Bioprocess Biosyst Eng (2013) 36:809–817
DOI 10.1007/s00449-013-0907-5

ORIGINAL PAPER

Effect of heterologous xylose transporter expression in *Candida tropicalis* on xylitol production rate

Woo Young Jeon · Woo Yong Shim · Sung Hyeon Lee · Joon Ho Choi · Jung Hoe Kim

Received: 26 December 2012 / Accepted: 15 January 2013 / Published online: 15 February 2013

Abstract Xylose utilization is inhibited by glucose uptake in xylose-assimilating yeasts, including *Candida tropicalis*, resulting in limitation of xylose uptake during the fermentation of glucose/xylose mixtures. In this study, a heterologous xylose transporter gene (*At5g17010*) from *Arabidopsis thaliana* was selected because of its high affinity for xylose and was codon-optimized for functional expression in *C. tropicalis*. The codon-optimized gene was placed under the control of the GAPDH promoter and was integrated into the genome of *C. tropicalis* strain LXU1 which is *xyl2*-disrupted and NXRG (codon-optimized *Neurospora crassa* xylose reductase) introduced. The xylose uptake rate was increased by 37–73 % in the transporter expression-enhanced strains depending on the glucose/xylose mixture ratio. The recombinant strain LXT2 in 500-mL flask culture using glucose/xylose mixtures showed a xylose uptake rate that was 29 % higher and a xylitol volumetric productivity (1.14 g/L/h) that was 25 % higher than the corresponding rates for control strain LXU1. Membrane protein extraction and Western blot analysis confirmed the successful heterologous expression and membrane localization of the xylose transporter in *C. tropicalis*.

Keywords Xylitol · *Candida tropicalis* · Xylose transporter · Codon optimization

Introduction

The asporogenic diploid yeast *Candida tropicalis* is a useful organism in industrial applications and academic studies. Its capacity to use *n*-alkanes and fatty acids as carbon sources has been utilized for the production of long-chain dicarboxylic acids, which are important raw materials for the preparation of polymers, adhesives, and perfumes, by the metabolic engineering of peroxisomal enzymes [1]. *C. tropicalis* is also distinguished by its great xylose-assimilating ability and has, therefore, been extensively studied in relation to xylitol production [2].

Xylitol is a five-carbon sugar alcohol that has economic importance worldwide. It is used as an alternative natural sweetener in the food and confectionary industry, has an anti-cariogenic (cavity-reducing) effect, and has the same order of sweetness as sucrose and can replace it on an equal-weight basis. Xylitol does not require insulin for its metabolic regulation and is, therefore, used as an insulin-independent sugar substitute in diabetic patients [3]. Xylitol can also be used as a chemical building block for the production of economically valuable chemicals such as xylaric acid and glycols [4].

Industrially, xylitol is produced by the chemical hydrogenation of xylose, which is derived mainly from biomass hydrolysates using Raney nickel catalysts. This

W. Y. Jeon and W. Y. Shim contributed equally to this study.

W. Y. Jeon
Biotechnology Process Engineering Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), 125 Gwahangno, Yuseong-gu, Daejeon 305-806, Korea

W. Y. Shim · S. H. Lee · J. H. Kim (✉)
Department of Biological Sciences, Korea Advanced Institute of Science and Technology, 373-1, Guseong-dong, Yuseong-gu, Daejeon 305-701, Korea
e-mail: kimjh@kaist.ac.kr

J. H. Choi
Department of Food Science and Biotechnology, College of Life Science and Natural Resources, Wonkwang Research Institute for Food Industry, Wonkwang University, Iksan, Chonbuk 570-749, Korea

chemical production method is a subject of environmental concerns, because it requires high-pressure hydrogen gas and a toxic catalyst. Therefore, many studies have focused on "eco-friendly" biological methods for xylitol production that utilize xylose-assimilating yeast species such as *C. tropicalis* and *C. parapsilosis* [5–7], or metabolically engineered strains of *Saccharomyces cerevisiae* that harbor the *XYL1* gene from *Pichia stipitis* [8, 9]. Glucose is a potentially desirable co-substrate for efficient xylitol production, because it can be utilized directly from industrial biomass hydrolysates.

Many studies have addressed xylose transport systems in yeasts, including *S. cerevisiae* and *P. stipitis*. These studies have revealed the existence of two transport systems: a low-affinity facilitated diffusion system and a high-affinity xylose–proton symport system [10]. In *S. cerevisiae*, sugars (including xylose) enter the cell via the *HXT* family of sugar transporters [11, 12]. *S. cerevisiae* is able to take up xylose through both low-affinity and high-affinity glucose transport systems [13]; however, only the high-affinity system was detected after incubation with xylose.

Three genes, *SUT1*, *SUT2*, and *SUT3*, that encode glucose transporters were cloned and characterized in *P. stipitis* [14]. These transporters have high affinity for glucose but low affinity for xylose. In general, glucose and xylose compete for the common transport machinery in yeasts, and hence, glucose is utilized first followed by xylose mainly because the transport machinery has a higher affinity for glucose than xylose. During fermentation of glucose/xylose mixtures using *P. stipitis*, xylose uptake was suppressed by increasing glucose concentration [15]. Glucose uptake similarly inhibited xylose utilization in xylose-assimilating yeasts such as engineered *S. cerevisiae* and *P. stipitis*.

Recent studies have reported the enhancement of xylose uptake using xylose-assimilating *S. cerevisiae* for ethanol production. Sut1, the glucose transporter of *P. stipitis*, was expressed in xylose-assimilating *S. cerevisiae* and shown to enhance the fermentation of xylose to ethanol [16]. The heterologous expression of a xylose transporter from *Arabidopsis thaliana* in recombinant xylose-assimilating *S. cerevisiae* increased xylose consumption [17].

In a recent study, we overcame the suppressing effect of glucose on xylose reductase (XR), which catalyzes the reduction of D-xylose to xylitol, by expressing codon-optimized XR of *Neurospora crassa* (termed NXRG) with the GAPDH promoter in *C. tropicalis* [18]. The resulting strain displayed a xylitol production rate 73 % higher than that of the control strain. Glucose uptake still inhibited xylose utilization and xylitol production during the initial period of fermentation. These findings suggested that the xylose uptake may be the limiting factor in xylitol production using glucose/xylose mixtures in *C. tropicalis*.

In the present study, we introduced a heterologous xylose transporter into *C. tropicalis* to enhance the xylose uptake during fermentation using glucose/xylose mixtures. A xylose transporter from *A. thaliana* was selected because of its high affinity for xylose [17], and functional expression of this transporter in *C. tropicalis* was achieved by codon optimization. The codon-optimized xylose transporter was expressed in the NXRG-expressing *C. tropicalis* strain LXU1 using a GAPDH promoter. Xylose uptake analysis and fermentation experiments were performed using a recombinant *C. tropicalis* strain (LXT2) that expressed the constitutive xylose transporter, with glucose as co-substrate. The xylose uptake rate and volumetric productivity of xylitol were compared with those of parental LXU1.

Materials and methods

Strains and media

The *C. tropicalis* strains used in this study are listed in Table 1. Strain LX, a uracil auxotroph derived from strain LXU1, was used as a host strain for transformation [7]. Genomic DNA of *C. tropicalis* ATCC 20336 was used as a source of the marker gene *URA3*. The media used for genetic manipulation of *C. tropicalis* were YM (3 g/L yeast extract, 3 g/L malt extract, 5 g/L Bacto Peptone, 20 g/L glucose), YNB (6.7 g/L yeast nitrogen base without amino acids, 20 g/L glucose), and YNB-5FOA (6.7 g/L yeast nitrogen base without amino acids, 20 g/L glucose, 0.1 g/L uracil, 0.1 g/L uridine, 0.8 g/L 5-fluoroorotic acid). *Escherichia coli* DH5α (RBC Bioscience, Taiwan) was used as a host strain for amplification and construction of plasmids and was cultured in Luria–Bertani (LB) medium.

Codon-optimization of heterologous xylose transporter gene

Codon-optimization of the heterologous xylose transporter gene *At5g17010* of *A. thaliana* was performed for

Table 1 *Candida tropicalis* strains used in this study

Strain	Genotype
LXU1	*ura3/ura3 xyl2Δ::PNAUA/xyl2Δ::hisG*
LX	*ura3/ura3 xyl2Δ::PNA/xyl2Δ::hisG*
LXT2	*ura3/ura3 xyl2Δ::PNA/xyl2Δ:: PT2U*
LXT2-V5[a]	*ura3/ura3 xyl2Δ::PNA/xyl2Δ:: PT2U*

PNAUA, P_{GAPDH}-NXRG-T_{GAPDH}-*ARG-URA3-ARG*; *PNA*, P_{GAPDH}-NXRG-T_{GAPDH}-*ARG*; *PT2U*, P_{GAPDH}-CoXT2-T_{GAPDH}-*URA3*

[a] The LXT2-V5 strain has CoXT2 with V5 epitope

Table 2 Primers used in this study

Primer	Sequence[a]	Restriction site(s)
Ura3F	AGATCTGATCTGGTTTGGATTGTTGGAGA	*Bgl*II
Ura3R	GGATCCGATCTTGAAGTCCTCGTTTGTG	*Bam*HI
His80-F2	GTT**AACCCTGCGCCGTCTTGACTTCGGCGGCTGCCGTTTATCGCTGGCAACA** **CCGGTTGACGAAGCCTGGGACGGCCCGGCCG**GGATCCCAAAGCATCAGCTCATCGAG	*Hpa*I, *Bam*HI
His80-R2	GTT**AACTTCGACTTCACGCAGGCCGTTAGCTTCAAGCGTCGCGCCGGTAGAGACCAA** **ATCGCAGATAGCGTCGGCCAGCCCCG**GGATCCAAAAGTTCGACAGCGTCTCC	*Hpa*I, *Bam*HI
HIS-F2	AACGTGCTGGAAGAAGAGCTACTCAA	
TgapR	GGATCCTCTGGTTTAGAAGTAGGGACTGTATG	*Bam*HI
CoXT2-F	TCTAGAACAAACATGGCCTTGGAC	*Xba*I
CoXT2-R-V5	CTCGAGCTTAATGGTGATGGTGATGATGACCGGTACG*CGTAGAATCGAGACCGAGGAG* *AGGGTTAGGGATAGGCTTACC*CAAACACTTAGCTTCGATTTCTTCCA	*Xho*I

[a] The restriction sites introduced into the primers are underlined. Boldface letters indicate homologous sequences for integration into *C. tropicalis*. Italic letters indicate V5 epitope sequence for recognition by anti-V5 antibody

functional expression in *C. tropicalis*. The amino acid sequences of heterologous transporter genes were back-translated using the preferred codons in *C. tropicalis* and the codon usage database based on NCBI-GenBank data (http://www.kazusa.or.jp/codon) (GENEART, Germany).

Construction of codon-optimized xylose transporter expression cassettes

A 0.5-kb hph gene fragment was amplified by PCR from pREP4 (Invitrogen, CA, USA) using the synthetic primers His80-F2 and His80-R2; each primer contained both the 80-bp *hisG*-homologous region and a *Bam*HI site. The PCR product was inserted into the pGEM-T Easy vector (Promega, WI, USA), which contained two homologous regions (Hisf2F and Hisf2R) for integration into the genome of *C. tropicalis* LX. The resulting plasmid was termed pGEM-Hisf2.

The *URA3* gene was amplified by PCR with genomic DNA of strain 20336 using primers Ura3F and Ura3R, which were designed using the *URA3* sequence (GenBank accession number AB006207). The *URA3* gene was inserted into the pGEM-T Easy vector, and the resulting plasmid was termed pGEM-URA3.

The GAPDH promoter and terminator [18] fragments were inserted into the *Bgl*II site of pGEM-URA3. The resulting plasmid contained the GAPDH promoter–terminator–URA3 cassette, which was then inserted into pGEM-Hisf2. pPTUHisf2 containing the Hisf2F–promoter–terminator–URA3–Hisf2R cassette was, thus, constructed.

The codon-optimized xylose transporter gene (CoXT2) was inserted into pPTUHisf2 between the *Xba*I and *Xho*I sites, and the resulting plasmid was termed pPTUHisf2-CoXT2. The constructed vector was digested with *Hpa*I to prepare an integrated expression cassette consisting of Hisf2F–promoter–CoXT2–terminator–URA3–Hisf2R.

Yeast transformation

Candida tropicalis was transformed using the lithium acetate (LiOAc) method with some modification [7]. Cells grown in YM medium were washed and resuspended in LiOAc solution (0.1 M LiOAc, 10 mM Tris–HCl, pH 8.0, 1 mM EDTA, pH 8.0). A mixture of 30 μL cell suspension, 30 μL transforming DNA, 5 μL salmon testis DNA (Sigma, MO, USA), and 325 μL polyethylene glycol 8000 solution (50 % polyethylene glycol 8000 in LiOAc solution) was incubated at 30 °C for 45 min. The cells were subjected to heat shock treatment at 42 °C for 15 min, washed with YNB medium, and spread onto YNB-coated plates. The cells were incubated for 2 days, and transformants were selected. Genetic modifications were confirmed by PCR with specific primers. The sequences of all primers used are listed in Table 2.

Analysis of xylose uptake

Cells of control and recombinant strains were grown in YM medium for 15 h at 30 °C until glucose was exhausted. Equal amounts of cell were harvested and transferred to incubation media consisting of mixture ratios of glucose (50 g/L) and xylose (50 g/L) of 1:1, 1:2, and 0:1. Xylose uptake was analyzed after incubation of cells in a 250-mL Erlenmeyer flask with 50 mL of incubation medium at 30 °C. Samples were collected at 30, 60, 90, and 120 min and xylose concentration was determined by high-performance liquid chromatography (HPLC).

Fermentation experiments and analytical methods

The fermentation medium contained 50 g/L D-xylose, 20 g/L D-glucose, 10 g/L yeast extract, 5 g/L KH_2PO_4, and

0.2 g/L $MgSO_4 \cdot 7H_2O$. Flask culture for xylitol production was performed in a 500-mL Erlenmeyer flask with 100 mL of fermentation medium in a shaking incubator, 30 °C, 200 rpm. After depletion of initial glucose, 2.5 g/L glucose was added at 14, 20, 26, and 32 h to ensure a continuous supply of NADPH.

The concentrations of D-glucose, D-xylose, and xylitol were analyzed by HPLC with a Sugar-Pak I column (6.5 × 300 mm, Waters, MA, USA) and a refractive-index detector (Waters). Degassed chromatography-grade water (Merck, Germany) was used as the mobile phase at a column temperature of 90 °C and a flow rate of 0.5 mL/min. Cell growth was determined spectrophotometrically at wavelength of 600 nm. One A_{600} unit was equivalent to 0.474 g dry cell weight/L.

Western blot analysis and membrane protein extraction

Heterologous xylose transporter expression was confirmed by Western blot analysis based on specific interaction between V5 epitope and anti-V5 antibody (Invitrogen). PCR was performed following the addition of V5 epitope to the C-terminal of CoXT2 using a specific reverse primer containing the V5 epitope sequence. CoXT2 tagged by V5 epitope was termed CoXT2-V5.

To determine whether engineered *C. tropicalis* was capable of expressing the heterologous xylose transporter, the recombinant strain was cultured with the control strain LXU1 for 12 h and cells were harvested by centrifugation at 12,000×*g* for 5 min. The pellet was washed with 50 mM potassium phosphate buffer (pH 7.0), suspended in the same buffer, and sonicated. The resulting crude cellular extract was collected by centrifugation at 12,000×*g* for 5 min and subjected to SDS-PAGE and Western blot analysis. Equal amounts of protein were loaded in each lane and resolved on SDS, 12.5 % polyacrylamide gels (Invitrogen). For the estimation of total protein content, proteins were visualized by Coomassie Brilliant Blue staining. For Western blot analysis, proteins were transferred to a PVDF membrane (Millipore, MA, USA). The membrane was incubated with a 1:5,000 dilution of anti-V5 antibody in T-TBS(+) overnight in a cold room, rinsed three times with T-TBS(+), incubated with 1:5,000 diluted goat anti-mouse IgG-HRP (Santa Cruz Biotechnology, CA, USA) for 1 h at room temperature, rinsed three times with T-TBS, and developed using a Supersignal West Pico Chemiluminescence substrate kit (Pierce, IL, USA).

Yeast membrane proteins were isolated using a Mem-PER Eukaryotic Membrane Protein Extraction Reagent Kit (Pierce) according to the manufacturer's instructions. The extracted samples were resolved on 12.5 % polyacrylamide gels and subjected to Western blot analysis using anti-V5 antibody as described above.

Results

Codon-optimization of the *A. thaliana* xylose transporter gene

The codon usage database indicates that the codon usage of *C. tropicalis* differs from that of *A. thaliana*; i.e., some amino acid residues of the *A. thaliana* xylose transporter are encoded by codons that are rarely used in *C. tropicalis* (Table 3). The CTG codon, which typically codes for leucine, is translated as serine in *C. tropicalis* [19]. For heterologous expression of the *A. thaliana* xylose transporter, the amino acid sequence (GenBank accession number AAT85724) was back-translated using the preferred codons in *C. tropicalis*. The codons that encode phenylalanine, leucine, serine, and histidine displayed dramatic changes. The codon-optimized xylose transporter gene was synthesized and termed CoXT2.

Construction of a CoXT2-expressing strain of *C. tropicalis*

We previously constructed a *C. tropicalis* strain (LNG2) that harbored two copies of NXRG [18]. However, it was not possible to delete the selection marker gene *URA3* in strain LNG2. To introduce a CoXT2 expression cassette, we constructed a new NXRG expression cassette that was capable of removing the *URA3* gene. This expression cassette was integrated into the first *hisG* fragment region in the genome of *C. tropicalis*, and the resulting strain was termed LXU1. LXU1 was incubated on YNB-5FOA plates to delete the *URA3* marker gene, and the resulting uracil auxotroph strain was termed LX.

Candida tropicalis LX, a uracil auxotroph derived from LXU1, was used as the host strain for transformation. The expression cassette, Hisf2F–promoter–CoXT2–terminator–URA3–Hisf2R, was integrated into the second *hisG* fragment region in the genome of *C. tropicalis* LX by homologous recombination (Fig. 1a). To confirm the site-specific insertion of the cassette, PCR was performed using specific primers. A particular transformant that harbored the CoXT2 expression cassette was termed LXT2 (Fig. 1b).

Xylose uptake in the CoXT2-expressing strain

For the analysis of xylose uptake, batch culture was performed in a 250-mL Erlenmeyer flask containing 50 mL of a glucose/xylose mixture at 30 °C. The mixture ratios of glucose and xylose were 1:1, 1:2, and 0:1. The xylose uptake rate of *C. tropicalis* LXT2 was 3.20 g/L/h in the 0:1 (xylose-only) medium. This value was 37 % higher than that for control strain LXU1 (Fig. 2c). For the 1:1 and 1:2

Table 3 Comparison of the codon usage between wild-type *A. thaliana At5g17010* (wt) and synthetic *At5g17010* (syn)

AA	Codon	Fraction		No. of codons		AA	Codon	Fraction		No. of codons	
		At	Ct	wt	syn			At	Ct	wt	syn
F	TTT	0.51	0.48	19	0	Y	TAT	0.52	0.39	10	2
	TTC	0.49	0.52	7	26		TAC	0.48	0.61	8	16
L	TTA	0.14	0.19	8	0	Stop	TAA	0.36	0.45	1	1
	TTG	0.22	0.65	12	75		TAG	0.20	0.48	0	0
	CTT	0.26	0.08	20	0		TGA	0.44	0.06	0	0
	CTC	0.17	0.05	10	0	H	CAT	0.61	0.39	3	0
	CTA	0.11	0.01	10	0		CAC	0.39	0.61	0	3
	CTG	0.11	0.02	15	0	Q	CAA	0.56	0.75	5	14
I	ATT	0.41	0.52	19	23		CAG	0.44	0.25	9	0
	ATC	0.35	0.42	7	15	N	AAT	0.52	0.36	5	1
	ATA	0.24	0.07	12	0		AAC	0.48	0.64	4	8
M	ATG	1.00	1.00	10	10	K	AAA	0.49	0.42	7	5
V	GTT	0.40	0.52	21	41		AAG	0.51	0.58	7	9
	GTC	0.19	0.33	10	9	D	GAT	0.68	0.51	5	6
	GTA	0.15	0.04	7	0		GAC	0.32	0.49	2	1
	GTG	0.26	0.11	12	0	E	GAA	0.52	0.79	11	25
S	TCT	0.28	0.28	8	1		GAG	0.48	0.21	14	0
	TCC	0.13	0.29	5	38	C	TGT	0.60	0.82	5	8
	TCA	0.20	0.15	18	2		TGC	0.40	0.18	3	0
	TCG	0.10	0.08	1	0	W	TGG	1.00	1.00	6	6
	AGT	0.16	0.12	7	0	R	CGT	0.17	0.1	1	0
	AGC	0.13	0.07	2	0		CGC	0.07	0.02	1	0
P	CCT	0.38	0.18	7	0		CGA	0.12	0.02	3	0
	CCC	0.11	0.05	2	0		CGG	0.09	0.01	1	0
	CCA	0.33	0.73	12	24		AGA	0.35	0.79	7	17
	CCG	0.18	0.04	3	0		AGG	0.20	0.05	4	0
T	ACT	0.34	0.41	7	2	G	GGT	0.34	0.7	22	56
	ACC	0.20	0.42	4	16		GGC	0.14	0.14	7	0
	ACA	0.31	0.12	7	0		GGA	0.37	0.11	18	0
	ACG	0.15	0.06	0	0		GGG	0.16	0.05	9	0
A	GCT	0.43	0.52	16	41						
	GCC	0.16	0.33	7	3						
	GCA	0.27	0.12	18	0						
	GCG	0.14	0.04	3	0		Total			504	504

The codon usage tables for *A. thaliana* (At) and *C. tropicalis* (Ct) were based on a codon usage database (http://www.kazusa.or.jp/codon)

media, the xylose uptake rates of LXT2 were 1.23 and 1.60 g/L/h, which were, respectively, 74 and 70 % higher than the corresponding rates for LXU1 (Fig. 2a, b).

Xylitol production by the CoXT2-expressing strain

For the analysis of xylitol production, flask culture was performed in a 500-mL Erlenmeyer flask containing 100 mL fermentation medium with glucose as a co-substrate for cell growth and co-factor (NADPH) regeneration. Following depletion of initial glucose, 2.5 g/L glucose was added at 14, 20, 26, and 32 h to ensure a continuous supply of NADPH. The xylose uptake rate of LXT2 was 29 % higher than that of LXU1 during 12–42 h (Fig. 3). The xylitol volumetric productivity of LXT2 was 1.14 g/L/h, which was 25 % higher than the rate for LXU1 (Fig. 3).

Expression and localization of the heterologous xylose transporter in *C. tropicalis*

Western blot analysis was performed to confirm the successful expression of the heterologous xylose transporter in *C. tropicalis*. To confirm expression of the protein, anti-V5 antibody was used to detect the C-terminal V5 epitope in

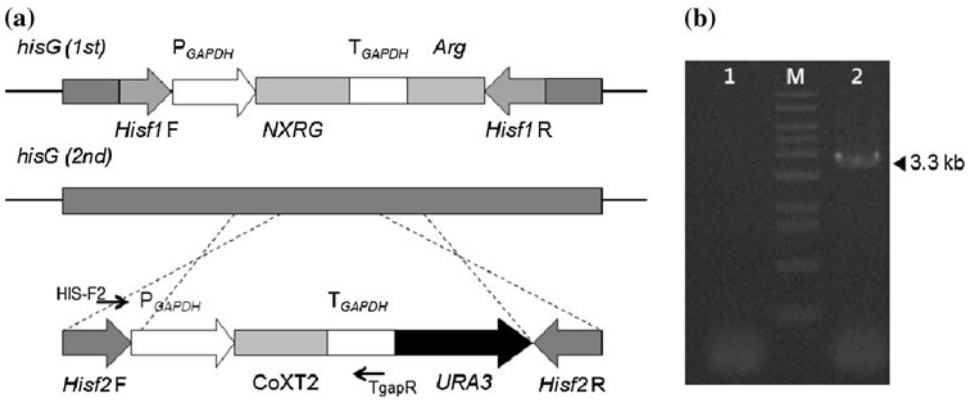


Fig. 1 Construction of the CoXT2-expressing strain LXT2. **a** Physical map of the expression cassette and integration of the cassette into the second *hisG* region of *C. tropicalis* LX. **b** PCR confirmation of the specific integration of the CoXT2 expression cassette. *Lanes 1* and *2* represent PCR with primers HIS-F2 and TgapR for the amplification of a 3.3-kb product. *Lane 1* host strain LX. *Lane 2* LXT2. *Lane M* DNA size marker

the transporter. Total protein isolated from an equal quantity of cells was loaded for strain LXT2-V5 and the negative control strain. The transporter protein (CoXT2) encoded by the codon-optimized *At5g17010* was predicted to have a molecular mass of 53.4 kDa. Western blot analysis showed that a protein of this size was expressed in *C. tropicalis* LXT2 (Fig. 4a).

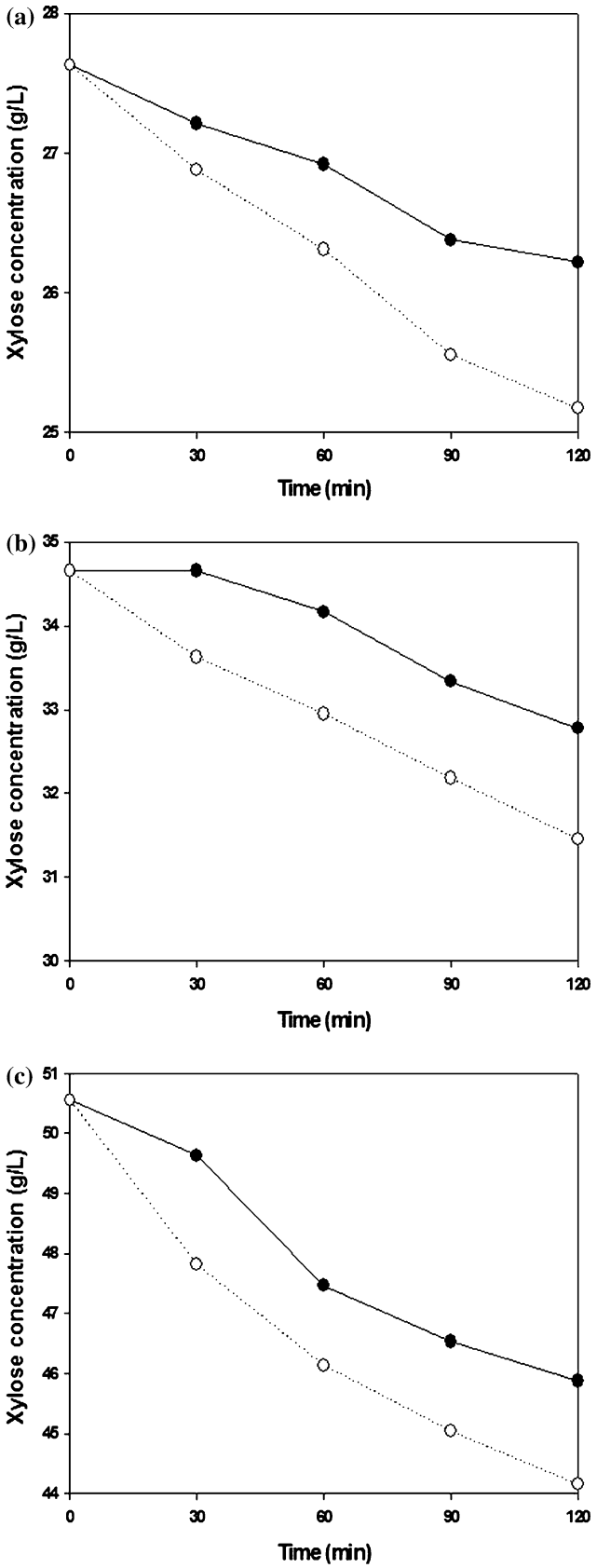
A Mem-PER Eukaryotic Membrane Protein Extraction Reagent Kit was used to confirm that the transporter was localized to the plasma membrane. Yeast proteins from *C. tropicalis* LXU1 and LXT2-V5 were solubilized and partitioned based on hydrophobic phase separation. Hydrophilic and hydrophobic (membrane protein) fractions were analyzed by Western blotting with anti-V5 antibody and HRP-labeled secondary antibody. A band of the correct size was observed in the lane that contained the hydrophobic fraction with CoXT2-V5 (Fig. 4b), indicating that the *A. thaliana* transporter encoded by CoXT2 was successfully expressed and localized in the plasma membrane in *C. tropicalis*.

Discussion

Many studies have addressed the use of xylose-assimilating yeasts such as *C. tropicalis* and *C. parapsilosis* as alternative methods for the biological production of xylitol [5, 6]. In some studies, metabolic engineering has been applied for the improvement of xylitol production. The *xyl1* gene in *C. parapsilosis* was placed under the control of an alcohol dehydrogenase promoter and integrated into the genome of *C. tropicalis*. The resulting recombinant strain of *C. tropicalis* showed higher yield and productivity than did the wild strain [20]. The *XYL2* gene encoding XDH was disrupted in *C. tropicalis* strain ATCC 20913. The resulting strain BSXDH-3 produced xylitol with a yield of 97 % under fully aerobic conditions [21]. Glycerol was determined as the best co-substrate for cell maintenance and NADPH regeneration by screening of various carbon sources [7]. In terms of economic feasibility, however, glucose must be considered as a desirable co-substrate, because it can be used directly from industrial biomass hydrolysates.

In our previous study, glucose suppression was overcome by the expression of the codon-optimized XR of *Neurospora crassa* (termed NXRG) with the GAPDH promoter in *C. tropicalis* [18]. The resulting strain showed a xylitol production rate higher than that of the control strain; however, the glucose uptake suppressed xylose uptake and xylitol production during the initial fermentation period. Such suppression has been observed in not only our *C. tropicalis* strain but also other xylose-assimilating strains of *S. cerevisiae* and *P. stipitis* [13, 15]. In addition, according to a recent study, pentose utilization was impeded by the simultaneous catabolism of hexose sugars as well as the inhibitory effects of glucose [22]. No previous studies have reported the improvement of xylitol production by enhancing xylose uptake in *C. tropicalis*.

In the present study, a heterologous xylose transporter was introduced into *C. tropicalis* for the enhancement of xylose uptake during fermentation with various glucose/xylose mixture ratios. The *A. thaliana* xylose transporter encoded by the gene *At5g17010* was selected because of its high affinity for xylose [17]. Codon-optimization was performed for functional expression of the transporter in *C. tropicalis*. The codon-optimized transporter gene (CoXT2) was introduced into NXRG-expressing *C. tropicalis* strain LXU1 with the GAPDH promoter. Both the resulting strain, LXT2 and LXU1 showed decreases in xylose uptake with the increase of glucose ratio. These data



◀**Fig. 2** Xylose uptake in *C. tropicalis* strains LXU1 and LXT2. **a** Glucose/xylose ratio 1:1. **b** Glucose/xylose ratio 1:2. **c** Glucose/xylose ratio 0:1 (xylose-only). *Filled circles* denote LXU1. *Open circles* denote LXT2

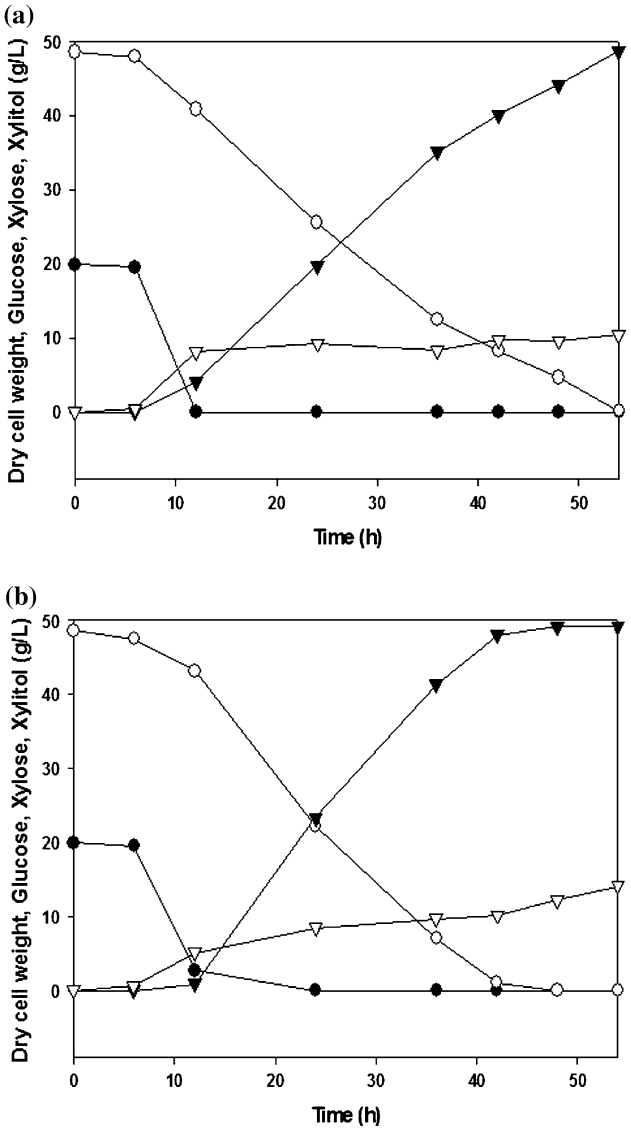


Fig. 3 Xylitol fermentation profiles of *C. tropicalis* strains LXU1 (**a**) and LXT2 (**b**). *Open triangles* denote dry cell weight. *Filled circles* denote glucose. *Open circles* xylose. *Filled triangles* denote xylitol

suggest that the xylose uptake is inhibited by the presence of glucose. However, LXT2 showed increases in xylose uptake that ranged from 37 to 73 % depending on the mixture ratios. Expression of the transporter enhanced the xylose uptake in not only xylose medium but also glucose/xylose mixtures. In 500-mL flask culture, the xylose uptake of LXT2 was 29 % higher than that of LXU1.

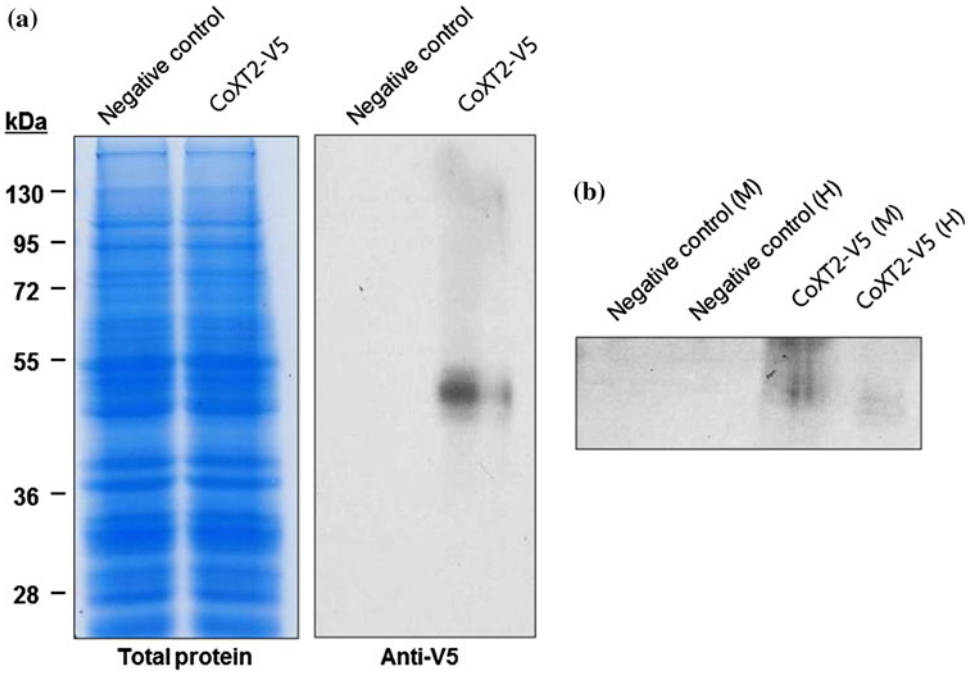


Fig. 4 Western blot and localization analysis of the *A. thaliana* xylose transporter expressed in *C. tropicalis*. **a** Coomassie Brilliant Blue-stained gel and Western blotting using anti-V5 antibody. Total protein isolated from an equal quantity of cells was loaded for strains LXU1 (negative control) and LXT2-V5. The transporter protein encoded by codon-optimized *At5g17010*, CoXT2, was predicted to have molecular mass 53.4 kDa. Western blot analysis showed that a protein of the correct size was expressed in LXT2. **b** Membrane protein extraction and Western blot analysis of the *A. thaliana* xylose transporter encoded by CoXT2. A Mem-PER Eukaryotic Membrane Protein Extraction Reagent Kit (Pierce) was used. The observation of a band at the correct size (53.4 kDa) indicates that the *A. thaliana* xylose transporter is localized to the plasma membrane of LXT2. CoXT2-V5 (M): hydrophobic fraction, solubilized membrane proteins. CoXT2-V5 (H): hydrophilic fraction

The volumetric productivity of LXT2 (1.14 g/L/h) in glucose and xylose mixtures was 25 % higher than that of LXU1. Both strains exhibited the same xylitol yield, because their xylitol dehydrogenase genes were deleted. Another *A. thaliana* xylose transporter encoded by *At5g59250* was also introduced into *C. tropicalis*. However, the resulting recombinant strain did not show significant enhancement of xylose uptake or xylitol production (data not shown). The successful heterologous expression and plasma membrane localization of the *A. thaliana* xylose transporter in *C. tropicalis* were confirmed by membrane protein extraction and Western blot analysis.

Our initial expectation was that xylose uptake would be enhanced during the initial fermentation period in glucose/xylose mixtures. However, xylitol production was slower in LXT2 than in LXU1 during the initial period. LXT2 did not consume glucose exhaustively and showed the lowest dry cell weight up to 12 h (Fig. 3b). These findings suggest that the expression of the heterologous transporter gene may have inhibited cell growth and xylitol production during the initial period. In contrast, LXT2 displayed a higher xylose uptake rate during the period after 12 h and a xylitol production rate of 1.57 g/L/h from 12 to 42 h, which was 30 % higher than the rate for LXU1 (Fig. 3b).

Previous studies of enhanced xylitol production using metabolic engineering focused mainly on XR [18, 20]. In contrast, we introduced a heterologous xylose transporter into *C. tropicalis* in the present study and observed significant enhancement of xylose uptake and xylitol production.

Acknowledgments This study was supported by a National Research Foundation of Korea (NRF) grant funded by the Korean government (MEST) (2011-0016840). We thank Dr. S. Anderson for English editing of the manuscript.

References

1. Picataggio S, Rohrer T, Deanda K, Lanning D, Reynolds R, Mielenz J, Eirich LD (1992) Metabolic engineering of *Candida tropicalis* for the production of long-chain dicarboxylic acids. Biotechnology 10:894–898
2. Kim SY, Kim JH, Oh DK (1998) Effect of redox potential on xylitol production by *Candida tropicalis*. Food Sci Biotechnol 7:282–285
3. Emodi A (1978) Xylitol: its properties and food application. Food Technol 32:20–32
4. Werpy T, Petersen G, Aden A, Bozel J, Holladay J, White J, Manhein A, Elliot D, Lasure L, Jones S, Gerber M, Ibsen K, Lumberg L, Kelly S (2004) Top value added chemicals from biomass. US DOE Biomass Program

5. Kim JH, Han KC, Koh YH, Ryu YW, Seo JH (2002) Optimization of fed-batch fermentation for xylitol production by *Candida tropicalis*. J Ind Microbiol Biotechnol 29:16–19
6. Oh DK, Kim SY, Kim JH (1998) Increase of xylitol production rate by controlling redox potential in *Candida parapsilosis*. Biotechnol Bioeng 58:440–444
7. Ko BS, Kim J, Kim JH (2006) Production of xylitol from d-xylose by a xylitol dehydrogenase gene-disrupted mutant of *Candida tropicalis*. Appl Environ Microbiol 72:4207–4213
8. Hallborn J, Walfridsson M, Airaksinen U, Ojamo H, Hahn-Hagerdal B, Penttila M, Kerasnen S (1991) Xylitol production by recombinant *Saccharomyces cerevisiae*. Biotechnology 9: 1090–1095
9. Kim YS, Kim SY, Kim JH, Kim SC (1999) Xylitol production using recombinant *Saccharomyces cerevisiae* containing multiple xylose reductase genes at chromosomal delta-sequences. J Biotechnol 67:159–171
10. Ghindea R, Csutak O, Stoica I, Tanase A-M, Vassu T (2010) Production of xylitol by yeasts. Roum Biotechnol Lett 15:5217–5222
11. Kruckeberg AL (1996) The hexose transporter family of *Saccharomyces cerevisiae*. Arch Microbiol 166:283–292
12. Boles E, Hollenberg CP (1997) The molecular genetics of hexose transport in yeasts. FEMS Microbiol Rev 21:85–111
13. Lee WJ, Kim MD, Ryu YW, Bisson LF, Seo JH (2002) Kinetic studies on glucose and xylose transport in *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 60:186–191
14. Weierstall T, Hollenberg CP, Boles E (1999) Cloning and characterization of three genes (SUT1-3) encoding glucose transporters of the yeast *Pichia stipitis*. Mol Microbiol 31:871–883
15. Agbogbo FK, Coward-Kelly G, Torry-Smith M, Wenger KS (2006) Fermentation of glucose/xylose mixtures using *Pichia stipitis*. Process Biochem 41:2333–2336
16. Katahira S, Ito M, Takema H, Fugita Y, Tasino T, Tanaka T, Fukuda H, Kondo A (2008) Improvement of ethanol productivity during xylose and glucose co-fermentation by xylose-assimilating *S. cerevisiae* via expression of glucose transporter Sut1. Enzyme Microb Technol 43:115–119
17. Ronald EH, Nasib Q, Stephen RH, Michael AC (2008) Expression of a heterologous xylose transporter in a *Saccharomyces cerevisiae* strain engineered to utilize xylose improves aerobic xylose consumption. Appl Microbiol Biotechnol 80:675–684
18. Jeon WY, Yoon BH, Ko BS, Shim WY, Kim JH (2012) Xylitol production is increased by expression of codon-optimized *Neurospora crassa* xylose reductase gene in *Candida tropicalis*. Bioprocess Biosyst Eng 35:191–198
19. Ohama T, Suzuki T, Mori M, Osawa S, Ueda T, Watanabe K, Nakase T (1993) Non-universal decoding of the leucine codon CUG in several *Candida* species. Nucleic Acids Res 21:4039–4045
20. Lee JK, Koo BS, Kim SY (2003) Cloning and characterization of the xyl1 gene, encoding an NADH-preferring xylose reductase from *Candida parapsilosis*, and its functional expression in *Candida tropicalis*. Appl Environ Microbiol 69:6179–6188
21. Ko BS, Rhee CH, Kim JH (2006) Enhancement of xylitol productivity and yield using a xylitol dehydrogenase gene-disrupted mutant of *Candida tropicalis* under fully aerobic conditions. Biotechnol Lett 28:1159–1162
22. Subtil T, Boles E (2012) Competition between pentoses and glucose during uptake and catabolism in recombinant *Saccharomyces cerevisiae*. Biotechnol Biofuels 5:14