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ORIGINAL RESEARCH PAPER

Enhancement of xylitol production by attenuation of intracellular xylitol dehydrogenase activity in *Candida tropicalis*

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Abstract To construct *Candida tropicalis* strains that produce a high yield of xylitol with no requirement for co-substrates, we engineered the yeast with an attenuated xylitol dehydrogenase (XDH) and then assessed the efficiency of xylitol production The mutants, strains XDH-5 (with only one copy of the XDH gene), and ARSdR-16 (with a mutated XDH gene) showed 70 and 40% of wild type (WT) XDH activity, respectively. Conversions of xylose to xylitol by WT, XDH-5, and ARSdR-16 were 62, 64, and 75%, respectively, with productivities of 0.52, 0.54, and 0.62 g l^{-1} h^{-1}, respectively. The ARSdR-16 mutant strain produced xylitol with high yield and high productivity in a simple process that required no co-substrates, such as glycerol. This strain represents a promising alternative for efficient and cost-effective xylitol production.

Keywords *Candida tropicalis* · Enzyme engineering · Fermentation · Xylitol · Xylitol dehydrogenase

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Introduction

Xylitol, a five-carbon sugar alcohol, is used as a natural sweetener in food industries. It inhibits the growth of the tooth-decaying bacterium *Streptococcus mutans*, conveying an anticariogenic effect (Mäkinen 1992). Xylitol can be produced from D-xylose by natural xylose-assimilating yeasts, including *Candida guilliermondii*, *C. parapsilosis*, and *C. tropicalis* (Barbosa et al. 1988; Kim et al. 1997; Kim et al. 1998). The majority of xylose-assimilating yeasts, including *C. tropicalis*, utilize D-xylose via two enzymatic oxidoreductive reactions involving xylose reductase and xylitol dehydrogenase (XDH) (Alexander et al. 1988). Xylose reductase requires NADPH as a cofactor, whereas XDH requires NAD^+. XDH in *C. tropicalis* is encoded by the *XYL2* gene (Ko et al. 2006a).

Recently, the two *XYL2* genes of *C. tropicalis* were disrupted in order to block the enzymatic pathway of xylitol to D-xylulose, for cell growth and maintenance (Ko et al. 2006b). This *XYL2*-disrupted mutant could produce xylitol with high productivity and a theoretical yield of nearly 97% (w/w). However, this mutant required a large amount of glycerol as a co-substrate to support cell growth and NADPH regeneration, consuming 0.3–0.4 g glycerol in the conversion of 1 g D-xylose to xylitol (Ko et al. 2006c). Furthermore, sterilized xylose and glycerol must be added separately to the fermentation tank, and the residual glycerol after xylitol production complicates the purification of xylitol. Thus, the use of glycerol may result in higher production costs. A new method of xylitol production that does not require a co-substrate would be advantageous.

The decrease of XDH activity in *C. tropicalis* may provide an alternative approach as the strain should be able to utilize xylitol without the need for a co-substrate. In this study, we engineered *C. tropicalis* strains with reduced XDH activity by reducing the XDH gene copy number and by creating site-directed mutations of the XDH gene. We assessed the efficiency of xylitol production in the mutant strains with reduced XDH activity.

Materials and methods

Site-directed mutagenesis of the *XYL2* gene

Genomic DNA from *C. tropicalis* ATCC 20336 was used as a template for PCR, and a mutation was introduced via overlapping PCR (Penning and Jez 2001). First-strand DNA was amplified with the primers 2XDH-FNde1 (5′-CCC ATA TGA CCC CAA ACC CAT CCT TAG-3′) and XDH-ARSdR-R (5′-AAC TTT CTG TCG GAT CTG GCA ACA ACC ATG ACT CTC TTG GCA C-3′), and second-strand DNA was amplified with the primers XDH-ARSdR-F (5′-TTG TTG CCA GAT CCG ACA GAA AGT TGA AGA TGG CCG GAG AC-3′) and 2XDH-REcoR2 (5′-ATG AAT TCT CTG GAC CGT CAA TCA AAC ATT TG-3′). As the final amplification step, the purified overlapping PCR products were used as templates, with 2XDH-FNde1 and 2XDH-REcoR2 as primers. The D207A/I208R/F209S/N211R (ARSdR) mutant of *XYL2* was cloned using a RBC TA cloning vector kit, and the resultant plasmid was designated as pRBC-ARSdR. The WT *XYL2* gene was amplified from *C. tropicalis* ATCC 20336 genomic DNA by PCR using the primers 2XDH-FNde1 and 2XDH-REcoR2. The WT *XYL2* was cloned into the RBC TA cloning kit. The resulting plasmid was designated as pRBC-2XDH.

Recombinant XDH expression, purification, and activity assay

Both pRBC-2XDH and pRBC-ARSdR were digested with *Nde*I and *Eco*RI and the fragments were ligated into pET21a, an expression vector harboring a *C*-terminal His-tag sequence. The respective plasmids were used to transform *E. coli* XL1 blue, and the selected plasmids containing WT *XYL2* and mutant *XYL2* were designated as pET-XDH and pET-ARSdR, respectively. These plasmids were used to transform *E. coli* BL21 for the expression of WT XDH and ARSdR XDH, respectively.

Cultures of the recombinant *E. coli* strains were sonicated to prepare crude enzyme solutions from which the XDH proteins were purified using nickel nitrilotriacetic acid–agarose (Qiagen). XDH activity was determined by monitoring the change in absorbance at 340 nm, reflecting NAD^+ reduction or NADPH oxidation, at 25°C. The XDH assay mixture contained 25 mM carbonate buffer (pH 9.5), 0.2 mM $NADP^+$, 20 mM xylitol, and enzyme solution. One unit of enzyme was defined as the amount of enzyme required to convert 1 μmol $NADP^+$ per min. Activity is reported as specific activity, units $(\text{mg protein})^{-1}$. Each measurement was repeated three times.

Construction and characterization of *C. tropicalis* strains with reduced XDH activity

A *XYL2*-disrupted mutant of *C. tropicalis*, BSXDH-3, was used as the host strain for yeast transformation (Ko et al. 2006c). The WT *XYL2* gene and ARSdR mutant *XYL2* gene were amplified from pRBC-2XDH and pRBC-ARSdR by PCR using the primers XDH-F (5′-ATG ACC CCA AAC CCA TCC TTA G-3′) and XDH-R (5′-GGA CCG TCA ATC AAA CAT TT G-3′), respectively. The PCR products were introduced into *C. tropicalis* BSXDH-3 via the lithium acetate method with a slight modification (Ito et al. 1983). The cells were spread on YNB-xylose plates

(6.7 g yeast nitrogen base without amino acids l^{-1}, 20 g D-xylose l^{-1}) and incubated for 3 days. To determine the intracellular XDH activity of the resultant yeast strains, enzyme solutions were prepared for each strain by sonicating 4.6 mg wt dry cell.

Xylitol fermentation experiments were performed by incubating the yeast in 250 ml Erlenmeyer flasks containing 50 ml xylitol fermentation medium (see Fig. 1) at 30°C with shaking at 180 rpm.

Analytical methods

Cell growth was monitored by the OD_{600}. Protein was measured by the Bradford method with BSA as standard. D-xylose, xylitol, and D-glucose were analyzed by HPLC with a Sugar–Pak I column (Waters) at 90°C with water as mobile phase at 0.5 ml min^{-1}.

Results and discussion

Engineering of recombinant XDH

The D207A/I208R/F209S/N211R (ARSdR) mutant of XDH from *Pichia stipitis* displays $NADP^+$-dependent activity (Watanabe et al. 2005), and XDH from *P. stipitis* and from *C. tropicalis* share high amino acid sequence identity (72%) (Ko et al. 2006a). Accordingly, it was anticipated that the approach used to engineer *P. stipitis* XDH could be applied to *C. tropicalis* XDH with similar results.

Four point mutations were introduced into the NAD^+-binding region of the *C. tropicalis* XDH gene by overlapping PCR, and this D207A/I208R/F209S/N211R mutant was designated as ARSdR. The ARSdR mutant *XYL2* gene was overexpressed in *E. coli*, and the recombinant protein was purified using Ni–NTA column chromatography. We determined the specific activity of the purified ARSdR mutant XDH for NAD^+ and $NADP^+$ (Table 1). The specific activity of the ARSdR enzyme for NAD^+ was only about 1% that of WT, whereas its specific activity for $NADP^+$ was about 48-fold that of WT. The specific activity of the ARSdR enzyme for $NADP^+$ was 34% that of WT XDH for NAD^+. This decrease in activity may be attributable to structural instability or reduced affinity for $NADP^+$ caused by the mutations.

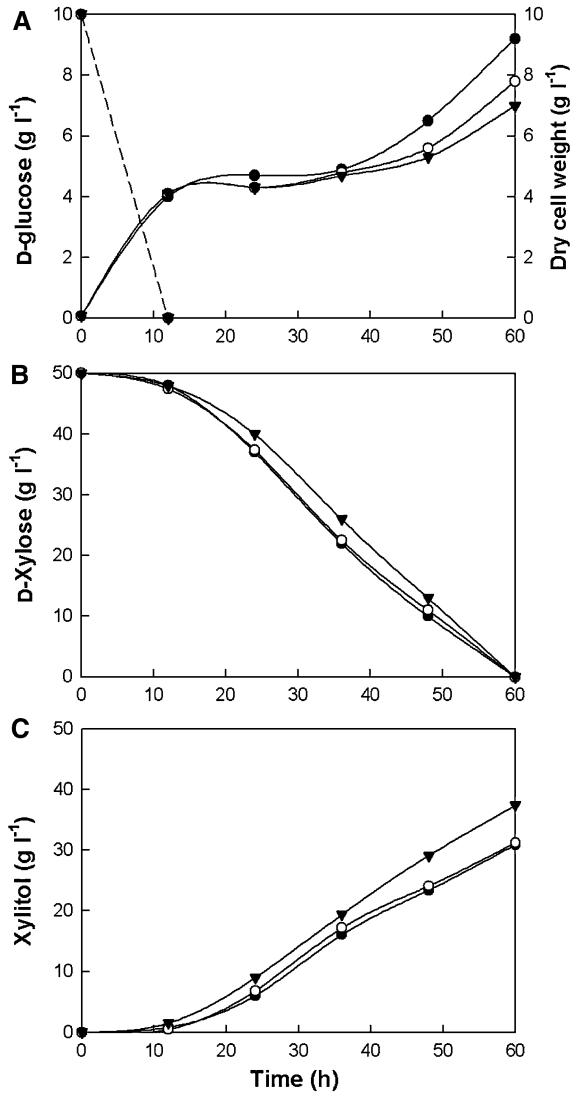

Fig. 1 Xylitol fermentation profiles of wild type, XDH-5 mutant, and ARSdR-16 mutant *C. tropicalis*. The fermentation medium for xylitol production consisted of 50 g D-xylose l^{-1}, 10 g D-glucose l^{-1}, 10 g yeast extract l^{-1}, 5 g KH_2PO_4, and 0.2 g $MgSO_4 \cdot 7H_2O$ l^{-1}. **a** Cell growth and glucose consumption profiles. *Solid* and *dashed lines* indicate the dry cell weight and glucose concentration, respectively. **b** D-xylose consumption profile. **c** Xylitol production profile. Symbols: *black circle* WT *C. tropicalis*, *white circle* XDH-5 mutant, *inverted black triangle* ARSdR-16 mutant

Construction and characterization of *C. tropicalis* strains with decreased XDH activity

To construct a yeast strain with decreased XDH activity, we introduced the ARSdR mutant XDH into BSXDH-3, a *XYL2*-disrupted mutant of *C. tropicalis*.

Table 1 Activities of wild type (WT) and ARSdR mutant xylitol dehydrogenase (XDH) specific for NAD^+ and $NADP^+$

Enzyme	Specific activity (U mg^{-1})		Activity ratio (%)[a]	Relative activity (%)[b]
	NAD^+	$NADP^+$		
WT	3.5 ± 0.02	0.025 ± 0.01	0.7 ± 0.05	100 ± 0.09
ARSdR	0.034 ± 0.001	1.2 ± 0.03	2.8 ± 0.08	34 ± 0.06

[a] The ratio of $NADP^+$-preferring to NAD^+-preferring activities of wild type XDH and the ratio of NAD^+-preferring to $NADP^+$-preferring activities of ARSdR XDH

[b] The specific activity of WT XDH for NAD^+ was used as the reference value

Table 2 Xylitol dehydrogenase (XDH) activities of the wild type (WT), XDH-5 mutant, and ARSdR-16 mutant of *Candida tropicalis*

Strain[a]	Specific activity (U mg^{-1})		$NADP^+/NAD^+$[b]	Relative activity (%)[c]
	NAD^+	$NADP^+$		
WT	1.9 ± 0.02	0.08 ± 0.04	0.043 ± 0.0004	100 ± 0.21
XDH-5	1.3 ± 0.02	0.037 ± 0.03	0.028 ± 0.0003	70 ± 0.12
ARSdR-16	0.052 ± 0.013	0.75 ± 0.032	14.6 ± 0.4	40 ± 0.11

[a] Yeasts were grown on YNB-xylose medium containing D-xylose as the sole carbon source

[b] The ratio of $NADP^+$-preferring to NAD^+-preferring activities of WT XDH, XDH-5, and ARSdR-16

[c] The XDH activity of WT *C. tropicalis* for NAD^+ was used as the reference value

We also constructed a transformant harboring the WT XDH gene as a control. The WT or ARSdR XDH gene integrated into the genomic DNA via homologous recombination at the position of the original WT *XYL2* gene prior to disruption. We selected one transformant expressing the WT XDH gene, designated as XDH-5, and one transformant expressing the ARSdR mutant gene, designated as ARSdR-16.

The XDH activities of the WT, XDH-5, and ARSdR-16 strains of *C. tropicalis* were determined after growth in xylose medium (Table 2). NAD^+-dependent XDH activity was detected in WT and XDH-5 *C. tropicalis*, whereas the ARSdR-16 mutant displayed $NADP^+$-dependent XDH activity. The NAD^+-preferring XDH activity of the ARSdR-16 mutant was only 7% of the $NADP^+$-preferring activity. The XDH activities of the XDH-5 and ARSdR-16 strains were 70 and 40% that of WT *C. tropicalis*, respectively. The activity of XDH-5 was reduced because this strain contains only one copy of the XDH gene versus two copies in WT *C. tropicalis*. In ARSdR-16, the mutated single copy of the XDH gene, which has only 34% of WT enzyme activity, accounts for its reduced XDH activity compared with WT activity. Furthermore, the growth rate of ARSdR-16 on YNB-xylose medium was slower than the growth of WT and XDH-5 (Fig. 2).

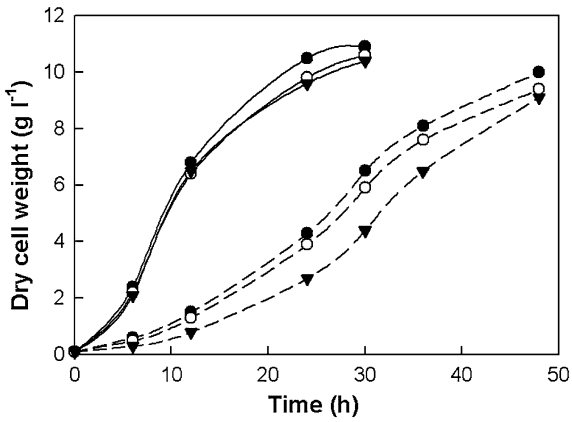

Fig. 2 Cell growth profiles of wild type, XDH-5 mutant, and ARSdR-16 mutant *C. tropicalis* on glucose or xylose medium. The medium consisted of 6.7 g yeast nitrogen base without amino acids and 20 g D-glucose or 20 g D-xylose l^{-1}. *Solid* and *dashed lines* indicate the growth profiles of cells grown on glucose medium and xylose medium, respectively. *black circle* WT *C. tropicalis*, *white circle* XDH-5 mutant, *inverted black triangle* ARSdR-16 mutant

Production of xylitol in recombinant yeasts

For xylitol production, the yeasts were grown in 250 ml flasks (Fig. 1). The WT, XDH-5, and ARSdR-16 *C. tropicalis* strains each produced xylitol using D-xylose as a substrate, with conversion yields of 62, 64, and 75%, respectively, and volumetric productivities of 0.52, 0.54, and 0.62 g l^{-1} h^{-1}, respectively. These results suggest that the reduction of net intracellular XDH activity in *C. tropicalis* caused an enhancement of xylitol production. This is likely explained by the drastically diminished ability to convert xylitol to xylulose owing to the reduced net intracellular XDH activity, using both NAD and NADPH. Despite this reduction, the XDH activity is still sufficient to allow for metabolism and growth without glycerol. In addition, the $NADP^+$-preference of ARSdR-16 XDH may be important for the enhanced xylitol production yield, because the additional NADPH regenerated by XDH activity could be supplied for xylitol production by xylose reductase.

The ARSdR-16 mutant achieved a 75% xylitol yield with no co-substrate such a glycerol. The absence of co-substrate after xylitol production may allow more cost-efficient purification of xylitol. Based on its high yield, high productivity, and cost-efficient process, the xylitol production method developed in this study appears to be the best production method available to date.

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