Introduction

Xylitol, which is a naturally occurring 5-carbon sugar alcohol, is found at low concentrations in fruits, mushrooms, and vegetables [44]. It is used as an alternative sweetener, and has a higher sweetening power than common polyols, such as sorbitol and mannitol [29]. Xylitol is known to reduce the incidence of dental caries by inhibiting the growth of bacteria that cause dental plaque and to assist weight loss without compromising taste [40]. The recognition of these advantages have resulted in a variety of applications of xylitol in the food and chemical industries [29].

Xylitol is produced from xylose, which is one of the major components in hemicellulosic biomass [27]. On an industrial scale, xylitol has been produced by the chemical hydrogenation of xylose by using Ni/Al₂O₃ as a catalyst under high temperature and pressure [1]. The formation of contaminants during the chemical reduction of xylose makes the costs of downstream processes high [37]. Xylitol can also be produced by biological methods that use xylose-utilizing yeasts, such as Candida guilliermondii, C. shehatae, C. tropicalis, C. utilis, and Scheffersomyces stipitis (formerly, Pichia stipitis) [2, 14, 16, 18, 33, 36]. The microbial production of xylitol is more favorable than the chemical process for industrial applications because it can be conducted under mild reaction conditions, such as at atmospheric pressure and ambient temperature [18]. However, the xylitol yield is relatively low for the natural xylose-fermenting yeasts, because xylitol is subsequently oxidized to xylulose by the enzymatic action of xylitol dehydrogenase and the xylulose is further metabolized by xylulokinase [4].

In an attempt to increase the xylitol production from xylose, a recombinant Saccharomyces cerevisiae strain that produces xylitol with a near-theoretical yield has been constructed [42]. Recombinant S. cerevisiae that was transformed by the xylose reductase of S. stipitis converted xylose into xylitol with a yield that was over 95% [2, 25].

Xylitol reductase (XR; E.C. 1.1.1.21), which is a member of the aldose reductase superfamily, catalyzes the formation of xylitol from xylose with the concomitant action of a

Cloning of the Xylose Reductase Gene of Candida milleri

Hyoun-Soo Sim†, Eun-Hee Park†, Se-Young Kwon, Sang-Ki Choi, Su-Han Lee*, and Myoung-Dong Kim*

1Department of Food Science and Biotechnology, Kangwon National University, Chuncheon 200-701, Republic of Korea
2Department of Food Technology and Service, Eulji University, Seongnam 461-713, Republic of Korea

The entire nucleotide sequence of the xylose reductase (XR) gene in Candida milleri CBS8195 sourdough yeast was determined by degenerate polymerase chain reaction (PCR) and genome walking. The sequence analysis revealed an open-reading frame of 981 bp that encoded 326 amino acids with a predicted molecular mass of 36.7 kDa. The deduced amino acid sequence of XR of C. milleri was 64.7% homologous to that of Kluyveromyces lactis. The cloned XR gene was expressed in Saccharomyces cerevisiae, and the resulting recombinant S. cerevisiae strain produced xylitol from xylose, indicating that the C. milleri XR introduced into S. cerevisiae is functional. An enzymatic activity assay and semiquantitative reverse transcription-PCR revealed that the expression of CmXR was induced by xylose. The GenBank Accession No. for CmXR is KC599203.

Keywords: Candida milleri, xylose reductase, xylitol, degenerate PCR, genome walking

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*Corresponding authors
M.-D.K.
Phone: +82-33-250-6458; Fax: +82-33-241-0508; E-mail: mdkim@kangwon.ac.kr
S.-H.L.
Phone: +82-31-740-7196; Fax: +82-31-740-7349; E-mail: shlee@eulji.ac.kr

†These authors contributed equally to this work.

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reducing power (cofactor), and it is the first enzyme in xylose metabolism in yeast [5, 26, 43]. XRs from a variety of microorganisms, such as S. stipitis, C. shehatae, and C. tropicalis, have been cloned, heterologously expressed, and characterized [2, 18, 25, 41]. XRs from S. stipitis, C. shehatae, and C. parapsilosis utilize both NADPH and NADH as a cofactor, whereas those from C. tropicalis, C. utilis, and Meyerozyma guilliermondii exclusively use NADPH [2, 19, 22, 25].

Although several XRs have been cloned and characterized, XR from C. milleri (formerly C. humilis) has never been cloned. C. milleri, which is a dominant yeast that is present in sourdoughs, is an acid-tolerant yeast that is genetically close to S. cerevisiae [34]. Accordingly, after the elucidation of the genetic features that underlie xylose utilization, this acid-tolerant yeast might be developed as a promising workhorse for the production of organic acid from the pretreated cellulosic biomass, which has a number of inhibitory acids [9, 10, 27]. In this study, as a first step toward strain development, the XR gene of C. milleri (CmXR) was cloned, and its sequence was analyzed. The functionality of the cloned CmXR gene was demonstrated by the production of xylitol in recombinant S. cerevisiae that had been transformed with the gene. In addition, the effects of carbon sources on the expression and enzymatic activity of XR in C. milleri were examined.

**Materials and Methods**

### Reagents, Strains, and Cultivation Conditions

All chemicals were of analytical grade or higher. The C. milleri CBS 8195 and the S. cerevisiae BY4742 gre3Δ mutants [MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 gre3Δ1::kanMX4] from the European Saccharomyces cerevisiae Archive for Functional Analysis (EUROSCARF) were used in this study. Escherichia coli TOP10 (Invitrogen, USA) was used for plasmid DNA preparation and grown at 37°C.

LB medium (5 g/l bacto-yeast extract, 10 g/l bacto-tryptone peptone, and 10 g/l NaCl) that was supplemented with 100 µg/ml ampicillin was used for plasmid DNA preparation. C. milleri cultures were grown in Yeast Extract Peptone Dextrose (YPED; 10 g/l bacto-yeast extract, 20 g/l bacto-proteose peptone, and 20 g/l glucose) medium in order to prepare the genomic DNA. In order to confirm the production of xylitol in S. cerevisiae, S. cerevisiae gre3Δ1 cells with the plasmid were grown in synthetic complete medium lacking uracil (SC-URA), which was supplemented with 20 g/l glucose and 20 g/l xylose. Yeast cultures were grown at 30°C unless stated otherwise.

### Cloning of the CmXR

C. milleri CBS8195 was cultured in YEPD medium at 30°C for 12 h, after which the genomic DNA was extracted as described in our previous report [34]. The degenerate oligonucleotide primers, XR-F and XR-R, that are listed in Table 1 were designed based on the core conserved regions of XRs (EKYPFGFY and RFNDPWDW) in yeasts [12, 16, 25, 46].

A polymerase chain reaction (PCR) was performed with 2.5 units of DyeMix-Tag polymerase (Enzymonics, Korea), 1 µg of template DNA, and 100 pmol of each primer (Table 1). The reaction conditions were programmed as 10 min at 94°C, followed by 35 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 55°C, and extension for 1 min at 72°C. The amplified fragment was cloned into the yT&A vector (iNtRON Biotechnology, Korea) according to the manufacturer’s protocol.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'-3')</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>XR-F</td>
<td>GARAARTAYCCWCCWGGWTYTA</td>
<td>Degenerate PCR</td>
</tr>
<tr>
<td>XR-R</td>
<td>TCAAGCCAGTCTGTCCAG</td>
<td>Gene walking</td>
</tr>
<tr>
<td>5'-CmXR-TSP1</td>
<td>CCACTCCAWGGRTCRTGAACTC</td>
<td>Degenerate PCR</td>
</tr>
<tr>
<td>5'-CmXR-TSP2</td>
<td>AACAACGGTGTCTGGACCAGGA</td>
<td>Gene walking</td>
</tr>
<tr>
<td>5'-CmXR-TSP3</td>
<td>TTCCACTCATCGACACTACCTACGT</td>
<td>Gene walking</td>
</tr>
<tr>
<td>3'-CmXR-TSP1</td>
<td>TCGCTCTATTITGAGCAGCCAC</td>
<td>Gene walking</td>
</tr>
<tr>
<td>3'-CmXR-TSP2</td>
<td>TGCTCCACCTCTAGTCTCCTTT</td>
<td>Gene walking</td>
</tr>
<tr>
<td>3'-CmXR-TSP3</td>
<td>GACTGTGGAAGGCGGCTGACC</td>
<td>Gene walking</td>
</tr>
<tr>
<td>5'-CmXR-TSP1-2nd</td>
<td>GAGCTTGGAAGCTTCCTCCA</td>
<td>Gene walking</td>
</tr>
<tr>
<td>5'-CmXR-TSP2-2nd</td>
<td>AAGCCGTCACACGAGCAGAGAT</td>
<td>Gene walking</td>
</tr>
<tr>
<td>5'-CmXR-TSP3-2nd</td>
<td>GGTACCGTCTGTCCAGGCGT</td>
<td>Gene walking</td>
</tr>
</tbody>
</table>

PCR, polymerase chain reaction; RT-PCR, reverse transcription-PCR.

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**Sequence Analysis**

The nucleotide and deduced amino-acid sequences were analyzed by the BLAST (The National Center for Biotechnology Information, NCBI, http://www.ncbi.nlm.nih.gov/BLAST/). The alignment of multiple sequences was conducted by the San Diego Supercomputer Center (SDSC) Biology Workbench (http://workbench.sdsc.edu/). The theoretical molecular mass of the protein was calculated with ExPaSy (http://au.expasy.org/compute_pi/). Multiple amino acid sequences were aligned with the CLUSTALW program [8], and the phylogenetic tree was constructed with MEGA 5.0 with the neighbor-joining method. A bootstrap analysis was performed with 1,000 replicates in order to test the relative support for the branches in the phylogenetic tree.

**Expression of CmXR in S. cerevisiae**

The p426GPD (URA3, 6.6 kb) plasmid containing the S. cerevisiae GPD promoter and the CYC1 terminator [32] was used for the expression of CmXR in the S. cerevisiae gre3.4 strain. CmXR was obtained by PCR with the CmiXR-426GPD-EcoRI-F and CmiXR-426GPD-XhoI-R primers (Table 1). Genomic DNA from C. milleri was used as the template. The amplified PCR products of the expected sizes were digested with the EcoRI and XhoI restriction enzymes and then inserted into the p426GPD plasmid in order to construct the pME1222 plasmid (7.6 kb).

**Transformation**

The S. cerevisiae gre3.4 strain was transformed with the lithium acetate method [11]. E. coli cells were transformed as described in the literature [39].

**Preparation of the Cell Extracts**

A single colony of C. milleri CBS8195 was grown in YEPD medium at 30°C to an A₆₀₀ of 0.8, harvested by filtration, shifted to Yeast Extract Peptone medium that was supplemented with different carbon sources for 1 h, and then collected by filtration for the preparation of the crude protein extract and total RNA. In order to prepare the protein extracts, the cells were resuspended in 1 ml of disruption buffer (20 mM Tris-HCl (pH 7.9), 10 mM MgCl₂, 1 mM ethylene diamine tetraacetic acid, 5% (v/v) glycerol, 1 mM dithiothreitol, 0.3 M ammonium sulfate, and 1 mM phenylmethanesulfonyl fluoride) and then broken down by vortexing with acid-washed glass beads (Sigma-Aldrich, USA) for 3 min [34]. Cell homogenates were centrifuged at 15,000 × g for 5 min at 4°C, and the supernatants were used as cell extracts. Cell extracts were prepared from 3 independent cultures. Protein concentrations were determined by a Bradford assay (Bio-Rad, USA) with bovine serum albumin used as the standard.

**Analytical Methods**

The cell growth of yeast cultures was measured with a spectrophotometer at 600 nm. The concentrations of glucose, xylose, ethanol, and xylitol were measured with high-performance liquid chromatography (Shimadzu, Japan) that was equipped with a HPX-87H column (Bio-Rad). The mobile phase consisted of a 5 mM H₂SO₄ solution.

The xylose reductase activity was determined as previously described [7, 46]. The standard assay volume of 200 µl contained 50 mM potassium phosphate buffer (pH 6.0), 0.2 mM NADPH (NADH), and 13.3 mM D-xylose. This reaction mixture was allowed to stand for 1 min in order to eliminate the endogenous oxidation of NADPH (NADH), and the reaction was started by the addition of 0.1 ml of substrate. One unit of XR activity was defined as the amount of enzyme that oxidized 1 µmole of NADPH (NADH) per minute at 30°C.

**RNA Isolation and Reverse Transcription-PCR (RT-PCR)**

Total RNA was prepared with TRIzol reagent (Invitrogen). RT-PCR was performed with the cDNA EcoDry Premix (Takara, Japan) according to the manufacturer’s instructions. Total RNA (1.0 µg) and full-length primers of CmXR [35] and CmXR cDNA (Table 1) were used for 30 cycles of RT-PCR amplification. Aliquots of the PCR product were separated on a 0.8% agarose gel.

**Statistical Analysis**

Statistical analyses were performed with Student’s t-tests [6] and were based on 3 independent experiments. The results were considered to be statistically significant when p values were less than 0.05.

**Results and Discussion**

**Sequence Analysis of the CmXR**

Through the alignment of the reported yeast XR genes [16, 46], degenerate PCR primers that targeted the core conserved region were designed (Table 1). A ~500 bp PCR product was obtained, cloned, and sequenced (data not shown). The alignment of the deduced primary structure of the cloned gene product with the reported XR genes suggested that the cloned product that was obtained with degenerate PCR contained conserved sequences of the XR gene. Based on the sequenced gene fragment, 8 gene-specific nucleotide primers were designed (Table 1). The entire nucleotide sequences of the CmXR gene, including 5’-upstream and 3’-downstream regions, were obtained with genome walking (GenBank Accession No. KC599203).

The CmXR gene consisted of an open-reading frame of 981 bp and putatively encoded a polypeptide with 326 amino acids (Fig. 1). The estimated molecular mass and pI were 36.7 kDa and 6.5, respectively. A sequence analysis indicated that the CmXR gene was not interrupted by an intron, and this was supported by the nucleotide sequence of the cDNA that was prepared from the C. milleri that was grown in medium that used xylose as a carbon source (data not shown). A 320 bp genomic DNA sequence containing a
The 5'-upstream flanking region of the CmXR gene was aligned and compared with the promoter sequences of other fungal XRxs (data not shown). The basal promoter elements, TATA and CAAT boxes [16], were located at the -119 and -143 nucleotide positions, respectively, in the 5'-flanking region of the XR gene. As with other xylose reductases in yeast and fungi, 2 sites of the putative stress-response element (STRE) sequence, 5'-AGGGG-3' [23, 24], were found 242 and 296 nucleotides upstream of the initiation codon, suggesting that the expression of XR in C. milleri might be regulated in a similar manner as other aldo-keto reductase genes in yeast [10]. The STRE sequence, AGGGG or CCCCT.

Fig. 1. Nucleotide sequence of the C. milleri XR gene and its deduced amino acid sequence. The untranslated and coding regions are represented by small letters and capital letters, respectively, and the asterisk indicates the stop codon. The underlined nucleotides represent the plausible TATA and CAAT boxes in the 5' region and the polyadenylation site and the transcription termination signal in the 3' region. The open boxes show the position at which the degenerate primers were designed. STRE represents stress reponse element.
Fig. 2. Multiple alignments of xylose reductase amino acid sequences from different organisms. Fully and strongly conserved residues are shaded in black and grey, respectively. Potential active sites (filled triangles) and substrate-binding residues (open circles) are shown. *C. milleri* (GenBank Accession No. KC599203); *C. shehatae* (AAF86345); *C. tenuis* (AAC25601); *S. stipitis* (CAA42072); *M. guilliermondii* (AAD09330); *C. tropicalis* (AEY80024); *C. parapsilosis* (AAO91803); *A. oryzae* (CAK42794); *K. lactis* (AAA99507).
[10, 24], is a general stress response system in *S. cerevisiae* that responds to nitrogen starvation, osmotic and oxidative stress, low pH, weak organic acids, and ethanol [3, 28, 38]. GRE3, which is a XR-like gene in *S. cerevisiae*, and the XR gene in *C. tropicalis* have 2 STREs 144 and 37 nucleotides upstream, respectively, of the initiation codon [2, 25]. The putative polyadenylation signal [16, 17], AATAA, was located 47 bp downstream from the stop codon. The TAG...TAN...ICT...TTT motif, which is known for its efficient termination of gene transcription [15, 16], was also found in the 3'-flanking region. As shown in Fig. 2, sequence alignment with other homologous XRs revealed that the deduced 326 amino acid protein of *C. milleri* XR had conserved regions; namely, an N-terminus region (LxxGxxPxxGxG), an active site region (GxxxxDxAxxY, which contains the conserved Asp-44 and Tyr-49), and a region harboring the active site (LxxxxxxxxDxxxxH, which contains the conserved His-111) [25, 36]. In addition, it was noteworthy that an LNSG motif [14, 18, 41] that is conserved in *C. tropicalis*, *C. shehatae*, and *S. stipitis* was not fully conserved in CmXR. The absence of the NAD(P)-dependent reductase with the characteristic GxGxxG motif (where x represents any amino acid) indicated that sequences other than the motif might have the ability for dinucleotide binding [13, 37]. The similarity of the CmXR to other yeast XRs was further revealed by phylogenetic analysis (Fig. 3). The homology of the amino acid sequence of CmXR was high compared with that of the XR of *Kluyveromyces lactis* (64.7%), and the levels of homology were followed by the sequences of *C. tropicalis* (56.2%), *M. guilliermondii* (55.9%), *C. shehatae* (54.8%), and *S. stipitis* (54.8%).

**Functional Verification of CmXR in S. cerevisiae**

Since it has been reported that the *S. cerevisiae* Gre3 has aldol-keto reductase activity [10, 20, 42] that converts xylose to xylitol, the *S. cerevisiae gre3* mutant was used as a host in order to verify the functionality of the CmXR. Profiles of cell growth, substrate consumption, and the xylitol production of *S. cerevisiae* transformants harboring an empty vector (p426GPD) or the pME1222 (p426GPD-CmXR) plasmid were compared in shake flask cultures with 20 g/l glucose and 20 g/l xylose. As shown in Fig. 4, the *S. cerevisiae* transformant harboring the p426GPD (empty vector) did not produce xylitol from xylose (Fig. 4A). However, the *S. cerevisiae* transformants that were transformed with the pME1222 plasmid utilized xylose to produce xylitol at a concentration of 2.1 g/l after 25 h of cultivation (Fig. 4B), indicating that the CmXR gene is functional as a xylose reductase in the

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**Fig. 3.** The phylogenetic tree of the *C. milleri* XR (CmXR) gene and other homologs from different organisms. On the basis of the full-length amino acid sequences of XR, the tree was constructed with the neighbor-joining method. The numbers on the nodes correspond to the percentages, with which a cluster appears in a bootstrap test based on 1,000 pseudoreplicates. The bars denote the relative branch length. The XRs are identified by their GenBank accession number in parentheses.
Xylose Reductase Gene of Candida milleri

Expression Analysis of the CmXR

An expression analysis for the different carbon sources of CmXR was done with CmURA3 [35], which was used as a control. As shown in Fig. 5, the expression of CmXR was detected on all carbon sources that were tested, albeit at different levels. The highest transcript level of CmXR was detected with xylose, and a lesser extent was detected with fructose and mannose as the carbon sources. Based on the analysis of the transcript level, the expression of CmXR was determined to be carbon-source dependent and was induced by xylose, as is the case of XR in S. stipitis [2] and Talaromyces emersonii [9]. XR enzymatic activity for xylose was the highest among the sources examined, and this was followed by fructose, mannose, and glucose. A significant increase in both the transcript levels and the XR enzymatic activity was observed when C. milleri was shifted to xylose from glucose, and these findings were compatible with those of previous reports [5, 30]. XR enzymatic activity was enhanced over 3-fold. In addition, it appeared that the expression of XR in C. milleri was seemingly derepressed by fructose and mannose. The findings of the XR enzymatic activity assay with different cofactors, NADPH and NADH, supported the idea that XR in C. milleri might prefer NADPH to NADH as a cofactor (Fig. 5). XR enzymatic activity in the presence of NADPH as a cofactor was approximately 1.6-fold higher than when NADH was used as a cofactor. In most yeasts, XR has a higher or even absolute preference for NADPH [37], although that from C. tropicalis prefers NADH or NADPH [25]. In contrast to C. tropicalis, the almost equivalent utilization of NADPH and NADH by XR has been reported in S. stipitis [41]. Further studies on the enzymatic properties, including cofactor preference, should be conducted with the purified XR protein of C. milleri.

In summary, the gene encoding xylose reductase in sourdough yeast C. milleri was isolated and identified. The function of the cloned gene was verified in S. cerevisiae. An expression analysis revealed that the transcription of CmXR was significantly unregulated in order to increase the XR enzymatic activity. This is the first report on an
expression analysis of XR in C. milleri. Further studies should explore the enzymatic properties of xylose reductase from C. milleri.

Acknowledgments

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