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ORIGINAL RESEARCH PAPER

L-Arabinose pathway engineering for arabitol-free xylitol production in *Candida tropicalis*

Byoung Hoon Yoon · Woo Young Jeon · Woo Yong Shim · Jung Hoe Kim

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Abstract Xylose reductase (XR) is a key enzyme in biological xylitol production, and most XRs have broad substrate specificities. During xylitol production from biomass hydrolysate, non-specific XRs can reduce L-arabinose, which is the second-most abundant hemicellulosic sugar, to the undesirable byproduct arabitol, which interferes with xylitol crystallization in downstream processing. To minimize the flux from L-arabinose to arabitol, the L-arabinose-preferring, endogenous XR was replaced by a D-xylose-preferring heterologous XR in *Candida tropicalis*. Then, *Bacillus licheniformis araA* and *Escherichia coli araB* and *araD* were codon-optimized and expressed functionally in *C. tropicalis* for the efficient assimilation of L-arabinose. During xylitol fermentation, the control strains BSXDH-3 and KNV converted 9.9 g L-arabinose l^{-1} into 9.5 and 8.3 g arabitol l^{-1}, respectively, whereas the recombinant strain JY consumed 10.5 g L-arabinose l^{-1} for cell growth without forming arabitol. Moreover, JY produced xylitol with 42 and 16% higher productivity than BSXDH-3 and KNV, respectively.

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B. H. Yoon · W. Y. Jeon · W. Y. Shim · 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

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Introduction

Xylitol, a five-carbon sugar alcohol, is used as an alternative natural sweetener in the food and confectionary industry. Xylose reductase (XR), a key enzyme in biological xylitol production, catalyzes the reduction of D-xylose to xylitol with accompanying NAD(P)H oxidation. XRs, which are commonly found in yeasts and filamentous fungi, have evolved to have broad substrate specificities for the efficient reduction of a number of sugars. The broad substrate acceptance of XR can adversely affect xylitol production. The hemicelluloses are a major source of xylose and also contain significant amounts of L-arabinose, which is reduced to arabitol by non-specific XRs. Arabitol, an epimer of xylitol, is difficult to remove during xylitol production and interferes with xylitol crystallization in the downstream processing used for purification. A hydroxyl group of arabitol causes steric hindrance and prevents the growth of xylitol crystals (Fernandes et al. 1999). Using protein engineering, Nair and Zhao (2008) established five point-mutations in *Neurospora crassa* XR (NcXR) that improved its substrate specificity for D-xylose.

Most bacteria can assimilate L-arabinose via a pathway consisting of three enzymes: L-arabinose isomerase (*araA*, AI), L-ribulokinase (*araB*, RK) and

L-ribulose-5-phosphate 4-epimerase (*araD*, RPE), which convert L-arabinose into L-ribulose, L-ribulose 5-phosphate, and D-xylulose 5-phosphate, respectively. Bacterial L-arabinose metabolic enzymes have been expressed in *Saccharomyces cerevisiae* for ethanol production (Becker and Boles 2003; Wiedemann and Boles 2008).

In our previous work, the *XYL2* gene encoding xylitol dehydrogenase was disrupted in *Candida tropicalis* for high-yield xylitol production. The resulting strain converted xylose into xylitol at nearly the theoretical maximum yield of 100% (Ko et al. 2006). However, a significant amount of arabitol was produced from the L-arabinose contained in the biomass hydrolysate. In the present study, we developed a *C. tropicalis* strain for arabitol-free xylitol production by introducing the L-arabinose metabolic pathway (Fig. 1). For this purpose, the *XYL1* genes encoding L-arabinose-preferring endogenous XR were disrupted completely in *XYL2*-disrupted *C. tropicalis*, and a functional D-xylose-preferring heterologous XR originating from *N. crassa* was expressed. Then, the bacterial L-arabinose utilization pathway was introduced into *C. tropicalis* for the efficient assimilation of L-arabinose. The L-arabinose pathway engineered *C. tropicalis* efficiently produced xylitol, without arabitol formation.

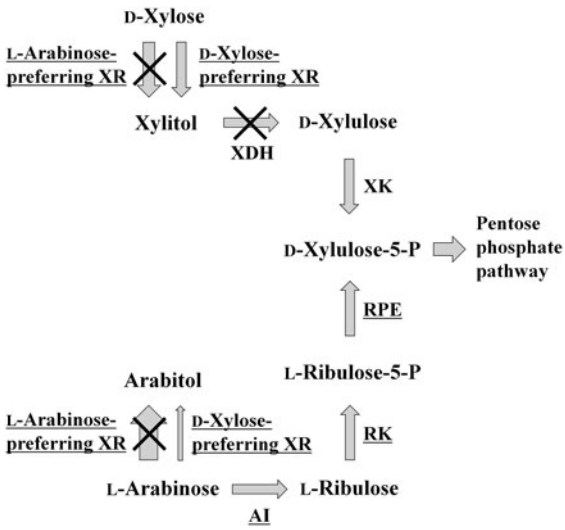

Fig. 1 Schematic of the D-xylose and L-arabinose metabolic pathway in metabolically engineered *C. tropicalis* JY. The engineering steps involved in this work are indicated by the *underlined* gene names. L-Arabinose-preferring XR was disrupted, and D-xylose-preferring XR was expressed instead. The bacterial L-arabinose utilization pathway, AI, RK, and RPE, were introduced into *C. tropicalis*. *XR* xylose reductase, *XDH* xylitol dehydrogenase, *XK* D-xylulose kinase, *AI* L-arabinose isomerase, *RK* L-ribulokinase, *RPE* L-ribulose-5-phosphate 4-epimerase

Materials and methods

Strains, media, and yeast transformation

Candida tropicalis L10 (*ura3/ura3*), a uracil auxotroph derived from *XYL2*-disrupted *C. tropicalis* BSXDH-3 (Ko et al. 2006), was used as the host strain for transformation. For genetic manipulation of *C. tropicalis*, YNB medium (20 g glucose l^{-1}, 6.7 g yeast nitrogen base without amino acids l^{-1}) and YNB-5FOA medium (20 g glucose l^{-1}, 6.7 g yeast nitrogen base without amino acids l^{-1}, 0.1 g uracil l^{-1}, 0.1 g uridine l^{-1}, 0.8 g 5-fluoroorotic acid l^{-1}) were used. *Escherichia coli* DH5α (RBC Bioscience, Taiwan) was used as the host for plasmid transformation and was cultured in Luria–Bertani (LB) medium. Glucose/xylose medium (20 g glucose l^{-1}, 50 g D-xylose l^{-1}, 10 g yeast extract l^{-1}, 5 g KH_2PO_4 l^{-1}, 0.2 g $MgSO_4 \cdot 7H_2O$ l^{-1}) was used to culture *C. tropicalis* for the XR activity assay. To confirm the viability of the constructed strain, L-arabinose minimal medium (20 g L-arabinose l^{-1}, 6.7 g yeast nitrogen base without amino acids l^{-1}) was used. The *C. tropicalis* was transformed using the lithium acetate (LiOAc) method with a slight modification (Ko et al. 2006).

Construction of the disruption cassettes for the *XYL1* gene of *C. tropicalis*

Four disruption cassettes for *XYL1*, which encodes *C. tropicalis* XR (CtXR), were constructed to knockout the endogenous XR activity. The *URA3* gene was amplified from the genomic DNA of *C. tropicalis* ATCC 20336 by PCR using primers Ura3F and Ura3R (Supplementary Table 1), which were designed from the *URA3* sequence (GenBank accession no. AB006207). The *URA3* gene was inserted into pGEM-T easy vector (Promega), and the resulting plasmid was designated pGEM-URA3. For pop-out of the *URA3* selection marker via DNA recombination, four DNA sequences [hph (reverse), hph, arg, and trp (reverse)] were used. The hph was

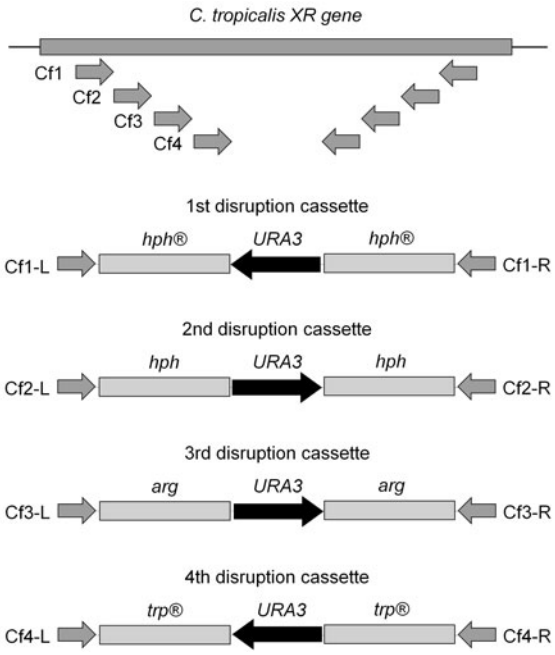

Fig. 2 The *C. tropicalis XYL1*-disruption cassettes. *Cf* CtXR fragments for insertion into *C. tropicalis XYL1*, ®, reverse direction of the DNA sequences

obtained from pREP4 (Invitrogen), and the arg and trp were from *Bacillus subtilis*. Identical genes were inserted into the *Bgl*II and *Bam*HI sites on either side of URA3 in pGEM-URA3. The four resulting plasmids contained the cassettes hph(reverse)-URA3-hph (reverse), hph-URA3-hph, arg-URA3-arg, and trp (reverse)-URA3-trp(reverse), respectively. These cassettes were inserted between four pairs of CtXR fragments for integration into the *C. tropicalis* genome. The constructed cassettes are shown schematically in Fig. 2.

Codon-optimization of mutated NcXR, *araA*, *araB*, and *araD*

The amino acid sequences of the NcXR harboring five point mutations (L102V, L107M, L109Q, I110C, and V114I) (Nair and Zhao 2008), *B. licheniformis araA*, *E. coli araB*, and *E. coli araD* were back-translated and synthesized using the preferred codons in *C. tropicalis* (GENEART, Germany), using the codon usage database (http://www.kazusa.or.jp/codon). The codon-optimized mutated NcXR, *araA*, *araB*, and *araD* were designated NXRG-Mut5, CoAraA, CoAraB, and CoAraD, respectively.

Construction of the expression cassettes for NXRG-Mut5, CoAraA, CoAraB, and CoAraD

NXRG-Mut5, CoAraA, CoAraB, and CoAraD were inserted between the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter and terminator of *C. tropicalis*, for heterologous expression. To pop-out the *URA3* marker, two genes (glu and arg) obtained from *B. subtilis* were used. Two glu and two arg genes were inserted into the *Bgl*II and *Bam*HI sites of pGEM-URA3, respectively. The resulting plasmids contained the cassettes glu-URA3-glu and arg-URA3-arg. Then, P_{GAPDH}-NXRG-Mut5-T_{GAPDH} and P_{GAPDH}-CoAraA-T_{GAPDH} were each ligated to glu-URA3-glu, and P_{GAPDH}-CoAraB-T_{GAPDH} and P_{GAPDH}-CoAraD-T_{GAPDH} were each ligated to arg-URA3-arg, to give the following four cassettes: P_{GAPDH}-NXRG-Mut5-T_{GAPDH}-glu-URA3-glu, P_{GAPDH}-CoAraA-T_{GAPDH}-glu-URA3-glu, P_{GAPDH}-CoAraB-T_{GAPDH}-arg-URA3-arg, and P_{GAPDH}-CoAraD-T_{GAPDH}-arg-URA3-arg. These four cassettes were inserted between four pairs of hisG, trp, hph, and hph (second) fragments for integration into the *C. tropicalis* genome. The constructed cassettes are shown schematically in Fig. 3.

RT-PCR analysis

Total RNA was purified from *C. tropicalis* K1, KNV, and JY (RNeasy purification kit; Qiagen). The cDNA

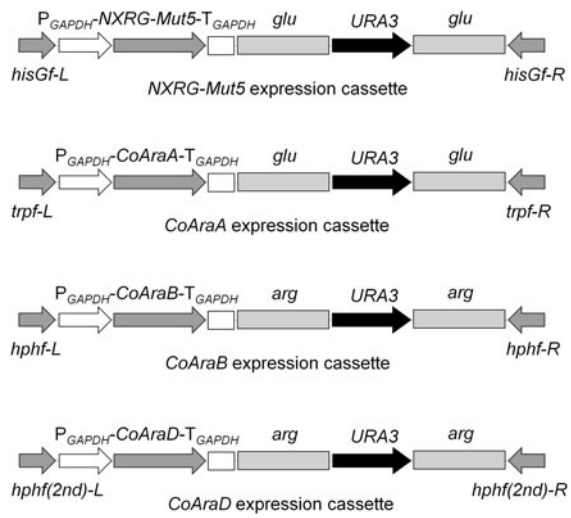

Fig. 3 NXRG-Mut5, CoAraA, CoAraB, and CoAraD expression cassettes. The hisGf, trpf, hphf, and hphf (2nd) are the hisG, trp, hph, and hph (second) fragments for insertion into *C. tropicalis*, respectively

was synthesized (AccuPower RT/PCR PreMix kit; Bioneer, Korea) and used subsequently for PCR. The protein-coding regions of NXRG-Mut5, CoAraA, CoAraB, and CoAraD were amplified from the cDNAs using four primer sets: NXRG-F/NXRG-R, CoAraA-F/CoAraA-R, CoAraB-F/CoAraB-R, and CoAraD-F/CoAraD-R, respectively (Supplementary Table 1).

XR activity assay

XR activity was determined by monitoring the change in A_{340} upon NADPH oxidation at 25°C. The cultured yeast cells were harvested by centrifugation (10,000×*g*, 5 min) and washed with 50 mM potassium phosphate buffer (pH 7.0). The cells were suspended in the same buffer and disrupted using glass beads. Cellular debris was separated by centrifugation (10,000×*g*, 5 min), and the supernatant was used to measure the enzyme activity. The XR assay mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.2 mM NADPH, 20 mM substrate (D-xylose or L-arabinose), and enzyme solution. Activities are expressed as specific activity (units per mg of protein), where one unit corresponds to the conversion of 1 μmol NADPH per min.

Xylitol fermentation experiment and analytical methods

Experiments to compare xylitol fermentation among the *C. tropicalis* strains were performed in a 500-ml Erlenmeyer flask with 100 ml fermentation medium at 30°C and shaking at 150 rpm. The fermentation medium contained 30 g D-xylose l^{-1}, 30 g L-arabinose l^{-1}, 20 g glucose l^{-1}, 10 g yeast extract l^{-1}, 5 g KH_2PO_4 l^{-1}, and 0.2 g $MgSO_4 \cdot 7H_2O$ l^{-1}. After the initial glucose was consumed, glucose additions of 2.5 g l^{-1} were made four times, at 14, 20, 26, and 32 h.

The concentrations of D-glucose, D-xylose, L-arabinose, arabitol, and xylitol were analyzed by HPLC with a Sugar-Pak I column and a refractive index detector. Distilled water was used as the mobile phase at 90°C and 0.5 ml min^{-1}. Cell growth was calculated from the OD_{600} value; an OD of 1 = 0.474 g dry cell wt l^{-1}.

Results and discussion

Construction of the *XYL1*-disrupted strain of *C. tropicalis*

Four strands of *XYL1* genes encoding L-arabinose-preferring endogenous CtXR were disrupted in *XYL2*-disrupted *C. tropicalis* (Fig. 4). L10 was transformed to uracil prototrophy with the first disruption cassette, and Ura^+ transformants were selected from YNB plates. One transformant was incubated on a YNB-5FOA plate to allow pop-out of the selection marker gene. The resulting uracil auxotroph strain was used as a host strain for integration of the second disruption cassette. The three additional strands of *XYL1* were disrupted sequentially using the same procedure. After deletion of the fourth strand, the resulting strain K1 did not show XR activity, indicating the complete knockout of XR (Table 1).

Construction of the NXRG-Mut5 expressing strain of *C. tropicalis*

The D-xylose-preferring NXRG-Mut5 containing five point mutations (Nair and Zhao 2008) was expressed in *C. tropicalis* K1 (Fig. 5). The NXRG-Mut5 expression cassette was integrated into the *hisG* region in the genome of K1. A Ura^+ transformant was selected from a YNB plate and designated KNV. To evaluate the expression of NXRG-Mut5, RT-PCR was used to detect the transcription of NXRG-Mut5. The mRNA transcript of NXRG-Mut5 was detected in KNV, but not in K1 (Supplementary Fig. 1).

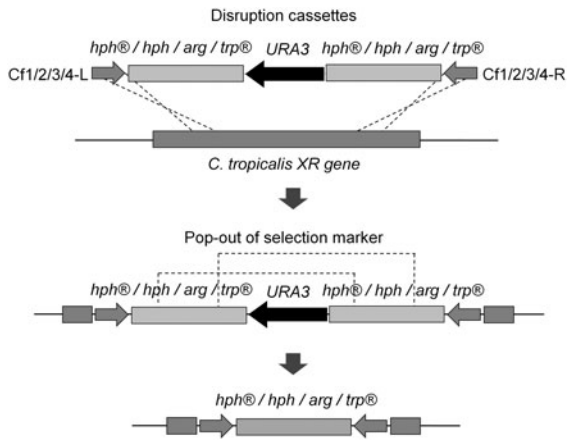

Fig. 4 Gene disruption of *XYL1* in *C. tropicalis*

Table 1 Comparison of xylose reductase (XR) activity between the parent strain BSXDH-3 and the *XYL1*-disrupted strain K1

Strains	Number of residual *XYL1* strands	Specific activity[a] (mU mg of protein^{-1})
BSXDH-3	4	177 ± 12
K1	0	ND[b]

[a] XR activity was determined using D-xylose as the substrate in cells grown in 50 ml glucose/xylose medium in a 250 ml flask for 24 h. Data represent the means of three experiments with standard deviation

[b] *ND* not detectable

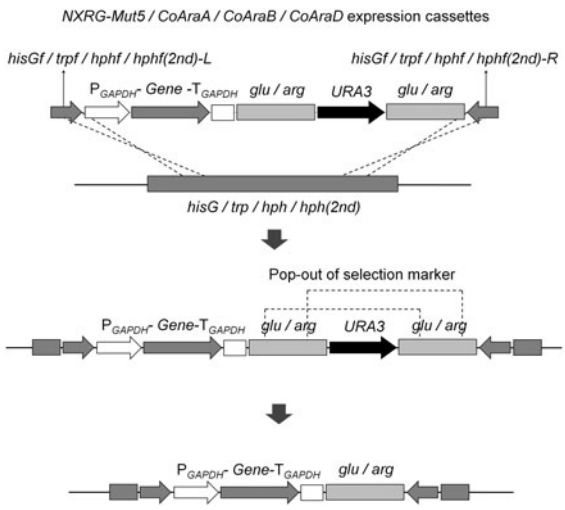

Fig. 5 Introduction of the NXRG-Mut5, CoAraA, CoAraB, and CoAraD expression cassettes into *C. tropicalis*

The XR activities were determined in BSXDH-3 and KNV on glucose/xylose medium. Compared with BSXDH-3, KNV showed 55% lower activity for D-xylose; however, the substrate preference for L-arabinose was decreased 95% (Table 2).

Construction of the CoAraA, CoAraB, and CoAraD expressing strain of *C. tropicalis*

In xylitol production, strict substrate specificities for arabinose isomerase (AI) and ribulose kinase (RK) are necessary. The AI of *E. coli* showed slight activity of 6% toward D-xylose as compared with L-arabinose (Patrick and Lee 1968), and this substrate specificity for D-xylose is undesirable for xylitol production. A novel AI that was recently cloned and characterized from *B. licheniformis* showed high catalytic efficiency and high substrate specificity for only L-arabinose (Prabhu et al. 2008). The *E. coli* RK has also been cloned and characterized (Lee et al. 1986, 2001). Although the enzyme exhibited activity toward several sugars, D-xylose, xylitol, and L-arabinose did not serve as substrates for *E. coli* RK (Lee et al. 1986).

The codon usage database indicated that the amino acid residues of bacterial L-arabinose-utilizing enzymes are encoded by codons that are rarely represented in *C. tropicalis*. Therefore, *B. licheniformis araA*, *E. coli araB*, and *E. coli araD* were codon-optimized for heterologous expression in *C. tropicalis*, resulting in CoAraA, CoAraB, and CoAraD, respectively.

The *URA3* selection marker of KNV was deleted for further engineering with this strain. KNV was incubated on a YNB-5FOA plate to pop-out the selection marker gene. The resulting uracil auxotroph strain was used as the host strain for integration of the CoAraA, CoAraB, and CoAraD expression cassettes. First, the CoAraB expression cassette was inserted into the resulting auxotroph strain, and then the *URA3* marker was deleted again on a YNB-5FOA plate. CoAraD was introduced using the same procedure, followed by the introduction of CoAraA (Fig. 5).

Table 2 Comparison of xylose reductase (XR) activity between BSXDH-3 and KNV

Strains	Specific activity[a] for D-xylose (mU mg of protein^{-1})	Specific activity[a] for L-arabinose (mU mg of protein^{-1})	Selectivity[b] (%)
BSXDH-3	177 ± 12	255 ± 22	144
KNV	80 ± 6.6	5.8 ± 0.6	7.3

[a] XR activity was determined using D-xylose or L-arabinose as the substrate in cells grown in 50 ml glucose/xylose medium in a 250 ml flask for 24 h. Data represent the means of three experiments with standard deviation

[b] Selectivity = specific activity for L-arabinose/specific activity for D-xylose (%)

The resulting strain was designated *C. tropicalis* JY. To evaluate the expression of CoAraA, CoAraB, and CoAraD, RT-PCR was used to detect transcription. The mRNA transcripts of CoAraA, B, and D were detected in JY, but not in the parental strains (Supplementary Fig. 1). When the *C. tropicalis* strains ATCC 20336 (parent strain of BSXDH-3), BSXDH-3, KNV, and JY were incubated on L-arabinose minimal plates, the parental strains (ATCC 20336, BSXDH-3, and KNV) did not grow, whereas JY grew utilizing L-arabinose as the sole carbon source. This indicates that L-arabinose was successfully connected to the pentose phosphate pathway via the bacterial L-arabinose utilization pathway in JY (Supplementary Fig. 2).

Xylitol fermentation with *C. tropicalis* JY

The fermentation profiles of *C. tropicalis* BSXDH-3, KNV, and JY were compared (Fig. 6). BSXDH-3 and KNV converted 9.9 g L-arabinose l^{-1} into 9.5 and 8.3 g arabitol l^{-1}, respectively, whereas JY consumed 10.5 g L-arabinose l^{-1} without forming arabitol (Fig. 6a, b). In addition, *C. tropicalis* JY grew to a higher cell density (17 g l^{-1}) than BSXDH-3 (7.8 g l^{-1}) or KNV (9.1 g l^{-1}) in 60 h, suggesting that the L-arabinose consumed by JY was converted into cell mass (Fig. 6c). All strains produced xylitol at nearly the theoretical maximum yield (97–99%) due to *XYL2* disruption. BSXDH-3 has endogenous CtXR, which is repressed during the early growth phase in the presence of glucose by catabolic repression. After depleting the initial glucose supply (12 h), BSXDH-3 began producing xylitol later than the other strains, which is one reason for its lower xylitol productivity (0.45 g l^{-1} h^{-1}) compared with KNV (Fig. 6d). Although JY and KNV have an identical NXRG-Mut5 gene under the control of the constitutive GAPDH promoter, JY produced xylitol with 16% greater volumetric productivity (0.64 g l^{-1} h^{-1}) than KNV (0.55 g l^{-1} h^{-1}), suggesting that the NADPH required for arabitol formation was also used for xylitol production in JY (Fig. 6d).

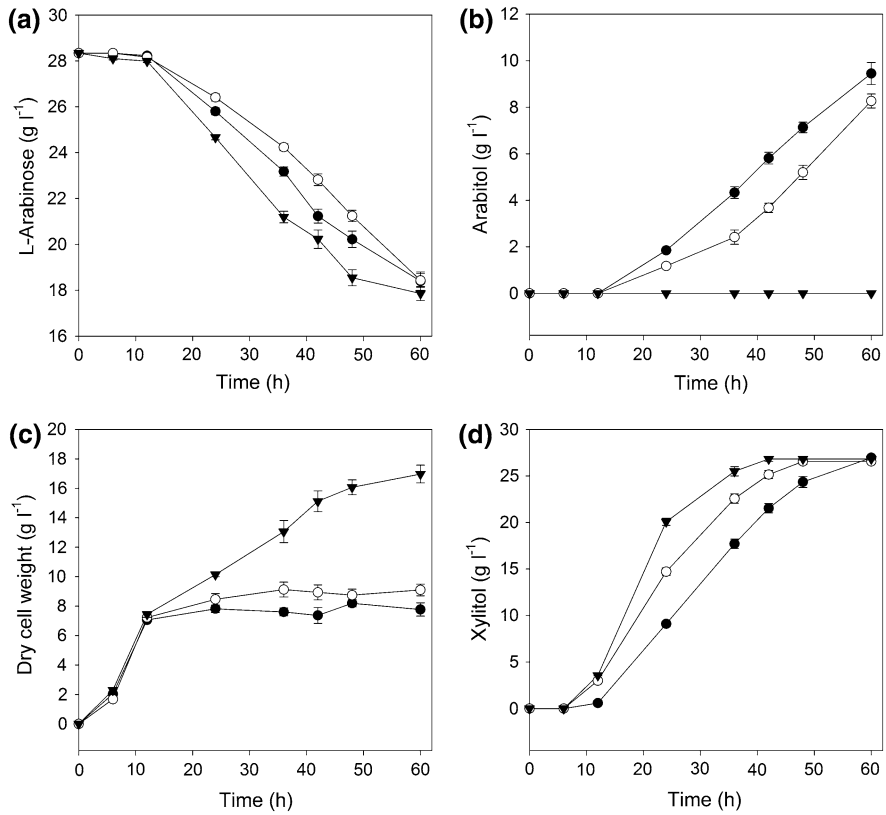

Fig. 6 Comparison of the fermentation profiles of *C. tropicalis* BSXDH-3 (*filled circle*), KNV (*open circle*), and JY (*filled triangle*). Data represent the means of three experiments with standard deviation. **a** L-arabinose consumption, **b** arabitol formation, **c** cell growth, **d** xylitol production

In conclusion: L-arabinose-preferring endogenous XR was replaced by D-xylose-preferring heterologous XR to minimize the flux from L-arabinose to arabitol in *C. tropicalis*. In addition, the bacterial L-arabinose utilization pathway was introduced into *C. tropicalis*. The resulting recombinant strain JY successfully assimilated L-arabinose for cell growth without arabitol formation, and xylitol production was enhanced. As L-arabinose is the second-most abundant hemicellulose-derived pentose, our result should contribute to the improved biological production of xylitol.

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