Activating and Elucidating Metabolism of Complex Sugars in *Yarrowia lipolytica*

Seunghyun Ryu,[a] Julie Hipp,[a] Cong T. Trinh[a,b,c]

Department of Chemical and Biomolecular Engineering[a] and Bredesen Center for Interdisciplinary Research and Graduate Education,[b] University of Tennessee, Knoxville, Tennessee, USA; Bioenergy Science Center (BESC), Oak Ridge National Laboratory, Oak Ridge, Tennessee, USA[c]

The oleaginous yeast *Yarrowia lipolytica* is an industrially important host for production of organic acids, oleochemicals, lipids, and proteins with broad biotechnological applications. Albeit known for decades, the unique native metabolism of *Y. lipolytica* for using complex fermentable sugars, which are abundant in lignocellulosic biomass, is poorly understood. In this study, we activated and elucidated the native sugar metabolism in *Y. lipolytica* for cell growth on xylose and cellobiose as well as their mixtures with glucose through comprehensive metabolic and transcriptomic analyses. We identified 7 putative glucose-specific transporters, 16 putative xylose-specific transporters, and 4 putative cellobiose-specific transporters that are transcriptionally upregulated for growth on respective single sugars. *Y. lipolytica* is capable of using xylose as a carbon source, but xylose dehydrogenase is the key bottleneck of xylose assimilation and is transcriptionally repressed by glucose. *Y. lipolytica* has a set of 5 extracellular and 6 intracellular β-glucosidases and is capable of assimilating cellobiose via extra- and intracellular mechanisms, the latter being dominant for growth on cellobiose as a sole carbon source. Strikingly, *Y. lipolytica* exhibited enhanced sugar utilization for growth in mixed sugars, with strong carbon catabolite activation for growth on the mixture of xylose and cellobiose and with mild carbon catabolite repression of glucose on xylose and cellobiose. The results of this study shed light on fundamental understanding of the complex native sugar metabolism of *Y. lipolytica* and will help guide inverse metabolic engineering of *Y. lipolytica* for enhanced conversion of biomass-derived fermentable sugars to chemicals and fuels.

Lignocellulosic biomasses, derived from agricultural residues or nonfood crops, are potential renewable feedstocks for sustainable microbial production of biofuels and biochemicals (1). Lignocellulosic biomass is more complex and recalcitrant than corn starch, containing mixed sugars such as C_6 sugars (e.g., glucose) and C_5 sugars (e.g., xylose) (2). Most microorganisms do not efficiently consume these mixed sugars due to the well-known carbon catabolite repression (CCR) effect (3). The underlying CCR mechanism is governed by complex enzymatic and transcriptional regulation of metabolic processes (e.g., sugar transporters, sugar-degrading enzymes, etc.) that make microbial cell factories preferentially use one sugar (e.g., glucose) instead of other sugars (e.g., xylose and cellobiose) (4). For instance, a higher-level CCR effect causes diauxic growth (5); a milder effect allows simultaneous sugar utilization but often makes the specific uptake rate of one sugar higher than that of others (6). For biotechnological application, it is highly desirable to engineer microorganisms as microbial cell factories that can efficiently convert complex biomass-derived sugars to desirable chemicals with minimal CCR effect (7, 8).

Fig. 1 shows assimilation pathways of glucose, xylose, and cellobiose in native yeasts. Most yeasts such as *Saccharomyces cerevisiae* can consume only C_6 sugars (9), while a few other yeasts such as *Pichia stipitis* (also known as *Scheffersomyces stipitis*) can assimilate both C_6 and C_5 sugars (10). To grow on xylose as a sole carbon source, yeasts need xylose-specific transporters to import xylose into the cytosol followed by conversion of xylose to xylitol mediated by a xylose reductase (XYL1), conversion of xylitol to xylulose mediated by a xylulose dehydrogenase (XYL2), and conversion of xylulose to xylulose-5-phospate mediated by a xylulose kinase (XYL3) and finally to other precursor metabolites of core metabolism required for cell growth (11). While native yeasts use XYL1 and XYL2 to convert xylose to xylulose, genetically engi-

neered yeasts expressing a bacterial isomerase for enhanced xylose utilization have also been successfully demonstrated (12). For growth on cellobiose, yeasts can use two possible routes. In route 1, cellobiose is first extracellularly degraded into glucose by a secreted β-glucosidase (BGL), and glucose is then imported into the cell (13). In route 2, cellobiose is first transported into the cell by a cellodextrin-specific transporter and subsequently converted to glucose by an intracellular BGL (14). Both routes were engineered in *S. cerevisiae*, and route 2 has been shown to be effective for simultaneous coutilization of xylose and cellobiose (15–17). For effective sugar assimilation, sugar-specific transporters have significant roles and are commonly regulated at both the transcriptional and enzymatic levels (18). Recent characterization of conserved structural motifs and amino acids responsible for xylose- and cellodextrin-specific transporters in *S. cerevisiae* provides useful insights into complex sugar utilization (19–22).

Yarrowia lipolytica, classified as an oleaginous yeast that is generally regarded as safe (GRAS), has recently emerged as a potential microbial cell factory with robust phenotypes advantageous for production of biofuels and biochemicals. *Y. lipolytica* not only can
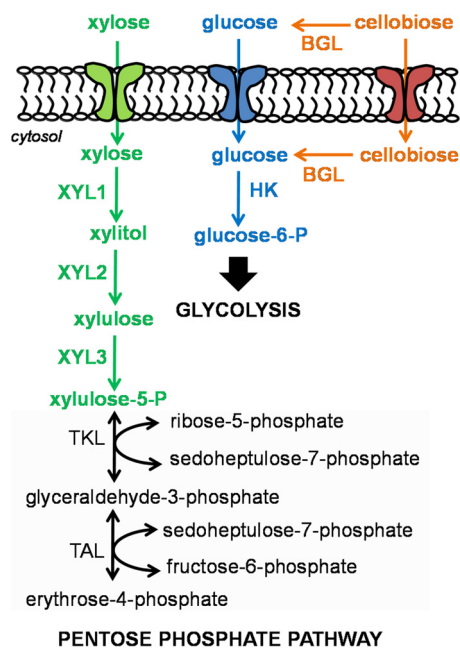
FIG 1 Degradation pathways of glucose (in blue), xylose (in green), and cellobiose (in orange) in yeasts. A simplified pentose phosphate pathway is presented in gray box. Abbreviations: XYL1, xylose reductase; XYL2, xylitol dehydrogenase; XYL3, xylulose kinase; TKL, transketolase; TAL, transaldolase; BGL, β-glucosidase.

be harnessed to produce large amounts of intracellular neutral lipids (>90% of dry cell weight [DCW]) (23, 24), oleochemicals (25), food supplements (e.g., omega-3 eicosapentaenoic acid) (26), high-value organics (e.g., citric, α-ketoglutaric, succinic, and pyruvic acids), and proteins (e.g., proteases and lipases) (27) but also is capable of assimilating complex substrates (e.g., organic acids, alcohols, triglycerides, and hydrocarbons) (27) as well as of thriving in a wide pH range (pH 2 to 11) (28) and in the presence of inhibitory acid-pretreated biomass hydrolysates (29) or high (>12% NaCl) salt concentrations (30) or even high (10% [vol/vol]) concentrations of ionic liquids (31). While native *Y. lipolytica* has been known for decades to use only some C6 sugars such as glucose, mannose, and fructose (32), its capability of assimilating other sugars such as xylose and cellobiose and their mixtures with glucose is poorly understood. For instance, the native xylose and cellobiose degradation pathways have not yet been successfully activated (33, 34) even though *Y. lipolytica* has putative metabolic enzyme and transport genes required for xylose and cellobiose degradation. Recent studies have focused on introducing the native xylose degradation pathway of *P. stipitis* in *Y. lipolytica* (34) as well as on developing the heterologous cellobiose degradation pathways in *Y. lipolytica*. Strategies for the latter include overexpression of (i) the heterologous cellodextrin transporter and intracellular BGL genes of *Neurospora crassa* for intracellular cellobiose degradation (15) and (ii) native extracellular BGL genes of *Y. lipolytica* for extracellular cellobiose degradation (33).

In this study, we activated and elucidated the sugar metabolism of *Y. lipolytica* for cell growth on xylose and cellobiose as well as on their mixtures with glucose. Through comprehensive metabolic and transcriptomic analyses, we identified sugar-specific putative transporters and metabolic degradation enzymes in *Y. lipolytica*

responsible for xylose and cellobiose assimilation. We discovered that *Y. lipolytica* exhibited enhanced sugar utilization for growth on mixed sugars, with strong carbon catabolite activation (CCA) for growth on the mixture of xylose and cellobiose and with mild CCR of glucose on xylose and cellobiose. The results sheds light on fundamental understanding of the complex native sugar metabolism of *Y. lipolytica* and will help guide inverse metabolic engineering of this microorganism for enhanced conversion of biomass-derived fermentable sugars to biofuels and biochemicals.

MATERIALS AND METHODS

Strains and plasmids. A list of the plasmids and strains used in this study is provided in Table S1 in the supplemental material. Plasmid pSL16-CEN1-1-227, containing the CYC1 terminator (T_{CYC1}) and leucine selection marker, was kindly provided by M. Matsuoka (Sojo University, Japan) (35). Plasmid pSR001 was created by inserting the *Y. lipolytica* TEF promoter (P_{TEF}) (36) in front of T_{CYC1} of pSL16-CEN1-1-227 as described elsewhere (31). The list of primers used in this study is shown in Table S2. Plasmid pSR002, carrying the native xylose dehydrogenase (XYL2) gene, was constructed by the use of Gibson gene assembly (37) of two DNA fragments, the XYL2 gene fragment amplified from the genomic DNA (gDNA) of *Y. lipolytica* using primers Xyl2$_{YL}$_Fwd/Xyl2$_{YL}$_Rev and the backbone amplified from pSR001 using primers pSR001_Fwd/pSR001_Rev. Likewise, plasmid pSR003, carrying xylose-specific/cellobiose-specific transporter YALI0D01111g (TRP7$_{YL}$), was constructed by the assembly of two DNA fragments, the TRP7$_{YL}$ gene amplified from the gDNA of *Y. lipolytica* using primers TRP7$_{YL}$_Fwd/TRP7$_{YL}$_Rev and the backbone amplified from pSR001 using primers pSR001_Fwd/pSR001_Rev.

The *Escherichia coli* TOP10 strain was primarily used for molecular cloning. *Y. lipolytica* strain ATCC MYA-2613 (or YlSR001), a thiamine, leucine, and uracil auxotroph, was obtained from the ATCC strain collection. The constructed plasmids, namely, pSR001, pSR002, and pSR003, were transformed into YlSR001 via electroporation (38) to create *Y. lipolytica* strains YlSR101, YlSR102, and YlSR103, respectively.

Medium and cell culturing. (i) Media. The complex Luria-Bertani medium, which contained 5 g/liter yeast extract, 10 g/liter tryptone, and 5 g/liter NaCl plus 100 µg/ml ampicillin for selection, was used for molecular cloning in *E. coli*. For characterization of *Y. lipolytica*, we used the defined Synthetic Complete Supplement Mixture (SC)-Leu media (pH 5.5), which contained yeast nitrogen base (catalog no. Y0626; Sigma-Aldrich, MO, USA), synthetic dropout amino acid mixture without leucine (Y1376; Sigma-Aldrich), and sugars. Each type of sugar (e.g., glucose, xylose, or cellobiose) was prepared with an initial concentration of 10 g/liter in media containing either single or mixed sugars.

(ii) Cell culturing. Growth of *Y. lipolytica* was conducted at 28°C and 190 rpm in 250-ml baffled flasks that contained defined media with working volumes of 25 ml. For detailed strain characterization, single colonies were inoculated in defined media until reaching the exponential phase (optical density at 600 nm [OD$_{600}$] of ~2, where an OD of 1 represents ~0.53 g/liter and ~3×10^7 viable cells/ml). Next, cells were centrifuged at $4,700 \times g$ for 3 min, washed with 10 mM phosphate-buffered saline (PBS), and resuspended in the fresh medium with an initial OD of 0.1. Cell growth was monitored throughout the cell culturing period, and samples were collected for high-pressure liquid chromatography (HPLC), real-time PCR (RT-PCR), and cell-free enzyme assays. Before the detailed strain characterization was performed in media containing xylose and/or cellobiose, we adapted cells through 5 rounds of culture transfers during the exponential growth to activate xylose and cellobiose degradation pathways. All experiments for the detailed strain characterization were performed in at least 4 biological replicates.

The (average) specific cell growth rate $\overline{\mu}$ (1/h) and sugar uptake rate $\overline{r}_s$ (mmol/gram dry cell weight [gDCW]/h) values were determined as follows:

$$\overline{\mu} = \frac{1}{N} \cdot \sum_{i=1}^{N} \frac{\ln X_i - \ln X_{i-1}}{\Delta t_i} \qquad (1)$$

$$\overline{r}_s = \frac{1}{N} \cdot \sum_{i=1}^{N} \frac{S_i - S_{i-1}}{\frac{1}{2}(X_i + X_{i-1})} \cdot \frac{1}{\Delta t_i} \qquad (2)$$

where S_i (g/liter) and X_i (g/liter) are the substrate and biomass concentrations, respectively, at time t_i (h).

Analytical methods. (i) HPLC. To quantify extracellular metabolites (e.g., sugars, xylitol, and xylulose), 1 ml of culture supernatants was filtered through 0.2-μm-pore-size filters prior to the HPLC run. A Shimadzu HPLC system, equipped with a refractive index detector (RID) and a UV light detector (Shimadzu Scientific Instruments, Inc., MD, USA) and an Aminex 87H column (catalog no. 1250140; Bio-Rad, CA, USA), was used to run samples. The running method used 10 mN H_2SO_4 as a mobile phase and operation at a flow rate of 0.6 ml/min and an oven temperature set at 48°C (39).

(ii) Transcriptomics by RT-PCR. To extract mRNA samples for RT-PCR, exponentially grown cells were first treated with RNA Protect cell reagent (catalog no. 76526; Qiagen Inc., CA, USA). About 2×10^7 cells were mixed with acid-washed glass beads and were then mechanically disrupted by the use of a Mini-Beadbeater-16 cell disrupter (model 607; BioSpec Product Inc., OK, USA). Disrupted cell samples were centrifuged at $17,000 \times g$ for 2 min, and supernatants were collected for mRNA purification using a Qiagen RNeasy minikit (catalog no. 74104; Qiagen Inc., CA, USA). The mRNA samples were first treated with DNase to remove gDNA contamination by the use of an RNease-free DNase kit (catalog no. 79254; Qiagen Inc., CA, USA) and were subsequently used to synthesize cDNA using a QuantiTect reverse-transcription kit (catalog no. 205311; Qiagen Inc., CA, USA).

The RT-PCR samples were prepared from the synthesized cDNA using a QuantiTect SYBR green PCR kit (catalog no. 204143; Qiagen Inc., CA, USA) and were run on a StepOnePlus real-time PCR system (Applied Biosystems, CA, USA) to quantify mRNA expression levels of targeted genes. Primers used for RT-PCR are listed in Table S2 in the supplemental material. For the analysis, the mRNA expression level of a targeted gene [e.g., $2^{-\Delta CT} = 2^{-(CT, \text{ target} - CT, \text{ actin})}$, where C_{Ti} is the critical threshold cycle value for gene i] in each sample was normalized with respect to that of the housekeeping actin gene (e.g., YALI0D08272g). To compare the relative levels of gene expression occurring under two different growth conditions, we used the C_T $\log_2$ ratio (e.g., $\log_2 2^{-\Delta\Delta CT} = -\Delta\Delta C_T = -(\Delta C_{T\text{condition1}} - \Delta C_{T\text{condition2}})$) of the normalized mRNA expression levels for the targeted gene for comparisons of condition 1 (e.g., growth on xylose) to condition 2 (e.g., growth on glucose as a reference) (40). The relative mRNA expression level for each gene under a given growth condition was reported as the average result ± 1 standard deviation from a data set of at least 4 biological replicates. Student's t test was performed to evaluate statistical significance.

Enzyme assays. To prepare samples for cell-free enzyme assays, 1×10^7 cells in late exponential growth were collected and centrifuged at $4,700 \times g$ for 3 min. Cell pellets were resuspended in Y-PER yeast protein extraction reagent (catalog no.78990; Thermo Scientific, IL, USA), which contained EDTA-free pierce protease inhibitors (catalog no. 88266; Thermos Scientific, IL, USA), and were incubated with agitation at room temperature for 20 min. Lysed cell samples were then centrifuged at $17,000 \times g$ for 10 min, and the soluble fractions were collected for enzyme assays. Unless specified, all the enzyme assay reactions were conducted in 384-well plates with a 50-μl working reaction volume at 28°C. Kinetics measurement was conducted with a BioTek Synergy HT microplate reader, and data were processed using Gen5 software (BioTek Instruments, Inc., VT, USA). Protein concentrations were measured by the Bradford assay (41). All enzyme assay experiments were performed with at least 4 biological replicates.

BGL assay. The extracellular and intracellular β-glucosidase (BGL) activities were measured in the cell supernatants and whole-cell lysates, respectively. The BGL assay reaction was conducted at pH 6 and 28°C in 25 mM sodium phosphate buffer that contained 10 mM p-nitrophenyl-β-D-glucopyranoside and 1.5 μg total proteins from either cell supernatants or whole-cell lysates. The BGL activities were determined by the emission of p-nitrophenol (p-NP) at 400 nm. One unit of the BGL activity was defined as 1 μmol of p-NP reduced per mg protein per min (42).

XYL1 assay. The xylose reductase (XYL1) assay was conducted at pH 6 and 28°C in 25 mM sodium phosphate buffer that contained 0.5 mM NAD(P)H, 1.5 μg total proteins from whole-cell lysates, and 300 mM xylose. The XYL1 activity was measured by the oxidation of NAD(P)H at 340 nm. One unit of XYL1 activity was defined as 1 μmol of NAD(P)H consumed per mg protein per min (43).

XYL2 assay. The xylitol dehydrogenase (XYL2) assay was conducted at pH 6 and 28°C in 25 mM sodium phosphate buffer that contained 0.5 mM NAD(P)$^+$, 1.5 μg total proteins from whole-cell lysates, and 300 mM xylitol. The XYL2 activity was measured by the reduction of NAD(P)$^+$ at 340 nm. One unit of XYL2 activity was defined as 1 μmol of NAD(P)H produced per mg protein per min (43).

XYL3 assay. A bioluminescence assay kit (catalog no. K254-200; Biovision, CA, USA) was used to measure xylulokinase (XYL3) activity. The XYL3 assay reaction was conducted at pH 6 and 28°C in 25 mM sodium phosphate buffer that contained 5 μl ATP, 5 μl ATP monitoring enzymes, 1.5 μg total proteins from whole-cell lysates, and 200 mM xylulose. One unit of XYL3 activity was defined as 1 relative light unit (RLU) per μg protein per s.

Bioinformatics. We applied the BLASTP tool (44) to search for putative sugar transporters and the metabolic enzymes of the xylose- and cellobiose-degrading pathways in *Y. lipolytica*. To find sugar transporters, we used the glucose transporter of *S. cerevisiae* (HXT7) (45), the xylose transporter of *P. stipitis* (XUT4) (46), the cellobiose transporter of *N. crassa* CDT1 (NCU00801) (14), and the xylobiose transporter of *Streptomyces thermoviolaceus* (BxlF) (47) as the templates. Using the MEGA5.2 tool, we performed ClustalW alignment for the BLASTP-searched putative sugar transporters and identified conserved structural domains and amino acid residues (48). The alignment results were also edited using the Jalview 2.8 tool (49) for enhanced visual presentation. To find the metabolic enzymes responsible for the xylose and cellobiose degradation, we used the XYL1, XYL2, and XYL3 proteins of *P. stipitis* (50, 51) as well as BGLs of *A. niger* (BGL1; Q30BH9) (52), *A. fumigatus* (BTGE; B0Y9Q9) (53), and *Candida wickerhamii* (BGLA; Q12602) (54) as the templates. We further applied the PSORTII tool (55) to predict the cellular localization of the BLASTP-searched putative BGL genes of *Y. lipolytica*.

To evaluate whether the xylose and cellobiose degradation pathways in our characterized *Y. lipolytica* strain, ATCC MYA-2613 (taxid 4952), are conserved across different isolated *Y. lipolytica* strains, we used the BLASTN tool (chosen due to intron interference) to search for sequence homology against WSH-Z06 (taxid 1437815) and CLIB122 (taxid 284591), whose genome sequences are available.

RESULTS

***Y. lipolytica* is capable of using xylose as a sole carbon source.** Genome mining confirmed that *Y. lipolytica* has the putative xylose-degrading metabolic enzymes (XYL1, XYL2, and XYL3) required for xylose assimilation (see Table S3 in the supplemental material). To activate the xylose degradation pathway, we adapted *Y. lipolytica* to grow on defined medium containing 10 g/liter xylose as a sole carbon source. We controlled the xylose growth adaptation to ensure that it would last no more than 15 generations (see Fig. S1A in the supplemental material) because this short adaptation was sufficient to achieve stable growth. After the adaptation, *Y. lipolytica* grew on xylose with a specific growth rate of 0.10 ± 0.02 (1/h) and a specific xylose uptake rate of 1.71 ± 0.42 mmol/gDCW/h (Table 1). After 72 h, *Y. lipolytica* had consumed about 2.23 ± 0.10 g/liter xylose and had produced 0.18 ± 0.02 g/liter xylitol as a major byproduct (Fig. 2B).

TABLE 1 Specific sugar uptake rates of *Y. lipolytica* for growth on single and mixed sugars[a]

Parameter	Uptake rate (mmol/gDCW/h)					
	Single sugar			Mixed sugars		
	Glc	Xyl	Cel	Xyl + Glc	Xyl + Cel	Glc + Cel
r_{Glc}	2.98 ± 0.78			2.13 ± 0.50		2.13 ± 0.52
r_{Xyl}		1.71 ± 0.42		0.94 ± 0.39	4.68 ± 0.90	
r_{Cel}			0.95 ± 0.32		1.68 ± 0.25	0.16 ± 0.07

[a] Each data point represents the average ± 1 standard deviation of the results from at least 4 biological replicates. Glc, glucose; Xyl, xylose; Cel, cellobiose.

Consistently, we detected the enzymatic activities of XYL1, XYL2, and XYL3 for growth of *Y. lipolytica* on xylose (Table 2). XYL1 and XYL2 exhibited activities toward NAD(P)H and NAD(P), respectively, as shown by the use of the whole-cell lysates (see Table S4 in the supplemental material). In addition, we confirmed that the XYL1, XYL2, and XYL3 genes were upregulated 1.57 ± 0.06-fold, 2.27 ± 0.27-fold, and 1.94 ± 0.23-fold higher for growth on xylose than on glucose, respectively (Fig. 3). Both the
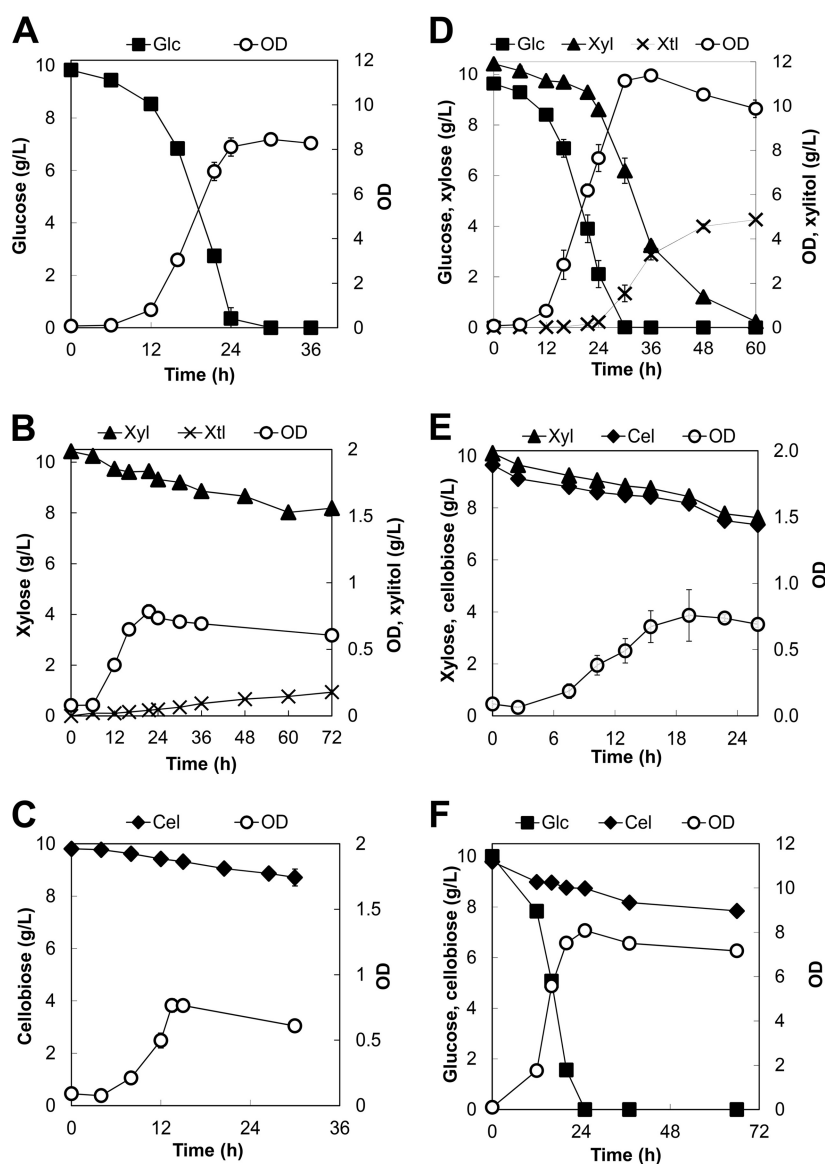
FIG 2 Profiles of cell growth and metabolites for growth of the parent *Y. lipolytica* strain on single sugars, including (A) glucose, (B) xylose, and (C) cellobiose, as well as on mixed sugars, including (D) xylose and glucose, (E) xylose and cellobiose, and (F) glucose and cellobiose. Each data point represents an average value ± 1 standard deviation calculated from the results of 3 of 6 biological replicate experiments that were conducted in parallel. Abbreviations: Glc, glucose; Xyl, xylose; Cel, cellobiose; Xtl, xylitol.

TABLE 2 Enzyme activities of xylose reductase, xylitol dehydrogenase, and xylulose kinase of *Y. lipolytica* growing on xylose and its mixtures with glucose and cellobiose[a]

Substrate(s)	Enzyme activity (U)		
	XYL1	XYL2	XYL3
Xyl	7.63 ± 1.56	3.75 ± 1.79	15.70 ± 3.51
Xyl + Glc	12.22 ± 3.16	41.42 ± 9.45	53.76 ± 13.60
Xyl + Cel	4.74 ± 0.00	40.38 ± 24.0	12.01 ± 5.83

[a] The measurement of the xylose reductase (XYL1) and xylitol dehydrogenase (XYL2) *in vitro* activity levels shown in the table used the NADH and NAD cofactors, respectively. Each data point represents the average ± 1 standard deviation of the results from 4 biological replicates. XYL3, xylulose kinase.

transketolase (TKL) and transaldolase (TAL) genes of the pentose phosphate pathway, immediately downstream of the xylose degradation pathway (Fig. 1), were also upregulated, which further supports the idea of the assimilation of xylose for growth in *Y. lipolytica*. In comparison with growth on glucose (Fig. 2A), *Y. lipolytica* reached a much lower plateau OD of 0.78 ± 0.02, produced a high yield of xylitol, and did not completely consume xylose after 24 h (Fig. 2B). This observed phenotype suggests that the xylitol dehydrogenase (XYL2) step might have been limiting and hence might have impeded efficient xylose assimilation.

***Y. lipolytica* is capable of using cellobiose as a sole carbon source.** To determine whether *Y. lipolytica* is capable of utilizing cellobiose as a sole carbon source, we first performed genome mining to identify putative BGLs. We found that *Y. lipolytica* has a total of 11 putative BGLs, of which 5 are secreted extracellularly, 3 are located in the cytosol, and 3 are located in the nucleus (see Table S3 in the supplemental material). As in the xylose experiments, to activate the cellobiose degradation pathway, we adapted *Y. lipolytica* to grow in defined medium containing 10 g/liter cellobiose as a sole carbon source (see Fig. S1B in the supplemental material). After adaptation, *Y. lipolytica* grew on cellobiose with a specific growth rate of 0.11 ± 0.02 (1/h) and a specific cellobiose uptake rate of 0.95 ± 0.32 (mmol/gDCW/h) (Table 1). However, the cellobiose assimilation was inefficient; *Y. lipolytica* consumed only 0.99 ± 0.22 g/liter cellobiose and achieved a relatively low plateau OD of 0.76 ± 0.02 after 15 h (Fig. 2C).

To elucidate how *Y. lipolytica* assimilated cellobiose, we characterized the mRNA expression levels of the 11 putative BGL genes (Fig. 4; see also Table S5 in the supplemental material). None of the 5 extracellularly localized putative BGL genes were upregulated during growth on cellobiose (Fig. 4A). Similarly, three of six putative intracellular BGL genes—*YALI0B14333g* (encoding cytosolic BGL), *YALI0E21109g* (encoding nucleus BGL), and *YALI0F01947g* (encoding nucleus BGL)—were not induced by cellobiose. In contrast, the three remaining putative intracellular BGL genes—*YALI0E20185g* (encoding cytosolic BGL), *YALI0F01672g* (encoding cytosolic BGL), and *YALI0F13299g* (encoding nucleus BGL)—were significantly upregulated at levels 1.71 ± 0.19-fold, 20.48 ± 0.61-fold, and 1.70 ± 0.11-fold higher for growth on cellobiose than glucose, respectively, and were likely responsible for cellobiose assimilation in *Y. lipolytica* (Fig. 4B). Consistently, we detected the intracellular BGL activity from the cell lysate as well as the extracellular BGL activity in the culture supernatant for growth on cellobiose; however, the extracellular BGL activity was low, at about 2 orders of magnitude lower than the intracellular activity (Table 3).

Elucidation of functional roles of sugar transporters in *Y. lipolytica*. (i) Identification of putative sugar transporters for glucose, xylose, and cellobiose. Sugar transporters play a significant role in cellular metabolism but are poorly understood in *Y. lipolytica*. To have a complete picture of complex sugar utilization, we first performed genome mining of *Y. lipolytica* to identify putative sugar transporters responsible for glucose, xylose, and cellobiose assimilation. We identified a total of 23 putative glucose transporter genes, 22 and 19 of which were also putative xylose and cellobiose transporter genes, respectively (Fig. 5A). These transporters, belonging to the major facilitator superfamily (MFS) sugar transporters (18, 56, 57), have 12 transmembrane (TM) domains and the well-known "diffused" motif between TM4 and TM5 that are known to be responsible for catalytic and structural functions (58). Since these transporters have short C-terminal tails, they are unlikely to function similarly to sugar sensors (59, 60). Among these 23 putative glucose transporter genes, YALI0A14212g was predicted to be unique to glucose whereas the other 3 genes, including YALI0B21230g, YALI0F18084g, and YALI0D00132g, were predicted to be unique for both glucose and xylose but not cellobiose.

To elucidate the functional roles of these 23 putative transporters for growth of *Y. lipolytica* on glucose, xylose, and cellobiose, we performed a comprehensive transcriptional analysis (Fig. 6; see also Table S6 in the supplemental material). By examining the relative mRNA expression levels of putative transporter genes that were downregulated or unchanged for growth of *Y. lipolytica* on xylose or cellobiose alone, we confirmed that 7 of the 23 putative glucose transporters were induced by glucose. We found that 16 of the 22 putative xylose transporter genes were overexpressed up to 3 orders of magnitude (>1,000-fold) higher for growth on xylose than glucose (Fig. 6). We verified that 4 of the 19 putative cellobiose transporter genes, comprised of *YALI0D01111g*, *YALI0C04730g*, *YALI0D00363g*, and *YALI0B00396g*, were expressed up to 62-fold higher for growth on cellobiose than glucose. These 4 putative cellobiose transporter genes were also highly upregulated by xylose induction.

(ii) Analysis of highly conserved structural motif and amino acid residues identified novel xylose-specific transporters of *Y. lipolytica*. While the structural topology and functional role of sugar transporters of eukaryotes can be classified with high confi-
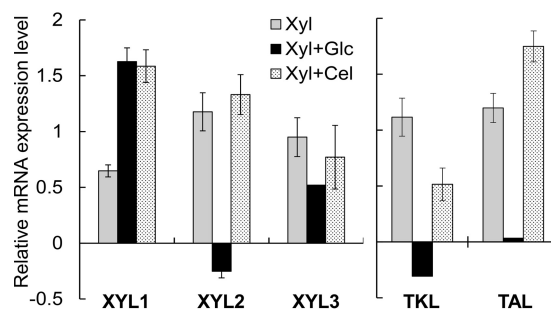

FIG 3 Relative mRNA expression levels (in $\log_2$ scale) of the xylose pathway (XYL1, XYL2, and XYL3) and pentose phosphate pathway (TKL and TAL) genes of the parent *Y. lipolytica* strain for growth on xylose (Xyl), a mixture of xylose and glucose (Xyl+Glc), and a mixture of xylose and cellobiose (Xyl+Cel). The reference condition for normalization is growth on glucose alone. Each data point represents an average value ± 1 standard deviation calculated from the results of 4 biological replicates.

FIG 4 Relative mRNA expression levels (in $\log_2$ scale) of (A) putative extracellular BGL genes and (B) putative intracellular BGL genes for growth of the parent *Y. lipolytica* on cellobiose (Cel) and on a mixture of xylose and cellobiose (Xyl+Cel) as well as on a mixture of glucose and cellobiose (Glc+Cel). The reference condition for normalization is growth on glucose alone. Each data point represents an average value ± 1 standard deviation calculated from 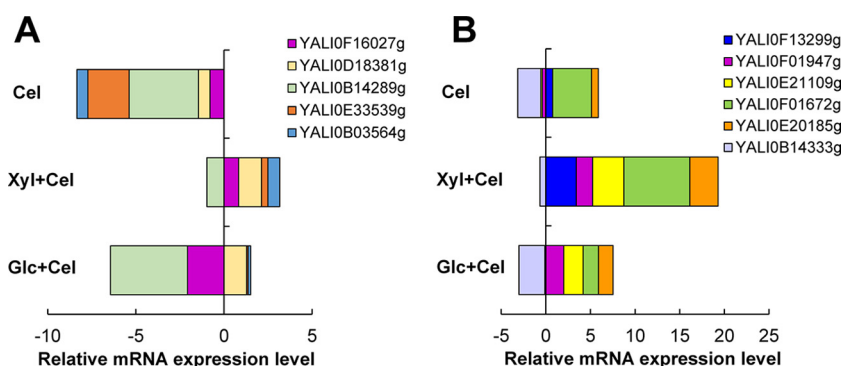the results of 4 biological replicates. Error data were not included in the figure due to the crowding effect but are presented in Table S5 in the supplemental material for reference.

dence by bioinformatics (18, 56, 57), fundamental knowledge regarding prediction of sugar specificity and transport efficiency is still lacking. Recent studies showed that the highly conserved structural motif G-G/F-X-X-X-G in the TM1 as well as the conserved T213 or N370 amino acid residue of the *S. cerevisiae* HXT7 in TM5 or TM8, respectively, can govern the specificity and efficiency of the xylose uptake over glucose in *S. cerevisiae* (20, 22). Motivated by these studies, we first investigated whether the G-G/F-X-X-X-G motif is conserved for the putative xylose transporters of *Y. lipolytica*. Among the 16 putative xylose transporters of *Y. lipolytica* that were upregulated for growth on xylose, we found that 8 of these transporters have the G-G/F-X-X-X-G motif containing the highly conserved L/F-L-F amino acids in place of X-X-X regions (Fig. 5B and Table 4), responsible for high-level xylose-specific uptake in *S. cerevisiae* (22). We also determined that 3 of the remaining 8 putative xylose transporters of *Y. lipolytica* have a similar but not identical G-G/F-X-X-X-G motif where variables occur at the first two amino acid residues, including G-V for YALI0F06776p, S-G for YALI0F23903p, and G-T for YALI0B21230p. These similar motifs were also found to be responsible for xylose-specific uptake in *S. cerevisiae* (22).

Since the replacement of T213 and N370 in *S. cerevisiae* HXT7 by a hydrophobic or small hydrophilic amino acid could change the substrate binding pocket and improve the xylose specificity (20, 22), we examined these conserved amino acid residues in the putative xylose transporters of *Y. lipolytica* (Fig. 5C). The results show that 8 of the 16 putative xylose-specific transporters induced

by growth on xylose have T213 replaced by N, Y, A, I, S, or V whereas 10 of the 16 putative xylose transporters of *Y. lipolytica* have N370 replaced by V, K, or Y (Table 4). Among the 8 putative xylose transporters of *Y. lipolytica* that contain the G-G/F-X-X-X-G motif, 1 transporter has both T213 and N370 replacement whereas the remaining 4 transporters have either T213 or N370 replacement. Altogether, these results suggest that *Y. lipolytica* likely has at least 5 putative xylose-specific transporters induced by xylose.

Synergistic effect of mixed-sugar utilization in *Y. lipolytica*. The CCR effect often causes inefficient utilization of mixed, complex fermentable sugars derived from lignocellulosic biomass for production of desirable biochemicals and biofuels (15). To investigate whether the CCR effect exists in *Y. lipolytica*, we characterized growth of *Y. lipolytica* on various mixtures of glucose, xylose, and cellobiose.

(i) Elucidation of growth characteristics of *Y. lipolytica* in a mixture of glucose and xylose. In a mixture of 10 g/liter glucose and 10 g/liter xylose, *Y. lipolytica* grew with a specific growth rate of 0.15 ± 0.01 (1/h). It consumed all glucose after 30 h (Fig. 2D) with a specific glucose uptake rate of 2.13 ± 0.50 mmol/gDCW/h (Table 1), which is similar to the growth phenotype seen with glucose alone without any observed inhibitory effect by xylose. Unlike growth on xylose alone, *Y. lipolytica* was able to consume all of the xylose after 60 h with a specific xylose uptake rate of 0.94 ± 0.39 mmol/gDCW/h. Even though xylose assimilation was significantly enhanced, it did not entirely contribute to biomass production because a relatively large amount of 4.87 g/liter xylitol was produced as a major product (Fig. 2D) together with other minor byproducts (e.g., pyruvic, succinic, and a-ketoglutaric acids; data not shown). This observed phenotype further supports the hypothesis that XYL2 is the rate-limiting step of the xylose degradation pathway in *Y. lipolytica*. Even though the xylose consumption rate was lower than that of glucose, *Y. lipolytica* did not exhibit the diauxic growth commonly caused by the severe CCR effect and was able to consume glucose and xylose simultaneously (Fig. 2D; see also Fig. S2A in the supplemental material).

To further elucidate the driving force for enhanced xylose assimilation for growth on the mixture of glucose and xylose, we performed transcriptomic and metabolic analyses of the xylose degradation pathway. Like growth on xylose alone, both XYL1

TABLE 3 Enzyme activity of intracellular and extracellular β-glucosidases of *Y. lipolytica* growing on single and mixed sugars[a]

Substrate(s)	Enzyme activity (U)	
	Intra BGL	Extra BGL
Glc	1.58 ± 0.26	ND
Xyl	ND	0.04 ± 0.00
Cel	1.94 ± 0.59	0.01 ± 0.00
Xyl + Glc	2.67 ± 1.46	0.03 ± 0.00
Xyl + Cel	2.71 ± 0.19	0.22 ± 0.00
Glc + Cel	3.98 ± 0.62	0.27 ± 0.00

[a] Each data point represents the average ± 1 standard deviation of the results from 4 biological replicates. Abbreviations: intra, intracellular; extra, extracellular; BGLs, β-glucosidases; ND, not detected.
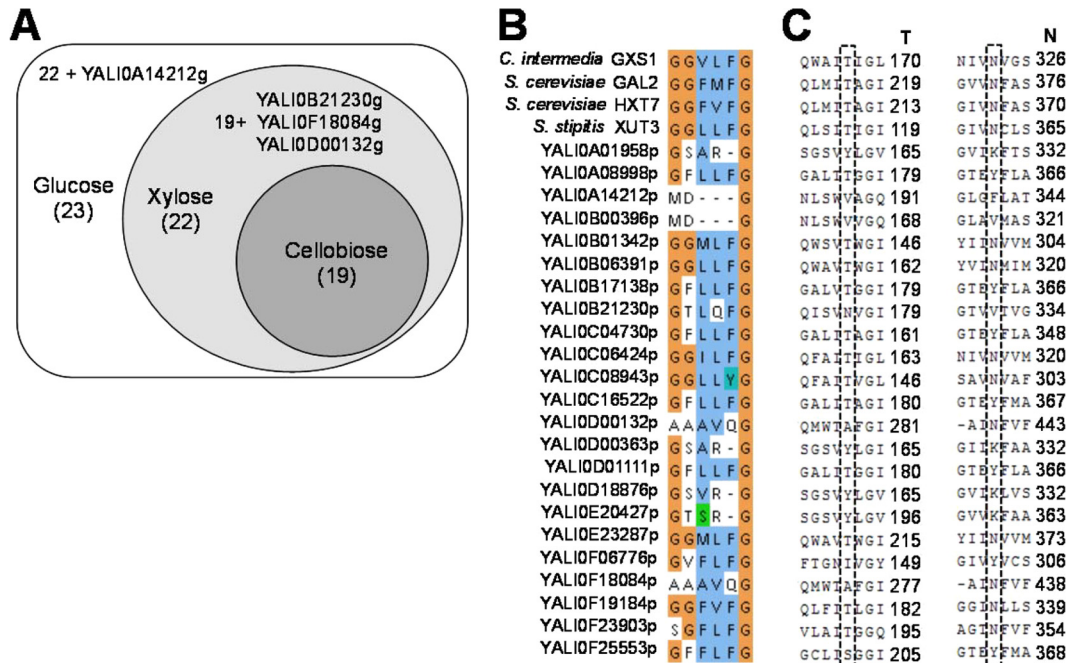
FIG 5 (A) Venn diagram of the putative glucose, xylose, and cellobiose transporter genes that were subjected to BLASTP searches in the parent *Y. lipolytica* strain. (B) The G-G/F-X-X-X-G consensus sequence. (C) The patterns of T213/N370 amino acid residues.
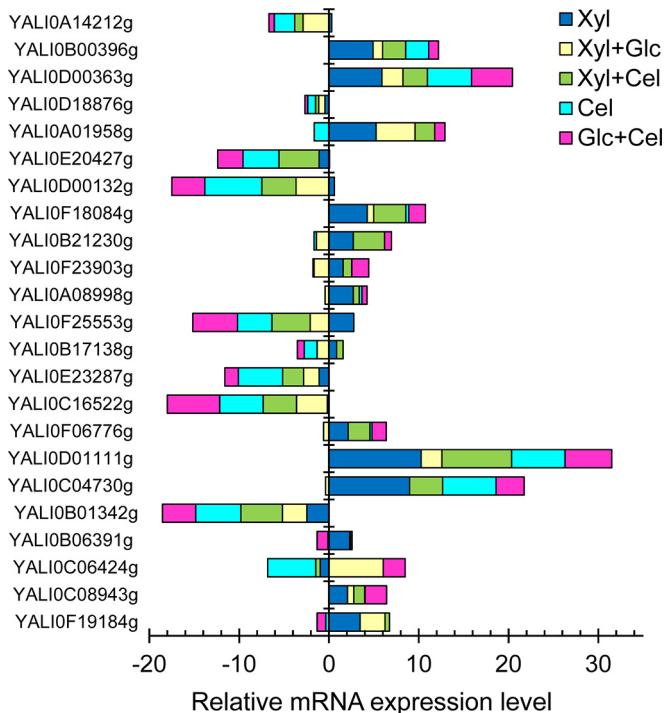

FIG 6 Relative mRNA expression levels (in $\log_2$ scale) of putative sugar transporter genes of *Y. lipolytica* that grow on single sugars, including glucose (Glc), xylose (Xyl), and cellobiose (Cel), as well as on mixtures of xylose and glucose (Xyl+Glc), of xylose and cellobiose (Xyl+Cel), and of glucose and cellobiose (Glc+Cel). The reference condition for normalization is growth on glucose alone. Each data point represents an average value ± 1 standard deviation calculated from the results of 4 biological replicates. Error data were not included in the figure due to the crowding effect but are presented in Table S6 in the supplemental material for reference.

and XYL3 genes were upregulated by 3.09 ± 0.26 and 1.43 ± 0.00-fold, respectively, higher for growth on mixed sugars than on glucose alone (Fig. 3). The observed gene upregulation correlated with relatively high enzyme activities of XYL1 and XYL3 (Table 2). In contrast to growth on xylose alone, the XYL2 gene was slightly repressed for growth on the mixture of glucose and xylose, which strongly correlated with the relatively high accumulation of xylitol. Like the XYL2 gene, the TKL gene, but not the TAL gene, was transcriptionally repressed (Fig. 3).

Consistent with the observed simultaneous assimilation of glucose and xylose, we found that some of the putative glucose and xylose transporters were not transcriptionally repressed by the CCR effect of glucose on xylose and vice versa. For instance, among the 7 putative glucose transporter genes induced by growth on glucose alone, the expression of *YALI0C06424g* was synergistically enhanced in the presence of xylose whereas both *YALI0E20427g* and *YALI0D18876g* remained unaffected (Table 4). Likewise, among the 16 putative xylose transporter genes, the expression levels of *YALI0F19184g* and *YALI0A01958g* were not affected by glucose (Table 4).

(ii) Elucidation of growth characteristics of *Y. lipolytica* in a mixture of xylose and cellobiose. During simultaneous saccharification and fermentation of lignocellulose, microbial cell factories, which can efficiently coutilize xylose and cellobiose, are desirable because they help eliminate external supply of BGLs, alleviate feedback inhibition of extracellular cellulases, and avoid the CCR effect exerted by glucose intracellularly. To investigate whether *Y. lipolytica* is capable of assimilating xylose and cellobiose simultaneously, we cultured *Y. lipolytica* in defined medium containing 10 g/liter xylose and 10 g/liter cellobiose. The results show that *Y. lipolytica* was able to simultaneously utilize xylose and cellobiose with a specific growth rate of 0.10 ± 0.01 (1/h) (Fig. 2E; see also Fig. S2B in the supplemental material). We observed that

TABLE 4 Classification of putative glucose transporters, putative xylose transporters, and putative cellobiose transporters of *Y. lipolytica* that are transcriptionally induced by their respective single sugars[a]

Transporter category and gene	G-G/F-X-X-X-G motif	Amino acid		Relative mRNA expression in indicated sugar mixture	
		T213	N370		
Putative glucose transporters				Xyl+Glc	Glc+Cel
YALI0C06424g	GGILFG	T	N	+++	+
YALI0A14212g	ND	V	F	-	*n.c.*
YALI0B01342g	GGMLFG	T	N	-	–
YALI0C16522g	GFLLFG	T	Y	–	—
YALI0E23287g	GGMLFG	T	N	-	-
YALI0E20427g	ND	Y	K	*n.c.*	-
YALI0D18876g	ND	Y	K	*n.c.*	*n.c.*
Putative xylose transporters				Xyl+Glc	Xyl+Cel
YALI0B21230g	GTLQFG	N	V	–	+
YALI0F19184g	GGFVFG	T	N	*n.c.*	-
YALI0A01958g	ND	Y	K	*n.c.*	-
YALI0A08998g	GFLLFG	T	Y	-	-
YALI0F23903g	SGFLFG	T	N	-	*n.c.*
YALI0C08943g	GGLLYG	T	N	-	*n.c.*
YALI0F18084g	AAAVQG	A	N	–	*n.c.*
YALI0B06391g	GGLLFG	T	N	-	-
YALI0D00132g	AAAVQG	A	N	–	—
YALI0F06776g	GVFLFG	I	Y	-	*n.c.*
YALI0B17138g	GFLLFG	T	Y	-	*n.c.*
YALI0F25553g	GFFLFG	S	Y	—	—
YALI0D01111g	GFLLFG	T	Y	—	-
YALI0C04730g	GFLLFG	T	Y	—	—
YALI0D00363g	ND	Y	K	–	-
YALI0B00396g	ND	V	V	–	-
Putative cellobiose transporters				Xyl+Cel	Glc+Cel
YALI0D01111g	GFLLFG	T	Y	+	*n.c.*
YALI0C04730g	GFLLFG	T	Y	-	-
YALI0D00363g	ND	Y	K	-	*n.c.*
YALI0B00396g	ND	V	V	*n.c.*	-

[a] For the relative mRNA expression levels of these transporters in sugar mixtures, qualitative scale designations corresponding to the effect of the presence of one sugar on the other in the mixture are defined as follows: +++, R (ratio or fold change) ≥ 20.0; ++, 10 ≤ R < 20, +, 1.5 ≤ R < 10; *n.c.* (no significant change), 0.5 ≤ R < 1.5, -, 0.1 ≤ R < 0.5; –, 0.05 ≤ R < 0.1; —, R < 0.05. The relative mRNA expression levels of putative transporter genes in each sugar transporter class were determined by the ratios of the normalized mRNA expression levels between growth in the mixtures and in the respective single-sugar experiments.

the assimilation of both xylose and cellobiose was synergistically enhanced, with the specific sugar uptake rates increased by 2.78 ± 0.22-fold and 1.90 ± 0.54-fold, respectively (Table 1).

We further performed comprehensive metabolic and transcriptomic analyses to elucidate the driving force for synergistically enhanced coutilization of xylose and cellobiose. For the xylose degradation pathway, the expression levels of all XYL1, XYL2, and XYL3 genes were upregulated to 3.01 ± 0.31-fold, 2.53 ± 0.32-fold, and 1.73 ± 0.35-fold (*P* value < 0.01) higher for growth on the mixture than on glucose alone (Fig. 3). The upregulation of TKL and TAL genes further supports the enhanced xylose assimilation in *Y. lipolytica*. The enhanced xylose utilization in the mixture also correlated with not only the upregulation of the xylose-degrading genes (Fig. 3) but also their enzyme activities (Table 2). Unlike the results seen with respect to growth on the mixture of xylose and glucose, the XYL2 gene was not transcriptionally repressed by cellobiose. Notably, in comparison with the growth seen on xylose alone, XYL1 and TAL genes were upregulated (Fig. 3) by 1.92 ± 0.20-fold and 1.47 ± 0.14-fold (*P* value < 0.01), respectively, which highly correlated with the enhanced xylose assimilation. For the cellobiose degradation pathway, the en-

hanced cellobiose utilization in the mixture correlated well with significant increases in the transcriptional expression of BGL genes and their enzyme activities of 166.04 ± 16.05-fold and 1.74 ± 0.24-fold, respectively, in comparison with growth on glucose alone and to 12.66 ± 1.30-fold and 1.51 ± 0.51-fold (*P* value < 0.01), respectively, in comparison with growth on cellobiose alone (Fig. 4 and Table 3). Strikingly, most of the extracellular BGL genes were also overexpressed, which was not observed for growth on cellobiose alone.

Transcriptional analysis of putative xylose and cellobiose transporters further supports the idea of improved simultaneous coutilization of xylose and cellobiose (Fig. 6 and Table 4). Among the 16 putative xylose transporter genes, the expression of *YALI0B21230g* was synergistically enhanced in the presence of cellobiose whereas the expression of *YALI0F23903g*, *YALI0C08943g*, *YALI0F18084g*, *YALI0F06776g*, and *YALI0B17138g* remained unaffected. Likewise, among the 4 putative cellobiose transporters, *YALI0D01111g* expression was synergistically enhanced whereas *YALI0B00396g* expression remained unaffected in the presence of xylose.

(iii) Elucidation of growth characteristics of *Y. lipolytica* in a mixture of glucose and cellobiose. To investigate whether *Y. li-*
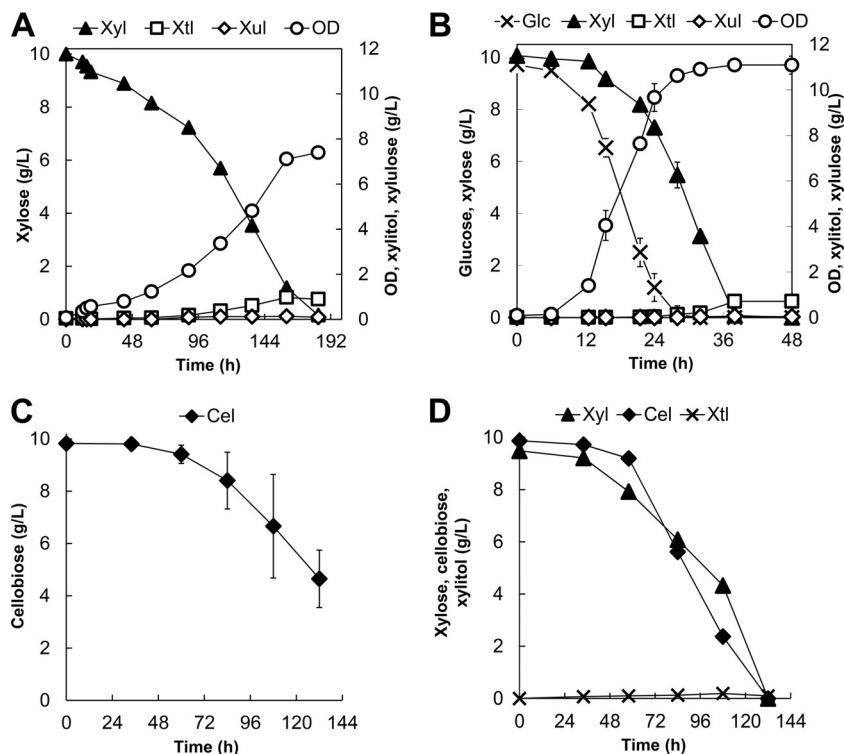
FIG 7 Profiles of cell growth and metabolites for (A) growth of YlSR102 on xylose, (B) growth of YlSR102 on the mixture of glucose and xylose, (C) growth of YlSR103 on cellobiose, and (D) growth of YlSR103 on the mixture of xylose and cellobiose. Each data point represents an average value ± 1 standard deviation calculated from the results of 3 of 6 biological replicate experiments that were conducted in parallel. Abbreviations: Xyl, xylose; Cel, cellobiose; Xtl, xylitol; Xul, xylulose.

polytica is capable of coutilizing glucose and cellobiose and whether it exhibits any CCR effect of glucose on cellobiose assimilation or vice versa, we characterized growth of *Y. lipolytica* in the mixture of 10 g/liter glucose and 10 g/liter cellobiose. *Y. lipolytica* grew with a specific growth rate of 0.19 ± 0.02 (1/h) and a specific glucose uptake rate of 2.13 ± 0.52 mmol/gDCW/h and was able to consume all of the glucose after 30 h (Fig. 2F). However, only 2.00 ± 0.12 g/liter of cellobiose was consumed after 96 h, with a specific cellobiose uptake rate of 0.16 ± 0.07 mmol/gDCW/h. The results also show that *Y. lipolytica* was able to simultaneously use glucose and cellobiose and did not exhibit a severe CCR effect of glucose on cellobiose assimilation (Fig. 2F; see also Fig. S2C in the supplemental material).

Transcriptional analysis shows that the expression of the BGL genes was slightly greater for growth on the mixture of glucose and cellobiose than on cellobiose alone at up to 5.71 ± 2.71-fold (Fig. 4), which correlated well with the increase in the total BGL activity by 2.15 ± 0.47-fold (Table 3). However, cellobiose consumption was not significantly enhanced as expected (Fig. 2F and Table 1), suggesting that the cellobiose transporters represent the rate-limiting step and likely that there is a CCR effect of glucose on cellobiose transporters. In fact, transcriptional analysis of the 4 putative cellobiose transporters shows that they were either downregulated or not affected in growth on the mixed sugars in comparison to growth on cellobiose alone (Table 4).

Alleviation of bottlenecks of xylose and cellobiose assimilation in *Y. lipolytica*. (i) Effect of overexpression of the native XYL2 on xylose assimilation. Characterization of growth of the parent *Y. lipolytica* YlSR101 strain on xylose alone and on its mix-

ture with glucose clearly suggests that the XYL2 step represents the most dominant bottleneck. To alleviate this bottleneck, we constructed strain YlSR102, which overexpressed the native XYL2 gene under the control of the constitutive TEF promoter. The strain characterization for growth on xylose alone clearly showed that YlSR102 has significantly improved growth on xylose, with an enhanced plateau OD of 7.39 ± 0.20, about 10-fold higher than that seen with the parent strain, and complete consumption of xylose as well as significantly lower accumulation of xylitol (Fig. 7A).

Likewise, for growth on the mixture of glucose and xylose, YlSR102 could consume all 10 g/liter xylose without xylitol accumulation within 36 h, about 1.7 times faster than YlSR101 (Fig. 7B). As expected, YlSR102 also exhibited the simultaneous coutilization of glucose and xylose (see Fig. S2A in the supplemental material).

Taken altogether, these results provide evidence that the XYL2 step is the most critical bottleneck affecting the efficient xylose assimilation in the native *Y. lipolytica* strain.

(ii) Effect of overexpression of the native cellobiose transporter on cellobiose assimilation. Growth characterization of the parent *Y. lipolytica* YlSR101 strain on cellobiose alone and on its mixture with either xylose or glucose clearly suggested that the cellobiose assimilation is inefficient, with less than 20% of cellobiose in the feed being consumed. We hypothesized that cellobiose transporters might have been the dominant rate-limiting step in efficient cellobiose assimilation in the parent *Y. lipolytica*. To test this hypothesis, we constructed the *Y. lipolytica* YlSR103 mutant, which overexpressed the *YALI0D01111g* putative cellobiose

transporter gene. This gene was chosen because it was induced at the highest levels by both cellobiose and xylose (Fig. 6).

For growth on cellobiose alone, YlSR103 improved both cell growth and cellobiose assimilation in comparison with the YlSR101 parent strain. YlSR103 reached a final biomass titer of 2.80 ± 0.70 gDCW/liter (or OD = ~5.25 ± 1.30) and was able to consume 5.18 ± 1.10 g/liter cellobiose (Fig. 7C). Unlike the parent strain, interestingly, YlSR103 exhibited a morphological change where cells formed pellets in the aqueous medium (61). For this reason, cell mass was analyzed only at the end of the experiments. In growth on the mixture of cellobiose and xylose, YlSR103 was capable of completely and simultaneously assimilating both cellobiose and xylose and outperformed the parent YlSR101 strain (Fig. 7D).

Taken altogether, these results clearly support the idea that the cellobiose transporter represents the rate-limiting step of cellobiose assimilation in the native *Y. lipolytica* strain.

DISCUSSION

In this study, we activated and elucidated the native sugar metabolism of *Y. lipolytica* in growth on xylose and cellobiose as well as on their mixtures with glucose through metabolic and transcriptomic analyses. We discovered that *Y. lipolytica* has putative sugar-specific transporters as well as metabolic enzymes to assimilate these sugars. We found that *Y. lipolytica* exhibited a mild CCR effect of glucose on xylose and cellobiose by exerting transcriptional repression of the xylose-degrading metabolic enzymes XYL2 and TKL and of the putative cellobiose transporters. In contrast, the CCA effect enhanced the coutilization of xylose and cellobiose. Regardless of the sugar mixture used, *Y. lipolytica* was able to simultaneously consume the sugars in the mixtures, likely due to mild CCR and beneficial CCA effects exerted on the sugar degradation pathways.

One important factor in activation of the xylose and cellobiose degradation pathways of the native *Y. lipolytica* strain is that of adapting it to grow on these sugars through serial culture transfers. During the first transfer, cell growth was very slow and was observed only after 3 days (see Fig. S1 in the supplemental material). Since studies by other groups did not investigate cell growth of the native *Y. lipolytica* after 2 days (34, 62), the xylose and cellobiose degradation pathways were not activated in those studies. In our study, since the adaptation period was conducted for less than 15 generations, which was sufficient to achieve relatively stable cell growth, the adapted phenotypes of the native *Y. lipolytica* were reversible (results not shown).

By demonstrating that native *Y. lipolytica* has 16 putative xylose transporters and xylose-degrading metabolic enzymes (e.g., XYL1, XYL2, XYL3, TKL, and TAL) induced by xylose, we have provided metabolic and genetic evidence of how the native *Y. lipolytica* could grow on xylose as a sole carbon source. However, the xylose consumption was inefficient because the XYL2 step was limiting and was also transcriptionally repressed by glucose. While the engineered XYL2-overexpressing YlSR102 strain proved very effective for overcoming this rate-limiting step, additional optimization is required to engineer the xylose degradation pathway of *Y. lipolytica* to operate as efficiently as those of the native yeast *P. stipitis* strain (63) and the recombinant *S. cerevisiae* strain (43).

Likewise, the native *Y. lipolytica* strain was able to grow on cellobiose as a sole carbon source because it has 4 putative cellobiose transporters as well as 11 BGLs induced by cellobiose.

Six of these 11 BGLs, including YALI0D18381, YALI0B14333, YALI0B14289, YALI0E20185, YALI0F01672, and YALI0F16027, were the same as those reported very recently (during the preparation of our manuscript) (33). Furthermore, we identified 5 additional BGLs, YALI0B03564p, YALI0E33539p, YALI0E21109p, YALI0F01947p, and YALI0F13299p, which also have functional roles in the presence of cellobiose. The transcriptional and enzymatic activities of BGL genes strongly depend on the types of sugars used for cell growth. For instance, most of intracellular BGL genes were activated by growth on cellobiose alone or on its mixture with glucose, suggesting that the native *Y. lipolytica* assimilated cellobiose intracellularly. In contrast, growth on the mixture of cellobiose and xylose resulted in overexpression of most of the extracellular and intracellular BGL genes, suggesting that the native *Y. lipolytica* strain could degrade cellobiose both intracellularly and extracellularly.

In addition, the native *Y. lipolytica* strain exhibited leaky expression of some BGL genes, as the BGL activity was detected regardless of whether the growth was on glucose or xylose or both (Table 3). This phenotype is often found in the many biomass-degrading fungi that tend to produce base-level cellulases regardless of growth conditions (64, 65). While native *Y. lipolytica* could grow on cellobiose as a sole carbon source, the cellobiose assimilation was inefficient compared to that seen with native *N. crassa* (14), native *A. niger* (66), recombinant *S. cerevisiae* (67), or recombinant *Y. lipolytica* (62), likely due to the limitation of the cellobiose transporter. With the knowledge of putative cellobiose transporters and BGL genes identified in this study, further optimization of the cellobiose degradation pathway is highly feasible.

Even though metabolic and transcriptomic analyses clearly demonstrate the activation of xylose and cellobiose degradation pathways, the catalytic activity and specificity for many metabolic enzymes/transporters need to be characterized in detail due to their potential novelty, which might be masked by transcriptional regulation of the native metabolism. For instance, complementary to the very recent studies that have successfully engineered xylose-specific transporters in *S. cerevisiae* (22), we discovered at least 5 novel, putative native xylose-specific transporters in *Y. lipolytica* that have both the G-G/F-X-X-X-G motif and T213/N370 replacement. Even though our study focused on activation and elucidation of the sugar metabolism in the native *Y. lipolytica* ATCC MYA-2613 strain (taxid 4952), we found that these sugar assimilation pathways are also conserved in other isolated *Y. lipolytica* strains, WSH-Z06 (taxid 1437815) and CLIB122 (taxid 284591) (see Table S7 in the supplemental material). This result suggests that, once activated, *Y. lipolytica* WSH-Z06 and CLIB122 can assimilate xylose and cellobiose.

In summary, this report sheds light on fundamental understanding of the complex sugar metabolism of *Y. lipolytica* and will help guide inverse metabolic engineering of this industrially important microorganism for enhanced conversion of biomass-derived fermentable sugars to chemicals and fuels with broad biotechnological applications.

ACKNOWLEDGMENTS

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