The Journal of Microbiology, June 2009, p. 351-357 Vol. 47, No. 3
DOI 10.1007/s12275-008-0225-9

Cloning, Expression, and Characterization of Xylose Reductase with Higher Activity from *Candida tropicalis*

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(Received September 16, 2008 / Accepted March 23, 2009)

Xylose reductase (XR) is a key enzyme in xylose metabolism because it catalyzes the reduction of xylose to xylitol. In order to study the characteristics of XR from *Candida tropicalis* SCTCC 300249, its XR gene (*xyl1*) was cloned and expressed in *Escherichia coli* BL21 (DE3). The fusion protein was purified effectively by Ni^{2+}-chelating chromatography, and the kinetics of the recombinant XR was investigated. The *Km* values of the *C. tropicalis* XR for NADPH and NADH were 45.5 μM and 161.9 μM, respectively, which demonstrated that this XR had dual coenzyme specificity. Moreover, this XR showed the highest catalytic efficiency (*kcat*=1.44×10^4 min^{-1}) for xylose among the characterized aldose reductases. Batch fermentation was performed with *Saccharomyces serivisiae* W303-1A:pYES2XR, and resulted in 7.63 g/L cell mass, 93.67 g/L xylitol, and 2.34 g/L·h xylitol productivity. This XR coupled with its dual coenzyme specificity, high activity, and catalytic efficiency proved its utility in *in vitro* xylitol production.

***Keywords*:** *Candida tropicalis*, xylose reductase, coenzyme specificity, catalytic efficiency

Xylose is a major pentose sugar that is found in hemicellulose, and it is one of the most abundant natural sugars in terms of biomass, as it is present in natural materials such as wood and agricultural residues (Ladisch *et al*., 1983). In xylose-assimilating yeast, xylose reductase (XR) (EC 1.1.1.21) catalyzes the reduction of xylose with concomitant NAD(P)H oxidation at the first step of xylose metabolism. Therefore, XR is important in fermenting xylose to produce xylitol. Based on sequence and structural similarities, yeast XRs were categorized into the aldose reductase (ALR) family, a member of the aldo-keto reductase (AKR) superfamily (Jez and Penning, 2001). They can be classified into two groups according to their coenzyme specificity (Mishra and Singh, 1993). Generally, XR is specific for NADPH, but in some cases, it can utilize both NADPH and NADH (Verduyn *et al*., 1985; Neuhauser *et al*., 1997; Hacker *et al*., 1999; Mayr *et al*., 2000), and may prefer NADH to NADPH (Lee *et al*., 2003).

Up to now, many XR genes have been characterized, and several of them have been cloned and expressed in a variety of hosts (Ho *et al*., 1990; Amore *et al*., 1991; Kuhn *et al*., 1995; Yokoyama *et al*., 1995b; Neuhauser *et al*., 1997; Hacker *et al*., 1999; Mayr *et al*., 2000; Kang *et al*., 2003; Lee *et al*., 2003; Nidetzky *et al*., 2003). In order to study their capability in fermentation, different cofactor specificities were proposed to maintain the redox balance between nicotinamide cofactors under a variety of growth conditions (Hahn-Hagerdal *et al*., 2001; Nidetzky *et al*., 2003), and the *C. tropicalis* strains exhibited the highest yield and rate of xylitol production (Oh and Kim, 1998). However, most reported XRs from *C. tropicalis* are NADPH-dependent, such as the reported XRs from *C. tropicalis* IF0 0618 which were clearly determined to be NADPH-dependent. In addition, catalytic efficiency is another significant parameter in xylitol production. However, the catalytic efficiencies of all of the reported XRs from other microorganisms, including those of *C. tropicalis*, are not very high (Oh and Kim, 1998). Therefore, screening for XRs with high catalytic efficiency is becoming more important for increasing the xylitol productivity.

In our previous work, we performed screening to determine the capacities of xylitol production in different yeast strains, and found that *C. tropicalis* SCTCC 300249 has relatively high xylitol production. In this study, in order to characterize the XR from *C. tropicalis* SCTCC 300249, a xylose reductase gene (*xyl1*) was cloned from the strain and expressed heterologously in *Escherichia coli* BL21(DE3). Purification was performed, and the purified XR was finally characterized. The XR showed dual coenzyme specificity, high activity, and, most importantly the highest catalytic efficiency among the XRs characterized thus far. The *xyl1* gene was subsequently subcloned into the pYES2 vector and transformed into *S. cerivisiae* W303-1A to evaluate its capability in xylitol production.

Materials and Methods

Strains and plasmids

C. tropicalis SCTCC 300249 strain was used as a source of the *xyl1* gene. *E. coli* BL21 (DE3) and *S. cerevisiae* W303-1A (His^-, Leu^-, $URA3^-$, Met^-) were used as host strains for XR gene expression. pMD18-T (TaKaRa, Japan) was used as a cloning vector, and the expression vectors used in this work

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were pET32a (Novagen, USA) and pYES2 (Invitrogen, USA).

Media and cultivation procedures

C. tropicalis SCTCC 300249 was cultivated in a 250 ml Erlenmeyer flask containing 50 ml YPX (the same as the YPD, except for the substitution of glucose by xylose) medium at 30°C. For electrotransformation, YPD medium and SC-U (prepared as described in the pYES2 manuals, http://tools.invitrogen.com/content/sfs/manuals/pyes2_man.pdf) agar media were used. Xylitol fermentation medium (xylose 100 g/L, glucose 10 g/L, yeast extract 10 g/L, KH_2PO_4 5 g/L, $MgSO_4 \cdot 7H_2O$ 0.2 g/L) was used for batch cultivation. *E. coli* was cultivated in Luria-Bertani medium at 37°C.

Cloning of *xyl1* gene from *C. tropicalis* SCTCC 300249

Total RNA was isolated from *C. tropicalis* SCTCC 300249 using the Trizol reagent, and the cDNA was synthesized with a Reverse Transcriptional kit (TaKaRa). Based on the two reported *xyl1* gene sequences of *C. tropicalis* IF0 0618, which were published on NCBI (Accession No: AB002106 and AB002105), primers P1; 5'-TCATCAATTACATACTATA GAACCAT-3' and P2; 5'-TAAACAAACACAGATCTTATTG AATT-3' were designed and used for amplification. The *Ex Taq*™ DNA Polymerase (TaKaRa) was used for amplification. The amplified DNA fragment that was extracted using a DNA Gel Extraction kit (TaKaRa) was ligated into the pMD18-T vector and sequenced at Invitrogen. The P3; 5'-CGGATCC ATGTCTACTACTG TTAATACT-3' and P4 primers; 5'-GC TCGAGTTAAACAAAGATTGGAATGT-3', which contained *Bam*HI and *Xho*I restriction sites (underlined), respectively, were used to clone the ORF of the *xyl1* gene and the previously noted DNA polymerase was used. The amplified ORF of the *xyl1* gene was digested with *Bam*HI (TaKaRa) and *Xho*I (TaKaRa). The digested fragment was purified and ligated into the pET32a and pYES2 vectors to generate the recombinant expression vectors, named pET32aXR and pYES2XR, respectively. The ligated sequences were confirmed by sequencing.

Protein expression and purification

Plasmid pET32aXR was transformed into *E. coli* BL21 (DE3) competent cells, and one of the positive colonies was inoculated into LB medium containing 50 μg/ml ampicillin and grown at 37°C. When OD_{600} reached 0.6, isopropyl-α-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.3 mM and incubation was continued for 4 h. The cells were harvested and resuspended in phosphate buffered saline (PBS), and were then lysed by sonication. The supernatant and lysate pellet fractions were separated by centrifugation at 10,000×g for 10 min. The supernatant was subsequently loaded onto Ni^{2+}-chelating column (Amersham), and bound proteins were then eluted from the column by a gradient of 5 to 200 mM imidazole in PBS buffer at a flow rate of 100 ml/h. All of the fractions flowing through the column were analyzed by SDS-PAGE, and the purified fusion protein was dialyzed against PBS for desalting. The resulting recombinant XR, which carried an artificial sequence of 43 amino acids, was cleaved with enterokinase at 25°C for 8 h. The reaction mixture was then loaded onto the Ni^{2+}-chelating column again (Cao *et al*., 2005). The purified protein was frozen in 10% glycerol (Woodyer *et al*., 2005) at -70°C. Protein concentrations were determined by the Bradford method (Bradford, 1976), with bovine serum albumin (BSA) as the protein standard.

Optimum pH, thermal, and chemical stability

The activity of recombinant XR was determined spectrophotometrically by monitoring the change in *A*340 upon oxidation of NAD(P)H in a spectrophotometric cuvette at room temperature. All spectrophotometric measurements were performed using a Beckman DU-650 instrument. Unless otherwise indicated, the XR assay mixture contained 100 mM potassium phosphate buffer, 0.01 mM 2-mercaptoethanol, 0.2 M D-xylose, 0.1 ml enzyme solution and 0.15 mM NAD(P)H (Yokoyama *et al*., 1995b). The reaction was started by the addition of 0.1 ml enzyme solution, and the reaction mixture was allowed to stand for 1 min to eliminate the the endogenous oxidation of NAD(P)H. One unit (U) of enzyme was defined as the amount of enzyme that caused a decrease of 1 μmol NAD(P)H per minute. Specific activity was expressed as units per milligram of protein (Yokoyama *et al*., 1995b).

The optimal temperature was studied by assaying XR activities at different temperatures ranging from 15 to 65°C. Its thermal stability was investigated at 60°C. The effects of pH on the enzyme activities and stabilities were evaluated at a pH range from 3.0 to 8.0 (Luciane *et al*., 2001), and the specific activity of recombinant XR in the presence of several chemicals, including 1 mg/ml BSA, 1 mM $CuSO_4$, 1 mM NaCl, 1 mM $MgSO_4$, and 1 mM DTT was measured.

Kinetics assay

Kinetic parameters for xylose were obtained from the initial velocity measured at two constant levels of NADPH (0.1 mM, 0.15 mM) using xylose as the varied substrate. The kinetic parameters for NAD(P)H were determined as previously indicated, except using xylose (0.1 M, 0.2 M) as constant and NAD(P)H as variable. Furthermore, the activities of the XR for several substrates were assayed with NADPH as the cofactor. All of the measurements were performed at optimal pH and optimal temperature. The results presented showed the means of triplicate assays, and all pass the test of significance at a significance level of 0.05.

Electrotransformation of yeast and xylitol fermentation

The expression vector pYES2XR, which contains the *xyl1* gene was transformed into *S. cerevisiae* W303-1A by electroporation (Becker and Guarente, 1991). After electric pulsing, the cells were added to 1 ml ice-cold YPD immediately and transferred into an Eppendorf tube. They were then incubated for 30~60 min at 30°C and plated as 100 μl aliquot on SC-U agar medium. The transformants were incubated at 30°C for 3 days, and the positive transformants were confirmed by PCR and designated *S. cerevisiae* W303-1A: pYES2XR.

The inoculum was prepared by transferring a loopful of cells from 3-day-old YPD agar medium into 20 ml of medium in a 100 ml flask plugged with foam, and was cultivated by shaking at 200 rpm for 48 h at 30°C. After a subsequent preculturing period in larger flasks (500 and 1,000

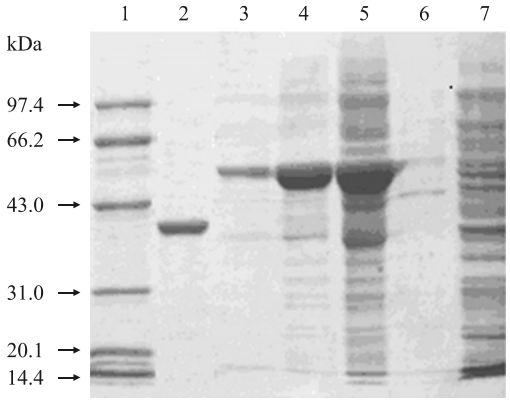

Fig. 1. Purification of heterologously expressed XR-His_6-tagged fusion protein. Lanes: 1, the molecular weight maker; 2, the final purified protein; 3, 4, 5, 6, protein eluted from the column by elution buffer containing 200 mM, 50 mM, 10 mM, 5 mM imidazole, respectively; 7, the flow through during purification.

ml), the cultures were centrifuged, washed twice with distilled water and used as inoculum. Batch cultures were carried out in a 5 L fermenter (BXBIO, China) with a 3 L working volume at 30°C. The agitation speed was set at 400 rpm, and an aeration rate of 1 vvm was maintained throughout the culture. Medium acidity was controlled at pH 5.0 by the addition of 1 M HCl or 1 M NaOH.

The final xylitol concentration, residual xylose, and glucose were analyzed by HPLC (Waters, USA) using a Hypersil NH2 column (4.6 mm i.d.×150 mm, 5 μm. Elite, China) with acetonitrile:water [83:17 (v/v)] as mobile phase at a column temperature of 30°C and a flow-rate of 1.0 ml/min. After washing with 50 ml of distilled water, the material was dried (105°C; 3 h). Dry cell mass were measured by filtering a 5.0 ml sample through a Millipore1 membrane (pore diameter=1.2 μm).

Results and Discussion

Cloning and sequence analysis of *xyl1* gene from *C. tropicalis* SCTCC 300249

Using primers P1 and P2, an ~1.1 kb DNA fragment was successfully amplified. The resultant sequence was cloned into the pMD18 vector and then sequenced prior to submission to the BLAST server (http://www.ncbi.nlm.nih.gov/BLAST/) for alignment. The alignment showed that the sequence had high homology (≥76% identity) with other XRs from *Candida* spp. yeasts, and it comprised an entire open reading frame (ORF). Based on this information, we postulated that the DNA fragment contained the ORF of the *xyl1* gene. In the following PCR, an ORF of the 975 bp *xyl1* gene from *C. tropicalis* SCTCC 300249 was cloned using primers P3 and P4, and its nucleotide sequence has been deposited at GenBank under accession number EU273285. The alignments for deduced amino acid sequences were performed between the XR from the present research and those previously reported in *C. tropicalis* (Yokoyama *et al*., 1995a), where the present XR with several altered amino acids showed significant homology (>97%) with those reported previously. In addition, these differences in sequence cannot be attributed to diverse mRNA splicing or post-translational modifications in *C. tropicalis*. Therefore, the XR in this paper must be novel, and is clearly different from the previously reported *C. tropicalis* XRs.

Protein expression and purification

The *xyl1* gene was cloned into pET32a and expressed in *E. coli* BL21(DE3) under IPTG induction. Compared with the uninduced cells, the IPTG-induced cells resulted in an additional protein band on SDS-PAGE gel, and expressed XR in a soluble form, which accounted for nearly 50% of the total cellular protein. The XR fusion protein had an enterokinase cleavage site immediately upstream to XR, which allowed the intact XR to be released from the purified fusion protein. The purified soluble fusion XR on SDS-PAGE was approximately 57 kDa (Fig. 1), which is consistent with its theoretical molecular weight. After cleavage with enterokinase and purification by a secondary pass through the Ni^{2+}-chelating column, the recombinant XR was obtained on SDS-PAGE gel with a molecular mass of around 37.1 kDa (Fig. 1). By means of this purification procedure, as shown in Table 1, a 7.1-fold purified XR was obtained with a recovery of 64.5% and a specific activity of 251.5 U/mg of protein.

Optimal pH, thermal, and chemical stabilities

The optimal pH for the turnover of xylose was determined by assaying the XR activities at different pH, ranging from 3.0 to 8.0. As shown in Fig. 2A, the optimal pH was around pH 5.5, slightly lower than that of XRs from *C. tropicalis* IF0 0618. The XR showed more tolerance to changes in pH, and more than 60% of the activity was retained in a pH range from 4.0 to 6.5. In order to determine the optimal temperature range for activity, the purified recombinant XR was treated at various temperatures, ranging from 15°C to 65°C. As shown in Fig. 2B, we can see that at higher temperatures, the enzyme was rapidly inactivated, while at lower temperatures, the inactivation rate decreased. The optimal temperature was approximately 45°C. After incubating XR at 60°C for 1 h, 80% of its activity was lost, with a half-life of around 15 min (Fig. 2C). Based on these findings, we

Table 1. Purification of xylose reductase from the cell extract of *C. tropicalis* SCTCC 300249

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg of protein)[a]	Purification (fold)	Yield (%)
Crude extract	119.4	4213.6	35.3	1	100
Purified enzyme	10.8	2716.2	251.5	7.1	64.5

[a] The specific activity of XR in this work was carried out at optimal pH and temperature, and with NADPH as a cofactor.

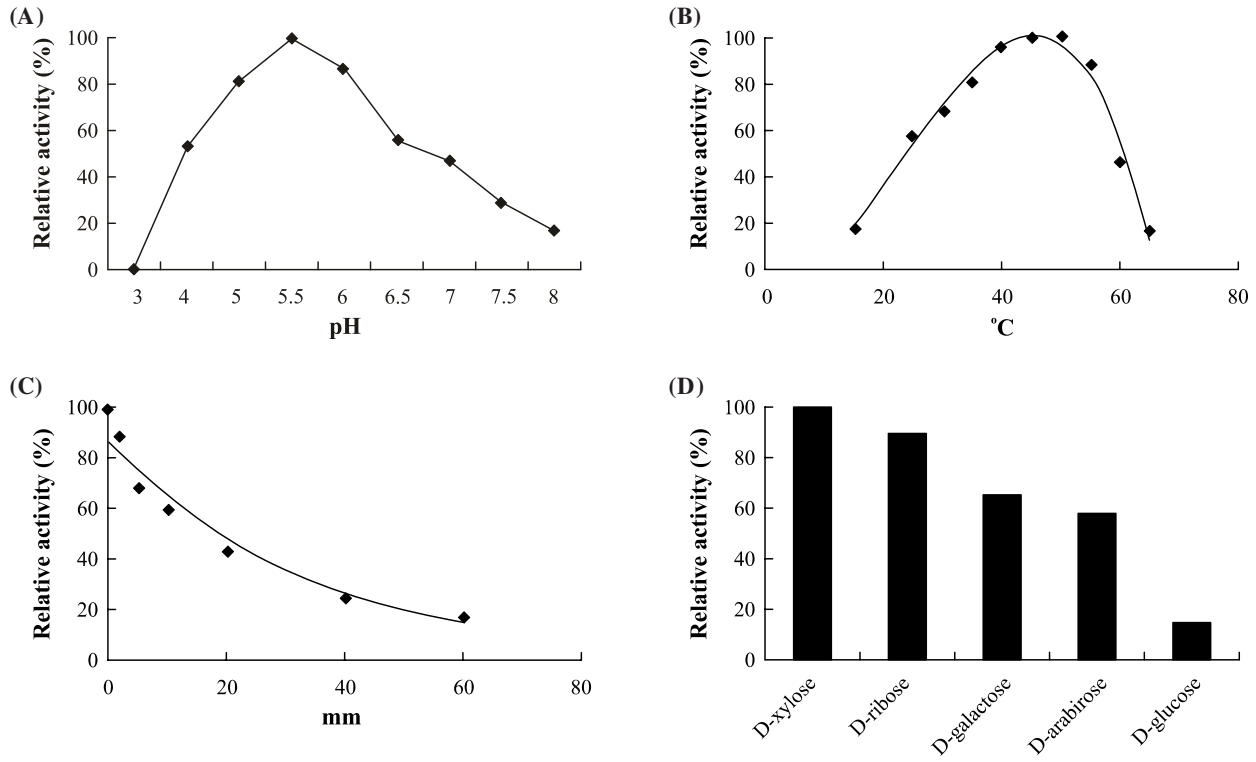

Fig. 2. The activities of XR were all determined with 0.2 M xylose and 0.15 mM NADPH. (A) Profile of pH effects on the XR activity. XR was assayed in a PBS buffer at a pH ranging from 3.0 to 8.0. (B) Thermal profile. The optimal temperature was determined by assaying the XR at pH 7.0 with different temperatures ranging from 15 to 65°C. (C) Thermal inactivation of XR was performed at 60°C. (D) Substrate specificity of XR was carried out at pH 5.5 and 45°C. The rate of xylose turnover was taken as 100%.

determined that the present XR showed more tolerance to pH and temperature, and these properties may act as an advantage in xylitol fermentation, especially when the fermentation is carried out with hemicellulose hydrolysate as its substrate.

In addition, the impacts of several chemicals on the activity of XR were also evaluated. XR activity increased by approximately 20% when bovine serum albumin was added at a concentration of 1 mg/ml, and its activity increased by 27% in the presence of 1 mM DTT, which suggested that sulfhy-

Table 2. Properties of XR from various organisms

Organism	*Km* for xylose (mM)[b]	*kcat* for xylose (min^{-1})[b]	*kcat*/*Km* for xylose (min^{-1} mM^{-1})[b]	*Km* for NADPH (μM)	*Km* for NADH (μM)
C. tropicalis[a] (this work)	31.5±3.7	14400±300	457.1	45.5±5.2	161.9±7.6
C. tropicalis (Yokoyama *et al.*, 1995b)	30-36	NR[d]	NR[d]	9-18	ND[e]
N. crassa[a] (Woodyer *et al.*, 2005)	34±4	3600±200	110	1.8±0.5	16±4
C. parapsilosis (Lee *et al.*, 2003)	31.5[c]	2835[c]	90[c]	36.5	3.3
P. stipitus (Verduyn *et al.*, 1985)	42	1500	36	9	21
C. tenuis (Neuhauser *et al.*, 1997; Hacker *et al.*, 1999)	72	1300	18	4.8	25.4

[a] Each value represents the Mean±SEM from three independent experiments
[b] With NADPH as a cofactor
[c] With NADH as a cofactor
[d] NR: not reported
[e] ND: not detectable

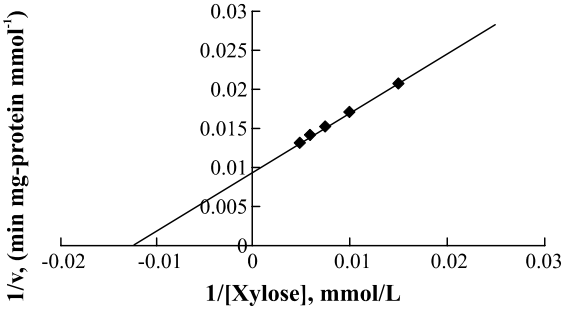

Fig. 3. Profile shows the Lineweaver-Burk plot of initial velocity versus various fixed xylose concentrations. The kinetics were determined by assaying the XR activities using the NADPH as a constant and xylose as a variable. Each value presented indicated the mean of triplicate assays, and the significance of each group was tested at a significance level of 0.05.

dryl compounds allow the enzyme to remain active in a reduced state. However, the activity of XR did not show any increase in the presence of 1 mM NaCl and 1 mM $MgSO_4$. It is even more unfortunate that the Cu^{2+} ions displayed an inhibitory effect on XR, as reported in *C. parapsilosis* (Lee and Kim, 2003).

Enzyme assay and kinetic parameters

The activity of the XR for several substrates was assayed with NADPH as a cofactor, and a profile was obtained (Fig. 2D). Fig. 2D shows that the activity for xylose was the highest among the five substrates examined, followed by D-ribose and D-galactose, while the XR was less active for D-arabirose and D-glucose. This suggested that pentose sugars acted as the best substrates, and this phenomenon was consistent with those of the reported XRs isolated from other sources (Kuhn *et al*., 1995; Neuhauser *et al*., 1997; Lee *et al*., 2003).

As shown in Fig. 3, the *Km* and v_{max} values of XR for xylose were evaluated by means of Michaelis-Menten kinetics, and were found to be 31.5 mM and 105.3 mmol/min·mg, respectively. Table 2 presents the characteristics of the XRs from *Candida tropicalis*, *Neurospora crassa*, *Candida parapsilosis*, *Pichia stipitus*, *Candida tenuis* (Verduyn *et al*., 1985; Yokoyama *et al*., 1995b; Neuhauser *et al*., 1997; Hacker *et al*., 1999; Lee *et al*., 2003; Woodyer *et al*., 2005), and those in the present study. The XRs from *C. tropicalis* are composed of two identical subunits (Yokoyama *et al*., 1995b; Lee *et al*., 2003), resulting in higher *Kcat* values compared with those from other microorganisms. As shown in Table 2, the *Kcat* and *Kcat*/*Km* of XR in this work with respect to xylose were 1.44×10^4 min^{-1} and 457.1 min^{-1} mM^{-1}, respectively, which were more than 4-fold that of the reported XR from *Neurospora crassa*, and were also higher than those of the other reported XRs. The parameters of *Kcat* and *Kcat*/*Km* for xylose were important in terms of the rate of xylose turnover and xylitol accumulation, Thus, this work indicated that XR is promising for its use in xylitol production. The *Km* values for NADPH and NADH were determined in the same manner, and were found to be 45.5 μM and 161.9 μM, respectively. Unlike the reported XRs from other *C. tropicalis* studies, the recombinant XR in this work exhibited dual coenzyme specificity.

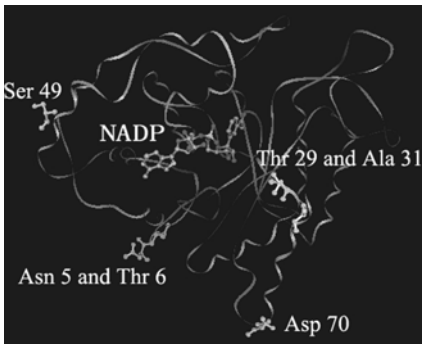

Fig. 4. The homology model of *C. tropicalis* XR with bound NADPH was built using the Insight II and MOE programs, and the changed amino acids and bound cofactor are displayed as balls and sticks.

Using the coordinates [Protein Data Bank (www.pdb.org)] for XR from *Candida tenuis* (PDB accession code 1MI3) (Kavanagh *et al*., 2002), a homology model was created using Insight II software, and the model was docked with NADPH by using the Molecular Operating Environment (MOE) program (Woodyer *et al*., 2005). The resulting model is depicted in Fig. 4, from which we can see that these changed amino acids are not within or close to the coenzyme-binding region. However, this is only a theoretical model, and the crystallographic characters of XRs from *C. tropicalis* have not yet been assayed. Therefore, we cannot definitively determine that those changed amino acids have no effect on coenzyme binding. In addition, the protein in our study is a recombinant XR, and the reported XRs from *C. tropicalis* IF0 0618 are native proteins. Furthermore, this phenomenon was also shown between the native protein from *N. crassa* NCIM 870 (Rawat and Rao, 1996) and the heterologously expressed fusion protein from *N. crassa* 10333 (Woodyer *et al*., 2005).

Xylitol production with *S. cerivisiae* W303-1A:pYES2XR

Catalytic efficiency was an important parameter in terms of the rates of xylose turnover and xylitol accumulation. In order to confirm that the purified enzymes have high catalytic efficiency, the XR gene was subcloned into the pYES2 vector and overexpressed in *S. cerevisiae* W303-1A. Batch fermentation was performed with *S. cerevisiae* W303-1A:pYES2XR in a 5 L fermenter containing 3 L xylitol fermentation medium. The experimental results are shown in Fig. 5, from which we can see that glucose was an essential co-substrate for supplying energy sources and was necessary for endogenous metabolism. Upon the depletion of glucose, xylose began to be taken up and converted into xylitol, after which the xylitol accumulated. As a result, xylitol was produced at up to 93.67 g/L at 40 h, and a xylitol yield of about 0.94 g/g (xylitol/xylose) was obtained. Meanwhile, the cell mass reached a maximum of 7.63 g/L. Additionally, a high xylitol productivity of 2.34 g/L·h was obtained in the batch fermentation at 40 h, which may result from the high catalytic efficiency of XR in this work.

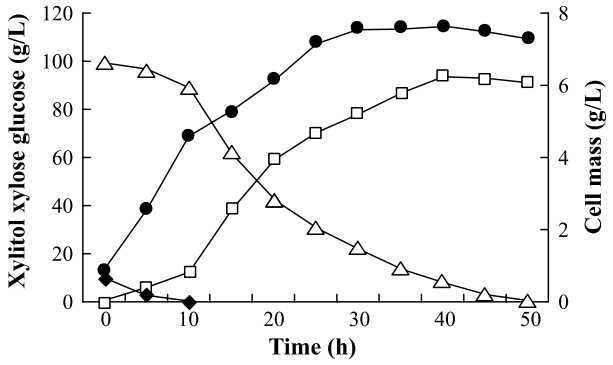

Fig. 5. Fermentation profile of *S. cerevisiae* W303-1A:pYES2XR, which was determined in batch xylose fermentations using glucose as a co-substrate. The results are representative of three replicates, of which the differences between the results were less than 5%. Symbols: (□) Xylitol, (▲) Xylose, (◆) Glucose, (●) Dry cell mass.

As shown in Table 2, the present XR displayed high catalytic efficiency, while in the batch fermentation performed with the recombinant *S. cerevisiae* strain, the xylitol productivity was lower than that of natural and engineered *Candida tropicalis* (Yahashi *et al*., 1996; Lee *et al*., 2003). These differences may occur for the following reason. As far as we know, coenzymes such as NADH and NADPH are essential for the enzymatic reduction of xylose with xylose reductase. However, the *Km* values of the present XR for coenzymes were higher than those of the other XRs (Table 2). In other words, this XR had a lower affinity for coenzymes, which might have an impact on the rate of xylose turnover and lower the xylitol productivity in the fermentation. However, regardless of what factors are responsible for these results, the xylitol productivity obtained in the fermentation performed with the recombinant *S. cerevisiae* was considerable.

In conclusion, the XR in this work exhibited dual coenzyme specificity, tolerance to wide pH range, and good thermal stability. More importantly, it showed a remarkably high catalytic efficiency, as shown in the batch fermentation. In addition, the results of this work provide a basis for further study in coenzyme specificity, and this XR can also be applied as an appropriate biocatalyst in xylitol production.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (NSFC 30740055, 30771312, 30871321), and the Foundation of Sichuan Province, China (04NG002-006-1).

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