporter (Figure 5). 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 [77,78]. 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 an incomplete fermentation.

In general, cellobiose fermentation by recombinant *S. cerevisiae* yeasts is much slower than the fermentation of other sugar (e.g., glucose), with also lower ethanol yields [26,27], see Table 3. As expected, the fermentation performance is influenced by the yeast strain background, the genes used, and the concentration of cellobiose. Is worth noting that, compared with other yeast cellobiose transporters (e.g., *HXT2.4* and *Ls120451*), the one cloned and engineered in this work (*MgCBT2*) gave better results (Table 3). In general, industrial strains perform better than laboratory strains, and mutant versions (or truncated versions) of the transporter genes also show interesting results. Several other strategies have been employed to increase cellobiose consumption and fermentation, including mutagenesis, direct evolution/selection (also called evolutionary engineering) [37,79–83], as well as increasing the copy number of transporter or β-glucosidase genes [84,85]. 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 [86], and thus it is also required to introduce/engineer transcriptional factors to increase cellobiose fermentation by this industrial yeast [87,88]. 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* [89,90]. 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.

Table 3. Examples of best-performing fermentation parameters by different recombinant *S. cerevisiae* strains on cellobiose.

Strain [a]	Transporter	β-Glucosidase	Cellobiose (g/L)	Cellobiose Consumption (%)	Incubation Time (h)	Ethanol Yield/g Cellobiose [b]	Reference
YPH499	Nc_CDT-1	Nc_gh1-1	20	100	150	0.44	[26]
BY4741	Nc_CDT-2	Nc_gh1-1	80-90	38–89	>170	*0.16–0.35*	[69]
	Nc_CDT-2 [c]	Nc_gh1-1	80	78	170	*0.23*	
INVSc1	Nc_CDT-2	Nc_gh1-1	22	91	30	0.35	[80]
	Nc_CDT-2 [d]	Nc_gh1-1	22	98	10	0.45	
D452-2	Nc_CDT-2 [d]	Nc_gh1-1	80	100	36	0.35	[82]
D452-2	Nc_CDT-1	Pc_BG	40	100	36	0.30	[27]
	Nc_CDT-1	Tt_BG	40	100	48	0.29	
	Pc_ST	Tt_BG	40	100	38	0.38	
CEN.PK2-1D	Ss_HXT2.4	Nc_gh1-1	80	90	54	0.40	[37]
	Ss_HXT2.4 [d]	Nc_gh1-1	80	94	36	0.45	
D452-2	Ss_HXT2.4	Nc_gh1-1	80	70	60	0.16	
	Ss_HXT2.4 [d]	Nc_gh1-1	80	100	24	0.45	
YSS1	Ls_120451	Nc_gh1-1	21	64	96	0.18	[38]
Still Spirits Turbo	Nc_CDT-1	Nc_gh1-1	86	100	56	0.40	[79]
	Nc_CDT-1 [d]	Nc_gh1-1 [d]	86	100	30	0.44	
Angel Yeast	Nc_CDT-1	Nc_gh1-1	45	92	96	0.34	[81]
Angel Yeast [e]	Nc_CDT-1	Nc_gh1-1	45	100	18	0.50	
MP-C5H1	Mg_CBT2	Sp_BGL7	20	55	192	0.47	This work
	Mg_CBT2ΔNΔC [c]	Sp_BGL7	20	94	144	0.38	

[a] Haploid laboratory strains are in normal characters, diploids in italics, and industrial strains are in bold. [b] Yields in italics were calculated from the published figures. [c] Truncated transporters (or mutants without lysine residues). [d] Mutant/evolved versions of the genes. [e] Evolved strain; no mutations found in the transporter/enzyme.

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 the growth of recombinant *S. cerevisiae* cells on this carbon source. While the *CBT2* transporter allowed an incomplete 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.

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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. [CrossRef]
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. [CrossRef] [PubMed]
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. [CrossRef] [PubMed]
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.; Parreiras, L.S.; Camargo, E.L.O.; Drezza, A.L.; Missawa, S.K.; et al. Second-generation ethanol: The need is becoming a reality. *Ind. Biotechnol.* **2016**, *12*, 40–57. [CrossRef]
5. Igwebuike, C.M.; Awad, S.; Andrès, Y. Renewable energy potential: Second-generation biomass as feedstock for bioethanol production. *Molecules* **2024**, *29*, 1619. [CrossRef]
6. Gao, M.; Ploessl, D.; Shao, Z. Enhancing the co-utilization of biomass-derived mixed sugars by yeasts. *Front. Microbiol.* **2019**, *9*, 3264. [CrossRef]
7. Broda, M.; Yelle, D.J.; Serwańska, K. Bioethanol production from lignocellulosic biomass-Challenges and solutions. *Molecules* **2022**, *27*, 8717. [CrossRef]
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. [CrossRef]
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. [CrossRef]
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. [CrossRef]

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. [CrossRef]
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. [CrossRef] [PubMed]
13. Pryor, S.W.; Nahar, N. β-glucosidase supplementation during biomass hydrolysis: How low can we go? *Biomass Bioenergy* **2015**, *80*, 298–302. [CrossRef]
14. Ferreira, R.G.; Azzoni, A.R.; Freitas, S. On the production cost of lignocellulose-degrading enzymes. *Biofuels Bioprod. Bioref.* **2021**, *15*, 85–99. [CrossRef]
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. [CrossRef] [PubMed]
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. [CrossRef] [PubMed]
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. [CrossRef]
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.; Treichel, H.; 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. [CrossRef]
19. Jeffries, T.W.; Van Vleet, J.R. *Pichia stipitis* genomics, transcriptomics, and gene clusters. *FEMS Yeast Res.* **2009**, *9*, 793–807. [CrossRef]
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. [CrossRef]
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. [CrossRef] [PubMed]
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.; Stambuk, B.U.; et al. Biochemical analysis of cellobiose catabolism in *Candida pseudointermedia* strains isolated from rotten wood. *Arch. Microbiol.* **2020**, *202*, 1729–1739. [CrossRef] [PubMed]
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. [CrossRef] [PubMed]
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. [CrossRef] [PubMed]
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. [CrossRef]
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. [CrossRef]
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. [CrossRef]
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. [CrossRef]
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. [CrossRef]
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. [CrossRef]
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. [CrossRef] [PubMed]
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. [CrossRef] [PubMed]
33. Oh, E.J.; Jin, Y.S. Engineering of *Saccharomyces cerevisiae* for efficient fermentation of cellulose. *FEMS Yeast Res.* **2020**, *20*, foz089. [CrossRef] [PubMed]
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. [CrossRef]
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. [CrossRef]

36. 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. [CrossRef]
37. 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. [CrossRef]
38. 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. [CrossRef]
39. 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*, 5492–5500. [CrossRef]
40. Lauwers, E.; Erpapazoglou, Z.; Haguenauer-Tsapis, R.; André, B. The ubiquitin code of yeast permease trafficking. *Trends Cell Biol.* **2010**, *20*, 196–204. [CrossRef]
41. 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. [CrossRef] [PubMed]
42. Meselson, M.; Yuan, R. DNA restriction enzyme from *E. coli*. *Nature* **1968**, *217*, 1110–1114. [CrossRef] [PubMed]
43. 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. [CrossRef] [PubMed]
44. 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. [CrossRef]
45. 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. [CrossRef]
46. Entian, K.; Kotter, P. Yeast genetic strain and plasmid collections. *Methods Microbiol.* **2007**, *36*, 629–666. [CrossRef]
47. Babrzadeh, F.; Jalili, R.; Wang, C.; Shokralla, S.; Pierce, S.; Robinson-Mosher, A.; Nyren, P.; Shafer, R.W.; Basso, L.C.; de Amorim, H.V.; et al. Whole-genome sequencing of the efficient industrial fuel-ethanol fermentative *Saccharomyces cerevisiae* strain CAT-1. *Mol. Genet. Genomics* **2012**, *287*, 485–494. [CrossRef]
48. 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. [CrossRef]
49. Pereira, I.O.; dos Santos, Â.A.; Gonçalves, D.L.; Purificação, M.; Guimarães, N.C.; Tramontina, R.; Coutouné, N.; Zanella, E.; Matsushika, A.; Stambuk, B.U.; 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. [CrossRef]
50. Mumberg, D.; Müller, R.; Funk, M. Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. *Gene* **1995**, *156*, 119–122. [CrossRef]
51. 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. [CrossRef]
52. 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. [CrossRef] [PubMed]
53. 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, NY, USA, 1995.
54. Butler, G.; Rasmussen, M.D.; Lin, M.F.; Santos, M.A.; Sakthikumar, S.; Munro, C.A.; Rheinbay, E.; Grabherr, M.; Forche, A.; Reedy, J.L.; et al. Evolution of pathogenicity and sexual reproduction in eight *Candida* genomes. *Nature* **2009**, *459*, 657–662. [CrossRef] [PubMed]
55. 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. [CrossRef] [PubMed]
56. 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, E.A.; et al. Comparative genomics of xylose-fermenting fungi for enhanced biofuel production. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 13212–13217. [CrossRef]
57. 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.; et al. A Cas9-based toolkit to program gene expression in *Saccharomyces cerevisiae*. *Nucleic Acids Res.* **2017**, *45*, 496–508. [CrossRef]
58. Stambuk, B.U. A simple experiment illustrating metabolic regulation: Induction versus repression of yeast α-glucosidase. *Biochem. Educ.* **1999**, *27*, 177–180. [CrossRef]
59. Hollatz, C.; Stambuk, B.U. Colorimetric determination of active α-glucoside transport in *Saccharomyces cerevisiae*. *J. Microbiol. Methods* **2001**, *46*, 253–259. [CrossRef]
60. Pirovano, W.; Feenstra, K.A.; Heringa, J. PRALINE^{TM}: A strategy for improved multiple alignment of transmembrane proteins. *Bioinformatics* **2008**, *24*, 492–497. [CrossRef]

61. Li, X.; 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: https://www.scienceopen.com/document?vid=cdb6582a-8d17-4b0a-aae0-7044b4d6363a (accessed on 30 November 2021).
62. 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. [CrossRef]
63. 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. [CrossRef] [PubMed]
64. Stambuk, B.U. Transcriptional and posttranslational regulation of a membrane nutrient transporter. *Biochem. Mol. Biol. Educ.* **2002**, *30*, 388–393. [CrossRef]
65. 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. [CrossRef] [PubMed]
66. 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. [CrossRef] [PubMed]
67. 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. [CrossRef]
68. 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. [CrossRef]
69. 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. [CrossRef]
70. 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. [CrossRef]
71. 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. [CrossRef]
72. 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. [CrossRef]
73. 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. [CrossRef] [PubMed]
74. 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. [CrossRef] [PubMed]
75. Martínez-Jimenez, F.D.; Pereira, I.O.; Ribeiro, M.P.A.; Sargo, C.R.; dos Santos, A.A.; Zanella, E.; Stambuk, B.U.; Ienczak, J.; Morais, E.; Costa, A. 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. [CrossRef]
76. 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. [CrossRef] [PubMed]
77. 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. [CrossRef]
78. 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. [CrossRef]
79. 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. [CrossRef]
80. 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*, 1521–1531. [CrossRef]
81. 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. [CrossRef]
82. 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. [CrossRef]
83. 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. [CrossRef] [PubMed]
84. 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. [CrossRef] [PubMed]
85. Kim, H.J.; Lee, W.H.; 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. [CrossRef] [PubMed]
86. 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. [CrossRef] [PubMed]

87. 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. [CrossRef]
88. Sullivan, S.F.; Shetty, A.; Bharadwaj, T.; Krishna, N.; Trivedi, V.D.; Endalur Gopinarayanan, V.; Chappell, T.C.; Sellers, D.M.; Kumar, R.P.; Nair, N.U. Towards universal synthetic heterotrophy using a metabolic coordinator. *Metab. Eng.* **2023**, *79*, 14–26. [CrossRef]
89. 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. [CrossRef]
90. 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. [CrossRef]