

Xylitol production is increased by expression of codon-optimized *Neurospora crassa* xylose reductase gene in *Candida tropicalis*

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Abstract Xylose reductase (XR) is the first enzyme in D-xylose metabolism, catalyzing the reduction of D-xylose to xylitol. Formation of XR in the yeast *Candida tropicalis* is significantly repressed in cells grown on medium that contains glucose as carbon and energy source, because of the repressive effect of glucose. This is one reason why glucose is not a suitable co-substrate for cell growth in industrial xylitol production. XR from the ascomycete *Neurospora crassa* (NcXR) has high catalytic efficiency; however, NcXR is not expressed in *C. tropicalis* because of difference in codon usage between the two species. In this study, NcXR codons were changed to those preferred in *C. tropicalis*. This codon-optimized NcXR gene (termed NXRG) was placed under control of a constitutive glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter derived from *C. tropicalis*, and integrated into the genome of xylitol dehydrogenase gene (*XYL2*)-disrupted *C. tropicalis*. High expression level of NXRG was confirmed by determining XR activity in cells grown on glucose medium. The resulting recombinant strain, LNG2, showed high XR activity (2.86 U (mg of protein)⁻¹), whereas parent strain BSXDH-3 showed no activity. In xylitol fermentation using glucose as a co-substrate with

xylose, LNG2 showed xylitol production rate 1.44 g L⁻¹ h⁻¹ and xylitol yield of 96% at 44 h, which were 73 and 62%, respectively, higher than corresponding values for BSXDH-3 (rate 0.83 g L⁻¹ h⁻¹; yield 59%).

Keywords Xylitol · *Candida tropicalis* · Xylose reductase · Glucose repression · Codon optimization

Introduction

Candida tropicalis, a diploid asporogenic yeast, is frequently utilized in industrial applications and research studies. Because of its ability to use *n*-alkanes and fatty acids as carbon sources, *C. tropicalis* can be used for production of long-chain dicarboxylic acids, and raw materials for preparation of polymers, adhesives, and perfumes, through metabolic engineering of peroxisomal enzymes [1, 2]. This species has potent xylose-assimilating ability and is useful for xylitol production [3, 4]. The universal leucine codon, CTG, is translated as serine in several *Candida* species, including *C. tropicalis* [5, 6].

Xylitol, a five-carbon sugar alcohol, is used as an alternative to sucrose, fructose, and various artificial sweeteners in the food and confectionary industries. It is roughly as sweet as sucrose, and can replace it on an equal-weight basis. Since xylitol does not require insulin for its metabolic regulation, it is useful as a sucrose substitute for diabetics [7]. The chemical method generally used for production of xylitol is hydrogenation of xylose using a Raney nickel catalyst. However, this method presents environmental and safety concerns because it requires high-pressure hydrogen gas, and a toxic catalyst.

Many studies have addressed safer biological methods of xylitol production using xylose-assimilating yeasts such

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as *C. tropicalis* and *C. parapsilosis* [4, 8], or metabolically engineered strains of *Saccharomyces cerevisiae* containing *XYL1* gene from *Pichia stipitis* [9, 10].

Xylose reductase (XR) catalyzes the first step in D-xylose metabolism, *i.e.*, reduction of D-xylose to xylitol accompanied by oxidation of NAD(P)H. XR (EC 1.1.1.21), a member of the aldose reductase (ALR) family and the aldo–keto reductase (AKR) superfamily, is found in most yeasts and filamentous fungi. XR genes have been cloned and characterized from various species, including *C. guilliermondii* [11], *C. parapsilosis* [12], *C. tenuis* [13], *Pichia stipitis* [14], and *Neurospora crassa* [15]. Catalytic activity and potential for the enzymatic production of xylitol are higher for XR of *N. crassa* than those of other species studied [15]. There is great interest in improving the efficiency of metabolic utilization of XR, since xylose, a major pentose sugar found in hemicelluloses, is the second most abundant naturally-occurring sugar.

In most xylose-assimilating yeasts, including *C. tropicalis*, D-xylose is the substrate for two successive enzymatic oxidoreductive reactions. XR catalyzes reduction of D-xylose to xylitol, while xylitol dehydrogenase (XDH) catalyzes oxidation of xylitol to D-xylulose, which is then converted into D-xylulose 5-phosphate by xylulose kinase, and enters the pentose phosphate pathway. Redox imbalance has been applied for xylitol production, using oxygen limitation to decrease the XDH reaction [16].

In a previous study, we achieved high-yield xylitol production under fully aerobic conditions in *C. tropicalis* by disrupting the *XYL2* gene encoding XDH [17]. Xylose was converted to xylitol with a theoretical maximum yield close to 100%, and glycerol was used as a co-substrate for cell growth and NADPH regeneration [18]. Glucose might seem more desirable than glycerol as a co-substrate for xylitol production, because glucose and xylose are the primary products of industrial biomass hydrolysis. However, glucose is not an adequate co-substrate, because it has a repressive effect on XR synthesis. When presented with a mixture of xylose and glucose, *C. tropicalis* uses glucose preferentially by repressing synthesis of xylose-assimilating enzymes, particularly XR [19]. Therefore, enhanced xylitol production requires high XR activity in the presence of glucose.

In the present study, we overcame the repressive effect of glucose on XR by constitutive expression of heterologous XR. XR from *N. crassa* (termed NcXR) was selected for high catalytic efficiency [15], and codon-optimization of NcXR was performed to achieve functional expression of preferred codons in *C. tropicalis*. A novel glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter region was isolated from *C. tropicalis* for constitutive XR expression in the presence of glucose. The codon-optimized NcXR gene was expressed in the *XYL2*-disrupted *C. tropicalis* mutant (BSXDH-3) using the GAPDH promoter. Fed-batch

fermentation was performed with the constitutive XR-expressing recombinant *C. tropicalis* LNG2, using glucose as co-substrate, and xylitol production rate and yield were compared with those of parental BSXDH-3.

Materials and methods

Strains and media

The five *C. tropicalis* strains used in this study are listed in Table 1. Strain L10 (*ura3/ura3*), a uracil auxotroph derived from strain BSXDH-3, was used as host strain for transformation [18]. Genomic DNA of strain ATCC 20336 was used as source of *URA3* marker gene. For genetic manipulation of *C. tropicalis*, we used YM medium (3 g/L yeast extract, 3 g/L malt extract, 5 g/L Bacto Peptone, 20 g/L glucose), YNB medium (6.7 g/L yeast nitrogen base without amino acids, 20 g/L glucose), and YNB-5FOA medium (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). Plasmids were amplified in *Escherichia coli* DH5 α (RBC Bioscience, Taiwan), which was cultured in Luria–Bertani (LB) medium. Glucose medium (20 g/L D-glucose, 10 g/L yeast extract, 5 g/L KH₂PO₄, 0.2 g/L MgSO₄·7H₂O) was used to culture *C. tropicalis* for XR activity assay.

Construction of codon-optimized NcXR expression cassettes, and yeast transformation

A 0.5-kb hph gene fragment was amplified by PCR from pREP4 (Invitrogen, CA) using the synthetic primers His80-F1 and His80-R1, each of which contained the 80-bp *hisG*-homologous region and a *Bam*HI site. The PCR product was inserted into pGEM-T Easy Vector (Promega, WI), which contained two homologous regions (HisF and HisR) for integration into the genome of *C. tropicalis* L10. The resulting plasmid was termed pGEM-HisF1.

URA3 gene was amplified by PCR with genomic DNA of strain ATCC 20336 using primers Ura3F and Ura3R, which were designed from the *URA3* sequence (GenBank

Table 1 *Candida tropicalis* strains used in this study

Strain	Genotype
ATCC 20336	<i>URA3/URA3 XYL2/XYL2</i>
ATCC 20913	<i>ura3/ura3 XYL2/XYL2</i>
BSXDH-3	<i>ura3/ura3 xyl2Δ::hisG/xyl2Δ::URA3</i>
L10	<i>ura3/ura3 xyl2Δ::hisG/xyl2Δ::hisG</i>
LNG2	<i>ura3/ura3 xyl2Δ::NUN/xyl2Δ::hisG</i>

NUN, P_{GAPDH}-N_{XRG}-T_{GAPDH}-*URA3*-P_{GAPDH}-N_{XRG}-T_{GAPDH}

accession number AB006207). *URA3* was inserted into pGEM-T Easy Vector, and the resulting plasmid was termed pGEM-*URA3*.

The draft sequence of the promoter-GAPDH-terminator was amplified by PCR with genomic DNA of strain ATCC 20336 using primers GapF and GapR, which were designed from the conserved sequences of the 5'- and 3'-flanking genes based on the *C. tropicalis* MYA-3404 database (Broad Institute, MA). Two primer sets, PgapF-PgapR and TgapF-TgapR, were designed, based on the draft sequence, and were used to amplify the 1,455-bp promoter and 308-bp terminator regions of GAPDH, respectively, using PCR. These sequence data have been submitted to the GenBank database under accession number HQ171163.

The promoter and terminator fragments were inserted into the *Bgl*II site of pGEM-*URA3*. The resulting plasmid contained the promoter-terminator-*URA3* cassette, which was then inserted into pGEM-Hisf1. Finally, pCGUHisf containing the HisR-promoter-terminator-*URA3*-HisF cassette was constructed. pCGUHisf was used to test XR expression in *C. tropicalis*.

Codon-optimized NcXR (NXRG) gene was obtained from GENEART (Regensburg, Germany) by changing the original codons to the preferred codons in *C. tropicalis*, using the codon usage database (<http://www.kazusa.or.jp/codon>). NXRG was inserted into pCGUHisf between the *Xba*I and *Xho*I sites, and the resulting plasmid was termed pCGUHisf-NXRG.

To construct a strain with high NXRG expression, an additional promoter-NXRG-terminator cassette was inserted between *URA3* and HisF in pCGUHisf-NXRG. The resulting vector, termed pCGUHisf-NXRG2, was digested with *Hpa*I to prepare an integrated expression cassette, i.e., HisR-promoter-NXRG-terminator-*URA3*-promoter-NXRG-terminator-HisF.

C. tropicalis was transformed using the lithium acetate method with slight modification [18]. Transformants were selected on YNB plates, and genetic modifications were confirmed by PCR. Sequences of all primers used in this study are listed in Table 2.

Fermentation experiment and analytical methods

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 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. Fed-batch culture for xylitol production was performed in a 500-mL Erlenmeyer flask with 100 mL fermentation medium in a shaking incubator, 30 °C, 200 rpm. After the initial glucose was consumed, 2.5 g/L glucose was added at 14, 20, 26, and 32 h for continuous supply of NADPH.

Concentrations of D-glucose, D-xylose, and xylitol were analyzed by high-performance liquid chromatography with

a Sugar-Pak I column (6.5 × 300 mm, Waters, MA) and a refractive-index detector (Waters). Distilled water was used as the mobile phase, with column temperature 90 °C and flow rate 0.5 mL/min. Cell growth was determined spectrophotometrically by absorbance at 600 nm. One A_{600} was equivalent to 0.474 g (dry cell weight)/L.

XR activity assay

XR activity was determined spectrophotometrically by monitoring the change in A_{340} upon NADPH oxidation at 25 °C. Cultured cells were harvested by centrifugation (10,000×g, 5 min), washed with 50 mM potassium phosphate buffer (pH 7.0), suspended in the same buffer, and disrupted using glass beads. Cell debris was separated by centrifugation (10,000×g, 5 min) and the supernatant was used for measurement of enzyme activity. The XR assay mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.2 mM NADPH, 20 mM D-xylose, and enzyme solution. The assay mixture was allowed to stand for a 1 min to remove the endogenous oxidation of NADPH. The oxidation reaction was started with addition of the enzyme solution. Activities were expressed as specific activity [units (mg of protein)⁻¹], where one unit corresponds to conversion of 1 μmol NADPH per min. Each measurement was repeated three times.

Results

Isolation and analysis of the GAPDH promoter region

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a tetrameric NAD-dependent enzyme involved in the glycolytic pathway, and is expressed constitutively at high levels. A novel 1,455-bp GAPDH promoter region was isolated from *C. tropicalis* ATCC 20336 genomic DNA by PCR with primers designed from the conserved sequences of the 5'- and 3'-flanking genes based on the strain MYA-3404 database (Broad Institute, MA). Although the isolated promoter region was cloned based on the strain MYA-3404 database, the promoter region sequence (1,455 bp) of strain ATCC 20336 GAPDH and that (1,849 bp) of strain MYA-3404 GAPDH showed low sequence identity (48.9%).

Three possible CAAT boxes were found, 551, 509, and 307 bp upstream from the ATG start codon in the promoter region of strain ATCC 20336 GAPDH. A putative conventional TATA box (TATAAA) was also located at position -155. The GAPDH promoter for heterologous expression in *C. tropicalis* was evaluated with codon-optimized NcXR gene (NXRG) in cells grown on glucose medium. XR activity assay could be used as an expression test because no endogenous XR is expressed in cells grown

Table 2 Primers used in this study

Primer	Sequence ^a	Restriction site(s)
His80-F1	<u>GTAAACATGCCGATTGATATCCTGCGCGTGC</u> GTGATGATGACATTCCGGGTCTGGT <u>AATGGATGGCGTGGTTCGATCTCGGTAT</u> GGATCC CAAAGCATCAGCTCATCGAG	<i>HpaI</i> , <i>BamHI</i>
His80-R1	<u>GTAAACAACGTTTCGCTGCTGACCATGTGCATCGCCACGCGCTGTTGCTCGCCTGCC</u> <u>AGCGGCAGAATTGTCGGCCTTTCGGC</u> GGATCC AAAAGTTCGACAGCGTCTCC	<i>HpaI</i> , <i>BamHI</i>
Ura3F	<u>AGATCTGATCTGGTTTGGATTGTTGGAGA</u>	<i>BglIII</i>
Ura3R	<u>GGATCCGATCTTGAAGTCCTCGTTTGTG</u>	<i>BamHI</i>
GapF	CCCAAACCAATTGATGGAATGGTATGACC	
GapR	GGATCTTGGGATTTCCAAGTGGTG	
PgapF	<u>AGATCTAACGTGGTATGGTTGTAAGAAAC</u>	<i>BglIII</i>
PgapR	<u>GGATCCGCGTCTAGATGTTTAAATCTTTAATTG</u>	<i>BamHI</i> , <i>XbaI</i>
TgapF	<u>TCTAGATTGCTCGAGCTATCCAACAACTCTAG</u>	<i>XbaI</i> , <i>XhoI</i>
TgapR	<u>GGATCC TCTGGTTTAGAAGTAGGGACTGTATG</u>	<i>BamHI</i>
HIS-F	AGCGATGATTCACGAGAATTGCTG	
HIS-R	CAGATCACTCCATCATCTTCTTGATCG	
URA-F	GGTTTGGATTGTTGGAGAATTTCAAG	
URA-R	TGAAGTCCTCGTTTGTGTTGCTTG	

^a The restriction sites introduced into the primers are underlined. Boldface type in His80-F1 and His80-R1 indicates the homologous sequences for integration into *C. tropicalis* L10

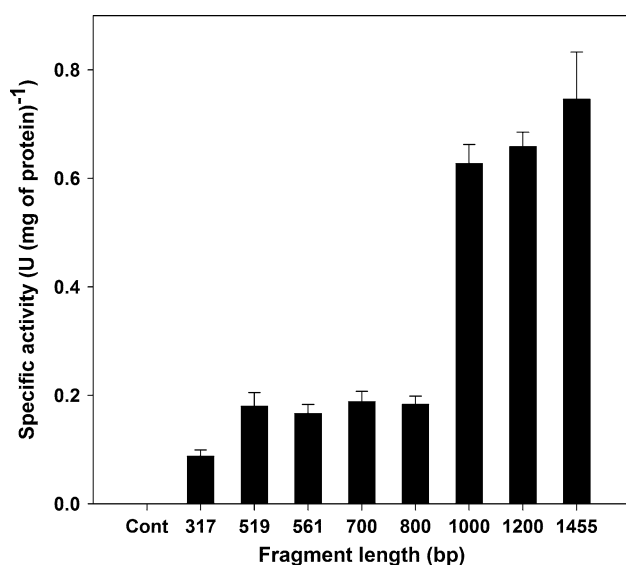


Fig. 1 XR expression levels with various lengths of the GAPDH promoter region. XR activity was determined in cells grown in 50 mL of glucose medium in a 250-mL flask for 12 h. *Cont* control strain BSXDH-3

on medium containing glucose as a carbon source. Various promoter lengths were evaluated to find the necessary minimum length for sufficient expression (Fig. 1). Promoter fragments of 317, 519, 561, 700, 800, 1,000, 1,200, and 1,455 bp were tested. Fragments shorter than 1,000 bp resulted in expression levels <26%, suggesting that there are enhancer elements in the 800-bp upstream region. The full 1,455 bp length was selected for maximum heterologous gene expression.

Table 3 XR activity of various *C. tropicalis* strains

Strains or expressed genes ^a	Specific activity ^b [U (mg of protein) ⁻¹]	Carbon source
BSXDH-3	ND	Glucose
NcXR gene	ND	Glucose
NXRE gene	ND	Glucose
NXRG gene	0.75 ± 0.09	Glucose
BSXDH-3 ^c	1.19 ± 0.16	Glycerol and xylose
BSXDH-3	ND	Glucose and xylose
LNG2	2.86 ± 0.10	Glucose and xylose

ND not detectable

^a NcXR gene, original XR gene from *N. crassa*; NXRE, CTG codon-changed NcXR; NXRG, codon-optimized NcXR

^b XR activity was determined in cells grown on 100 ml of sugar medium in a 500-mL flask

^c BSXDH-3 was cultured in glycerol and xylose medium

Codon-optimization of NcXR

CTG codon, a universal leucine codon, is translated as serine in several *Candida* species, including *C. tropicalis* [5]. Since NcXR has three CTG codons, these were changed to TTG codons, the leucine codons favored in *C. tropicalis*, using site-directed mutagenesis. Expression of the modified NcXR was tested using XR activity assay in cells grown on glucose medium. Although the CTG codon-changed NcXR (NXRE) was inserted in pCGUHisf and integrated in genome of strain L10, NXRE did not show XR activity (Table 3).

Table 4 Comparison of codon usage for wild-type NcXR (wt) and synthetic NXRG (syn)

AA	Codon	Fraction		No. of codons		AA	Codon	Fraction		No. of codons		AA	Codon	Fraction		No. of codons	
		Nc	Ct	wt	syn			Nc	Ct	wt	syn			Nc	Ct	wt	syn
F	TTT	0.35	0.48	2	2	P	CCT	0.23	0.18	5	0	N	AAT	0.28	0.36	0	0
	TTC	0.65	0.52	15	15		CCC	0.35	0.05	11	0		AAC	0.72	0.64	14	14
L	TTA	0.03	0.19	0	0	T	CCA	0.19	0.73	0	16	K	AAA	0.22	0.42	0	2
	TTG	0.18	0.65	0	31		CCG	0.23	0.04	0	0		AAG	0.78	0.58	20	18
	CTT	0.17	0.08	3	0	A	ACT	0.19	0.41	1	11	D	GAT	0.42	0.51	6	18
	CTC	0.32	0.05	25	0		ACC	0.41	0.42	9	0		GAC	0.58	0.49	13	1
	CTA	0.07	0.01	0	0	E	ACA	0.18	0.12	0	0	E	GAA	0.34	0.79	0	21
	CTG	0.22	0.02	3	0		ACG	0.23	0.06	1	0		GAG	0.66	0.21	21	0
I	ATT	0.31	0.52	3	20	A	GCT	0.24	0.52	7	26	C	TGT	0.3	0.82	0	2
	ATC	0.59	0.42	18	1		GCC	0.41	0.33	18	0		TGC	0.7	0.18	2	0
	ATA	0.09	0.07	0	0	R	GCA	0.14	0.12	1	0	R	CGT	0.14	0.1	2	0
M	ATG	1	1	5	5		GCG	0.2	0.04	0	0		CGC	0.29	0.02	13	0
V	GTT	0.23	0.52	2	18	Y	TAT	0.33	0.39	0	0	G	CGA	0.11	0.02	0	0
	GTC	0.42	0.33	16	2		TAC	0.67	0.61	13	13		CGG	0.14	0.01	0	0
	GTA	0.09	0.04	1	0	Stop	TAA	0.32	0.45	0	1	G	AGA	0.13	0.79	0	15
	GTG	0.26	0.11	1	0		TAG	0.28	0.48	1	0		AGG	0.19	0.05	0	0
S	TCT	0.15	0.28	4	0	H	TGA	0.4	0.06	0	0	G	GGT	0.26	0.7	7	23
	TCC	0.24	0.29	12	19		CAT	0.39	0.39	0	0		GGC	0.4	0.14	16	0
	TCA	0.11	0.15	0	2	Q	CAC	0.61	0.61	6	6	G	GGA	0.19	0.11	0	0
	TCG	0.18	0.08	2	0		CAA	0.39	0.75	1	14		GGG	0.15	0.05	0	0
	AGT	0.11	0.12	0	0	W	CAG	0.61	0.25	13	0	Total					
	AGC	0.21	0.07	3	0		TGG	1	1	7	7					323	323

The codon usage tables for *N. crassa* (Nc) and *C. tropicalis* (Ct) were based on the codon usage database (<http://www.kazusa.or.jp/codon>)

The codon usage database indicated that some amino acid residues of NcXR are encoded by codons that are rarely represented in *C. tropicalis* (Table 4). Three codons, CCC (P), CGC (R), and CTC (L), were expected to be particularly problematic in functional expression. For heterologous expression of NcXR, the amino acid sequence (GenBank accession number AAW66609) was back-translated using the preferred codons in *C. tropicalis*. The optimized NcXR gene (NXRG) was synthesized and expressed successfully in *C. tropicalis*. The NXRG-expressing strain displayed XR activity of 0.75 U (mg of protein)⁻¹, whereas no activity was detected in native NcXR or NXRE (Table 3).

Construction of NcXR-expressing strains of *C. tropicalis*

In our previous study, two copies of the *XYL2* gene encoding XDH were destroyed by inserting *hisG* fragment and *URA3* gene in the two *XYL2* gene positions [18]. The resulting strain, BSXDH-3, was incubated on YNB-5FOA plates for deletion of the *URA3* marker gene; the resulting uracil auxotroph strain was termed L10.

Strain L10 was transformed to uracil prototrophy with an *HpaI*-digested pCGUHisf-NXRG2, NXRG expression cassette (Fig. 2a). This cassette was integrated into the *hisG* fragment region in the L10 genome, and PCR was used to confirm site-specific insertion of the cassette into the transformants, one of which was termed LNG2 (Fig. 2b). NXRG was expressed in LNG2 by the *C. tropicalis* GAPDH promoter, and XR enzyme activity was determined in cells grown on a fermentation medium containing glucose as co-substrate. No XR activity was detected in BSXDH-3, because of glucose repression, whereas LNG2 showed high activity of 2.86 U (mg of protein)⁻¹, independent of glucose in the medium (Table 3).

Xylitol production by the NcXR-expressing strain

Fed-batch culture was performed in fermentation medium with glucose as a co-substrate for cell growth and co-factor (NADPH) regeneration (Fig. 3). In xylitol fermentation by BSXDH-3, no endogenous XR was expressed during the early growth phase in the presence of glucose (Fig. 3a). After glucose depletion, XR activity increased gradually up to a level of 0.21–0.23 U (mg of protein)⁻¹, and xylitol

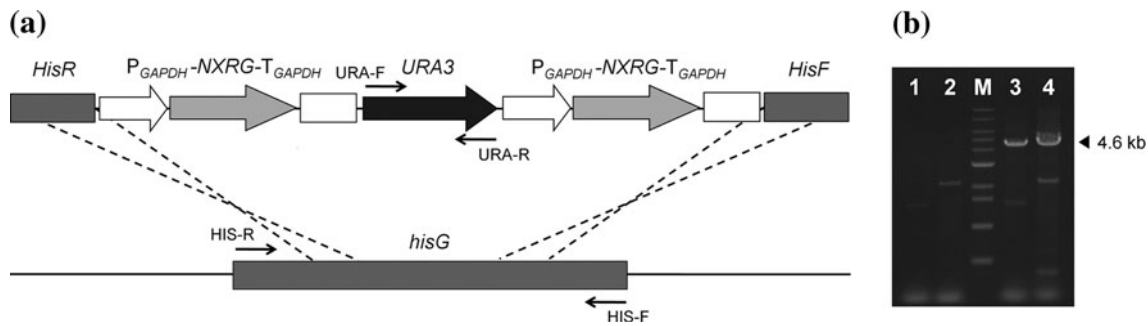


Fig. 2 Construction of NXRG-expressing strain. **a** Physical map of expression cassette, and integration of the cassette into *hisG* region of *C. tropicalis* strain L10. P_{GAPDH} , GAPDH promoter; T_{GAPDH} , GAPDH terminator. **b** PCR confirmation of specific integration of NXRG expression cassette. Lanes 1 and 3: PCR with primers URA-F

and HIS-F for amplification of 4.6-kb product. Lanes 2 and 4: PCR with primers HIS-R and URA-R for amplification of 4.6-kb product. Lanes 1 and 2: host strain L10. Lanes 3 and 4: strain LNG2. Lane M: DNA size marker

began to be produced up to a rate of $0.83 \text{ g L}^{-1} \text{ h}^{-1}$. BSXDH-3 gave a xylitol yield of 59% at 44 h. In contrast, LNG2 began to produce xylitol before depletion of initial glucose because of its high XR activity in the presence of glucose (Fig. 3b). XR activity was maintained from 1.91 to $2.86 \text{ U (mg of protein)}^{-1}$ during fermentation. LNG2 produced xylitol at a rate of $1.44 \text{ g L}^{-1} \text{ h}^{-1}$ and yield of 96%. These values were 73 and 62%, respectively, higher than those for BSXDH-3.

Discussion

Candida strains have been studied extensively for biological production of xylitol, because of their natural ability to take up and assimilate xylose [4, 20, 21]. Redox imbalance between NADPH-dependent XR and NAD-dependent XDH is the major factor controlling xylitol accumulation [22, 23], and research on *Candida* yeasts has therefore focused on controlling dissolved oxygen or redox potential in order to obtain high xylitol yield from D-xylose [8, 16]. Under oxygen-limited conditions, the electron transfer chain is not able to reoxidize all of the NADH generated. Increased intracellular NADH level decreases reaction of NAD-dependent XDH, leading to xylitol accumulation. Optimal concentration of dissolved oxygen for maximal xylitol production ranges from 0.8 to 1.2%, which is difficult to control in practice [16]. Furthermore, even when a co-substrate was used, xylitol yield did not reach the theoretical value using this approach with non-metabolically engineered yeasts [4, 20, 21]. In our previous study, 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 [17].

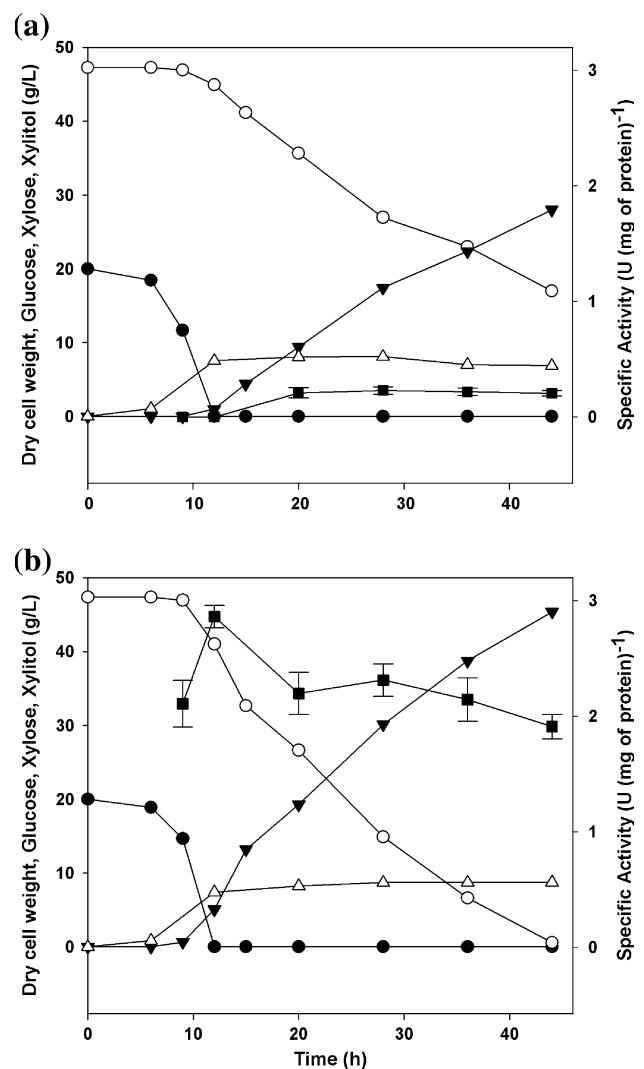


Fig. 3 Xylitol fermentation profiles of *C. tropicalis* strains BSXDH-3 (**a**) and LNG2 (**b**). Triangle dry cell weight, filled circle glucose, open circle xylose, inverted filled triangle xylitol, filled square XR activity

After screening various carbon sources, glycerol was selected as the best co-substrate for cell growth and NADPH regeneration [18]. However, glycerol has a disadvantage as a co-substrate compared with glucose. *i.e.*, the biomass used in biological xylitol production is hydrolyzed by mineral acid to produce a hydrolysate consisting primarily of glucose, xylose, and arabinose. The glucose contained in this hydrolysate can be used directly as a co-substrate; however, this situation does not apply to glycerol.

On the other hand, there are obstacles in using glucose as a co-substrate in xylitol production using *C. tropicalis*, one of which is the repressive effect of glucose on XR synthesis. Under most conditions, no xylose-metabolizing enzymes, including XR, are synthesized in the presence of a mixture of xylose and glucose, because of glucose repression [19]. For example, Table 3 illustrates the repressive effect of carbon source in medium on XR in strain BSXDH-3. BSXDH-3 showed no XR activity in xylose and glucose mixture, but did show activity ($1.19 \text{ U (mg of protein)}^{-1}$) in xylose and glycerol mixture. Previous reports have described attempts to minimize glucose repression of XR in *C. tropicalis* by supplying sodium chloride in culture medium, or by optimizing the fermentation process [24, 25]. However, these approaches do not guarantee permanent expression of XR in cells.

In the present study, in order to overcome glucose repression of XR, we expressed additional XR in *C. tropicalis*. NcXR was selected for expression because of its potent enzyme activity [15], and codon-optimization was performed to ensure heterologous expression. We tested some constitutive promoters of other yeasts, *e.g.*, *S. cerevisiae* ADH1 promoter and *Pichia pastoris* GAPDH promoter, for possible enhancement of functional expression of NXRG in *C. tropicalis*, but they had no significant effect (data not shown). Therefore, the endogenous GAPDH promoter of *C. tropicalis* was isolated and used for constitutive expression of NXRG in the presence of glucose. NXRG was introduced into *XYL2*-disrupted *C. tropicalis* with the GAPDH promoter. The resulting strain, LNG2, initiated production of xylitol before depletion of initial glucose, and produced xylitol at a 73% enhanced production rate ($1.44 \text{ g L}^{-1} \text{ h}^{-1}$), with xylitol yield of 96% in a glucose and xylose mixture.

Although XR was highly expressed in strain LNG2 during fermentation, LNG2 did not produce xylitol during the initial period (Fig. 3b). There is presumably another limiting factor that must be overcome before glucose can be successfully used as a co-substrate in xylitol production. This limiting factor may be xylose transport in the presence of glucose and xylose. Xylose is transported in yeasts by the facilitated sugar transport system, which has a 200-fold lower affinity for xylose than for glucose [26, 27]. Some sugar transporters from *Arabidopsis thaliana* and *P. stipitis*

have been expressed in recombinant xylose-assimilating *S. cerevisiae* for glucose and xylose co-consumption [28, 29]. It is possible that additional expression of xylose transporters in *C. tropicalis* will result in higher productivity and a shorter lag time for xylitol production.

In this study, glucose repression of XR was overcome successfully by heterologous expression of optimized NcXR with the GAPDH promoter in *C. tropicalis*. XR activity was maintained at a high level in the presence of glucose, and xylitol productivity and yield were enhanced significantly. Glucose has obvious economic and technological advantages as a co-substrate in industrial applications, and the present findings will contribute to improved methods for biological production of xylitol.

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