

Cloning and nucleotide sequence analysis of xylose reductase (XR) gene from thermotolerant methylotrophic yeast *Ogataea siamensis* N22

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ABSTRACT

The xylose reductase (XR) gene from thermotolerant methylotrophic yeast isolated in Thailand, *Ogataea siamensis* N22, was isolated using specific primers designed from the conserved regions of various yeast XR genes. A partial XR gene of *O. siamensis* N22, was obtained as a 516 bp PCR product and the deduced amino acid sequence showed 74% identity to aldose reductase gene of *Candida boidinii* and 67% identity to xylose reductase gene of *Kluyveromyces lactis* and *Pichia guilliermondii*. The 1-3 kb fragments of digested genomic DNA of *O. siamensis* N22 was cloned and transformed into *Escherichia coli*. The recombinant plasmid containing the partial nucleotide sequence of XR gene was obtained using PCR screening. Nucleotide sequence analysis of the partial *O. siamensis* N22 XR gene revealed 678 bp encoding 226 amino acids which showed 72% identity to aldose reductase from *Candida boidinii* and 69% identity to xylose reductase from *Pichia guilliermondii*. This is the first report on xylose reductase gene of *O. siamensis* N22.

Keywords: xylose reductase, gene cloning, *Ogataea siamensis*, thermotolerant yeast

INTRODUCTION

Xylose reductase (XR), the first enzyme of yeast xylose metabolism, is responsible for catalysis of xylose to xylitol with the use of either NADPH or NADH as a cofactor. However, the preference for NADPH was reported (Karhumaa *et al.*, 2007). Xylitol is subsequently oxidized to xylulose by xylitol dehydrogenase (XDH), the second enzyme of the pathway, which was found to exclusively use NAD⁺ as a cofactor. In the last step, xylulose is phosphorylated by xylulokinase (XK) to yield

xylulose-5-phosphate, channeled through the pentose phosphate pathway (Walfridsson *et al.*, 1997).

Yeasts those are capable to utilize xylose for their growth have been reported such as *Candida boidinii*, *C. shehatae*, *C. tropicalis*, *C. parapsilosis*, *C. tenuis*, *Kluyveromyces lactis*, *Pichia guilliermondii*, *P. stipitis*, and *Pachysolen tannophilus* (Billard *et al.*, 1995; Ko *et al.*, 2006; Vongsuvanlert and Tani, 1989). In addition, several xylose (aldose) reductase genes from yeasts have been cloned and characterized such as those of

Candida parapsilosis (Lee *et al.*, 2003), *C. tropicalis* (Yokohama *et al.*, 1995), *Kluyveromyces lactis* (Billard *et al.*, 1995), *Pachysolen tannophilus* (Bolen *et al.*, 1996) and *Pichia stipitis* (Takuma *et al.*, 1991). However, *Ogataea siamensis* N22 used in this study is a novel species of thermotolerant methylotrophic yeast that are able to utilize methanol as sole carbon source at high temperature (Limtong *et al.*, 2004; Limtong *et al.*, 2008). Characterization of genetic information particularly on xylose utilization pathway from this strain is therefore still unrevealed. Furthermore, it has been shown that *O. siamensis* N22 was a potent yeast strain for xylitol production from xylose at high temperature as 37°C (unpublished data).

The present report was aimed to isolate and characterize xylose reductase gene (*XR* gene) from *O. siamensis* N22, the thermotolerant methylotrophic yeast, newly isolated in Thailand. The *XR* gene isolation was performed by PCR amplification using specific designed primers followed by genomic cloning to obtain the full length of *XR* gene. As a result, the first report on nearly complete nucleotide sequence of the *XR* gene from *O. siamensis* N22 is presented.

MATERIALS AND METHODS

Microorganisms, media and DNA isolation

Ogataea siamensis N22, a source of the *XR* gene, were grown on YPD broth at 37°C. Yeast genomic DNA was isolated using the *Pichia* protocols described by Higgins and Cregg (1998). *Escherichia coli* XL1-Blue was used as a host for *XR* gene cloning. The wild-type and recombinant *E. coli* cells were grown on Luria-Bertani (LB) medium at 37°C supplemented with 100 µg/ml ampicillin as appropriate. Bacterial plasmid was extracted by the alkaline lysis method (Sambrook and Russell, 2001).

Isolation of Ogataea siamensis N22 xylose reductase gene

Xylose reductase gene (*XR* gene) was amplified by PCR directly from the genomic DNA of *O. siamensis* N22. A pair of specific primers for *XR* gene was designed from the alignments of xylose reductase amino acid sequences of various yeast species from GenBank database. The primers designated as F1 [(5' TT(C/T)GGCTG(C/T)TGGAAGGTCG 3')] and R2 [(5' TTG(C/T)TGCAAGTA(A/T)GGGTGGTGT 3')]. Identification of *XR* gene was performed by gradient PCR amplification (PX2 Thermo Cycle, Thermo electron corporation, USA.) using the specific primers. The PCR reaction mixture containing 20 ng of genomic DNA, 10 pmol of each primers, 2 µl of 10x PCR buffer (contained 15 mM MgCl₂), 0.5 U of *Taq* DNA polymerase, 0.02 mM of each dNTP and nuclease free water in the total volume of 20 µl was set up. The reaction was carried out at 94°C for 4 min followed by 30 cycles of 94°C for 1 min, 45-57°C for 1 min, 72°C for 1 min and the last cycle with 72°C for 4 min. PCR product was determined by electrophoresis and purified by Gel/PCR DNA Fragments Extraction Kit (Geneaid, Taiwan) for further nucleotide sequencing.

Cloning of Ogataea siamensis N22 xylose reductase gene

Genomic DNA of *O. siamensis* N22 was prepared and partial digested by *Bsp*143I. The digested DNA fragments appeared about 1-3 kb in size were collected and purified using QIAquick Gel Extraction Kit (QIAGEN, USA) and subsequently cloned into the pJET1.2/ blunting vector (CloneJET™ PCR cloning kit, Fermentas, USA), and were then introduced into *Escherichia coli* XL1-Blue competent cells prepared as described by Chung *et al.* (1989). Recombinant colonies appeared on LB agar medium containing 100 µg/ml ampicillin after incubation at 37°C were collected and subjected to plasmid DNA preparation using the alkaline lysis method

(Sambrook and Russell, 2001). *XR* gene insertion was screened by PCR amplification using the specific primers F1 and R2. The recombinant plasmids containing *XR* gene were analyzed by nucleotide sequencing.

Nucleotide sequence analysis of Ogataea siamensis N22 XR gene

O. siamensis N22 *XR* gene was analyzed for nucleotide sequence and deduced amino acid sequence. The protein was compared with related xylose (aldose) reductases available in the GenBank database using the BLAST program (<http://www.ncbi.nlm.nih.gov/blast.cgi>).

RESULTS AND DISCUSSION

Isolation of Ogataea siamensis N22 xylose reductase gene

XR gene from genomic DNA of *O. siamensis* N22 was amplified by gradient PCR using the designed primers F1 and R2. A 516 bp fragment of PCR product was obtained within the

T_a ranged 45.4–48.5°C (Fig. 1). The PCR product was then purified and subjected to DNA sequencing.

The nucleotide sequence of a 516 bp fragment was obtained and revealed a partial *XR* gene of *O. siamensis* N22 encoding 171 amino acid residues. The *XR* amino acid sequence appeared closely relate to the xylose (aldose) reductase of *Candida boinidii* (accession no. AAL47846) with 74% identity and 86% similarity, *Kluyveromyces lactis* (accession no. XP_454929) with 67% identity and 80% similarity, and *Pichia guilliermondii* (accession no. AAD09330) with 67% identity and 81% similarity.

Cloning of Ogataea siamensis N22 xylose reductase gene

Partial digestion of *O. siamensis* N22 genomic DNA was performed using *Bsp*143I. About 1–3 kb fragments were purified and ligated into pJET1.2/blunting vector prior to *E. coli* XL1-Blue transformation. As a result, 1308 recombinant clones were obtained on LB plates containing 100

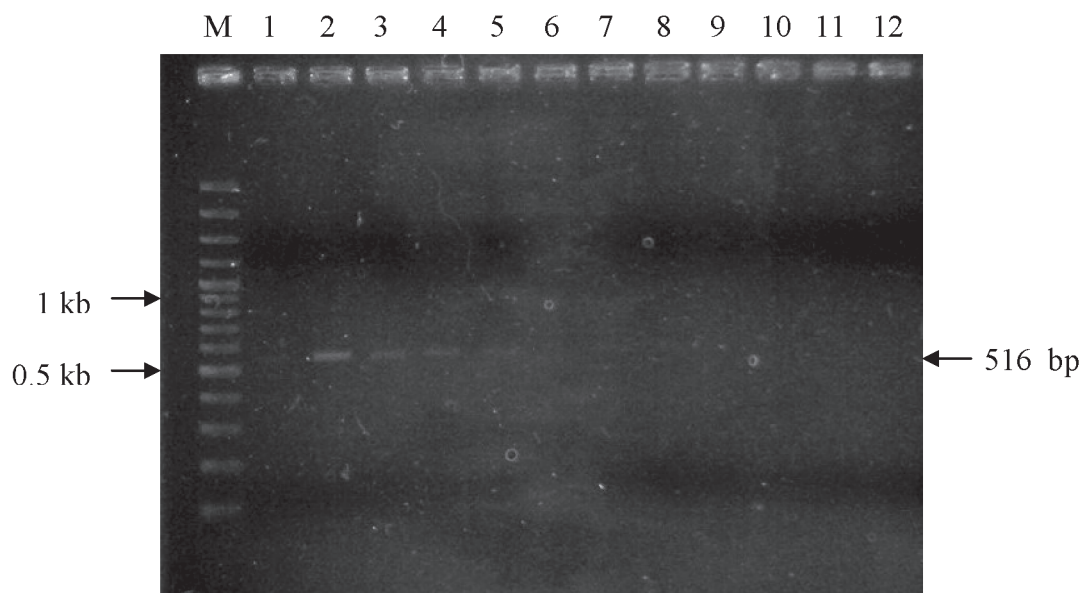


Figure 1 Isolation of an *XR* gene from *O. siamensis* N22 using gradient PCR with F1 and R2 primers at varied T_a . M, 100 bp DNA ladder; 1–12, T_a at 45.1, 45.4, 46, 47.2, 48.5, 50.1, 51.6, 53, 55.1, 56.2, 56.8 and 57.2°C, respectively.

µg/ml ampicillin. The recombinant plasmids of all clones were investigated by PCR screening using F1 and R2 primers. Results shown in Figure 2 indicated that clone no. 208 contained the *XR* gene insertion as the expected size of PCR product was observed (Fig. 2A). The recombinant plasmid of the clone no. 208 designated as pJET1.2/blunt-XR208 was digested with *Bgl*III and analyzed by gel electrophoresis. As a result, a 1.2 kb insertion fragment appeared in pJET1.2/blunt-XR208 (Fig. 2B).

***Nucleotide analysis of Ogataea siamensis* N22 xylose reductase gene**

Nucleotide sequence of *O. siamensis* N22 *XR* gene was analyzed. Results indicated that the DNA fragment from pJET1.2/blunt-XR208 which was expected to contain an entire *XR* gene, possessed 1,265 nucleotides. Analysis of an open reading frame (ORF) indicated that the partial sequence of *O. siamensis* N22 *XR* gene consisted of 678 nucleotides encoding 226 amino acids (Fig. 3). Comparing the amino acid sequences to various yeast xylose reductases in GenBank database revealed that xylose reductase of *O. siamensis* N22

possessed 72% and 69% identity with 85% and 84% similarity to xylose (aldose) reductases of *Candida boidinii* (accession no. AAL47846) and *Pichia guilliermodii* (accession no. AAD09330), respectively. The nucleotide sequence reported here has been submitted to GenBank database and assigned to the accession number FJ763639.

CONCLUSION

This is apparently the first report on cloning and characterization of xylose reductase gene from thermotolerant methylotrophic yeast, *O. siamensis* N22, isolated in Thailand. *O. siamensis* N22 is a potent yeast strain for xylitol production from xylose at high temperature. A partial *XR* gene of *O. siamensis* N22 consists of 864 nucleotides encoding for 287 amino acids. Investigation of the full sequence has been carrying out. Thereafter, an expression of *O. siamensis* N22 *XR* gene in suitable yeast expression vector will be performed. In addition, *XR* gene overexpression in either *O. siamensis* N22 or other appropriate yeast strains will also be focused to achieve high xylitol production by Thai yeast strain from D-xylose.

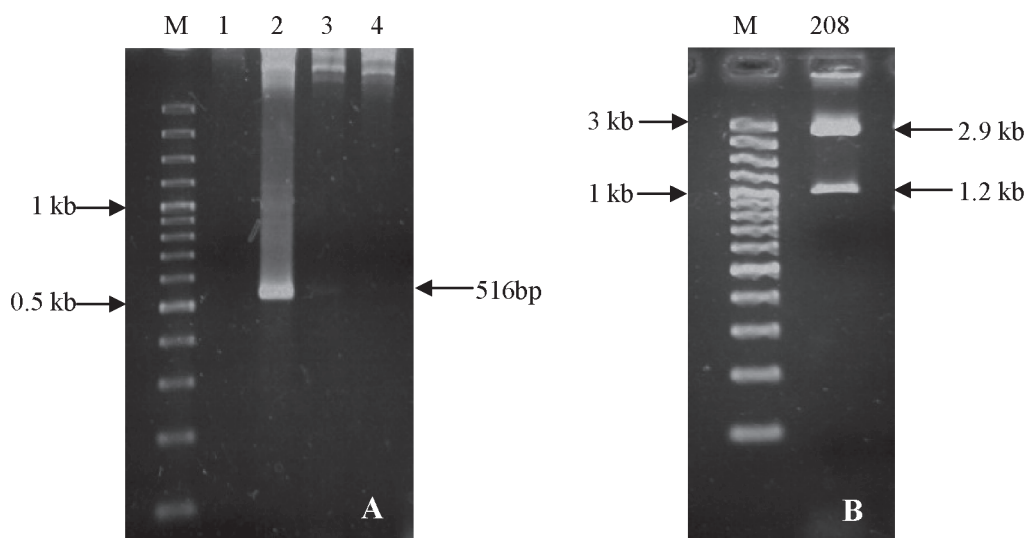


Figure 2 1.6% agarose gel electrophoresis of (A) PCR products from pJET1.2/blunt-XR of recombinant clones (B) Digestion of pJET1.2/blunt-XR208 by *Bgl*III. M, 100 bp DNA ladder; 1-4, clone no. 207-210, respectively; 208, clone no. 208 digested with *Bgl*III.

ATGGTTTCTAAAACAGTGAAGTTGAACAATGGCATTGAGATCCCAGTAGTTGGATTGGCTGCTGG	66
M V S K T V K L N N G I E I P V V G F G C W	22
AAAGTCGAAAAGTCAATCTGCGCTGACCAGATTTACGAAGCTATCAAAGTCGGATACCGTCTGTTC	132
K V E K S I C A D Q I Y E A I K V G Y R L F	44
GATGGCGCTATGGATTATGGTAATGAGAAGGAAATTGGTGAAGGCGTTGCTAGAGCAATCAAAGAT	198
D G A M D Y G N E K E I G E G V A R A I K D	66
GGATTGGTTAAGAGAGAGGAATTGGTTATGTCTCAAAGTTGTGGAACAGTTATCATCATCCTGAT	264
G L V K R E E L V I V S K L W N S Y H H P D	88
AACGTGAAGAAGGCAATTAGAAGAGTTCTGGATGACTTGAAGCTTGACTATCTCGACATCTTTTAC	330
N V K K A I R R V L D D L K L D Y L D I F Y	110
ATCCATTTCCTGTGCTGCTCAAAAATTTGTGCCATTTGAAGAGAAGTATCTCCAGGGTTGTACTGT	396
I H F P V A Q K F V P F E E K Y P P G L Y C	132
GGTCCTAATGGATGGGAGTATGAAGACGTTCTTTAGCAGTCACATGGAAGGCTATGGAAGGTTTA	462
G P N G W E Y E D V P L A V T W K A M E G L	154
GTTGAAGAAGGTCTTGTAAATCGATTGGTATCTCTAACTTCTCTGGAGCACTCAGGACCTG	528
V E E G L V K S I G I S N F S G A L I Q D L	176
TTGAGAGGATGCAAGATTAAGCCCCAATTGCTTCAAATTGAACACCATCCATACTTGACCCAGGAA	594
L R G C K I K P Q L L Q I E H H P Y L T Q E	198
AGATTGGTCAAGTATGTTTCAGGCTCAAGAAATTGCAGTTGTGGCTTACTCCTCATTCGCCCCGAA	660
R L V K Y V Q A Q E I A V V A Y S S F G P Q	220
TCATTGTGTAAGTTGATCA	
S F V E L D	

Figure 3 Nucleotide and deduced amino acid sequences of the partial *XR* gene of *Ogateae siamensis* N22.

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