0273-2289/03/105-108/0265/$20.00

# Molecular Characterization of a Gene for Aldose Reductase (*CbXYL1*) from *Candida boidinii* and Its Expression in *Saccharomyces cerevisiae*

**Min Hyung Kang,[1] Haiying Ni,[1] and Thomas W. Jeffries*,[1,2]**

*[1]Department of Bacteriology, University of Wisconsin-Madison, 1550 Linden Drive, Madison, WI 53706, E-mail: twjeffri@facstaff.wisc.edu; and [2]Forest Products Laboratory, One Gifford, Pinchot Drive, Madison, WI 53705*

## Abstract

*Candida boidinii* produces significant amounts of xylitol from xylose, and assays of crude homogenates for aldose (xylose) reductase (XYL1p) have been reported to show relatively high activity with NADH as a cofactor even though XYL1p purified from this yeast does not have such activity. A gene coding for XYL1p from *C. boidinii* (*CbXYL1*) was isolated by amplifying the central region using primers to conserved domains and by genome walking. *CbXYL1* has an open reading frame of 966 bp encoding 321 amino acids. The *C. boidinii* XYL1p is highly similar to other known yeast aldose reductases and is most closely related to the NAD(P)H-linked XYL1p of *Kluyveromyces lactis*. Cell homogenates from *C. boidinii* and recombinant *Saccharomyces cerevisiae* were tested for XYL1p activity to confirm the previously reported high ratio of NADH:NADPH linked activity. *C. boidinii* grown under fully aerobic conditions showed an NADH:NADPH activity ratio of 0.76 , which was similar to that observed with the XYL1p from *Pichia stipitis XYL1*, but which is much lower than what was previously reported. Cells grown under low aeration showed an NADH:NADPH activity ratio of 2.13. Recombinant *S. cerevisiae* expressing *CbXYL1* showed only NADH-linked activity in cell homogenates. Southern hybridization did not reveal additional bands. These results imply that a second, unrelated gene for XYL1p is present in *C. boidinii*.

**Index Entries:** *Candida boidinii*; *Saccharomyces cerevisiae*; aldose reductase; *CbXYL1*; xylose reductase; NADH; NADPH; gene cloning; gene expression.

*Author to whom all correspondence and reprint requests should be addressed.

## Introduction

D-Xylose is one of the major components of lignocellulosic biomass. Alcoholic fermentation of this renewable carbon source is essential if the overall process of lignocellulose bioconversion is to be economical *(1)*. Since the discovery that some naturally occurring yeasts cause pentose fermentation *(2,3)*, considerable interest in the study of the xylose catabolic pathway has arisen *(4–6)*. Yeasts convert xylose to xylulose through sequential reduction (either NADH or NADPH-linked xylose [aldose] reductase, XYL1p) and oxidation (NAD-dependent xylitol dehydrogenase, XYL2p) *(7,8)*. The subsequent phosphorylation of xylulose by D-xylulokinase *(9,10)* allows entry of the sugar phosphate into the pentose phosphate pathway.

Xylose (aldose) reductase (XYL1p) catalyzes the reduction of xylose to xylitol, which is the first step for xylose metabolism in yeasts. Most commonly, xylose reductase (XR) is specific for NADPH. However, the main XR of *Pichia stipitis* has significant activity with NADH as a cofactor *(7)*. Its NADH:NADPH activity ratio is 0.7. A second cryptic gene in *P. stipitis* is NADPH dependent *(11)*. NADH activity is critical to the anaerobic metabolism of xylose because the second step in the pathway, xylitol dehydrogenase (XYL2p), is specific for NADH, and cofactor imbalance can occur unless some means exists to regenerate $NAD^+$ *(12)*. Most known XYL1p enzymes favor NADPH over NADH. Interestingly, xylose-fermenting yeasts display different ways to avoid cofactor imbalances in the cellular redox system with NADH + $H^+$ being accumulated at the expense of NADPH + $H^+$ during anaerobic or oxygen limited growth on xylose. They either express multiple forms of XRs with different coenzyme specificities as in *Pachysloen tannophilus (13)* or only one single enzyme with dual cofactor specificity as in *Pichia stipitis (7)*.

In one xylitol-producing yeast, *Candida boidinii*, the CbXYL1p NADH:NADPH activity ratio has been reported to range between 2.0 and 5.9, depending on the aeration rate *(14)*. When two separate aldose reductases with different NAD(P)H activities are induced to different extents, the ratio can vary. The lower ratio was observed at higher aeration rates, which is consistent with the higher respiratory function of NADPH-linked enzymes under those conditions *(15,16)*. This finding suggests that if one were to replace or supplement NADPH-linked aldose (xylose) reductase with the CbXYL1p, aldose (xylose) reductase activity would be less dependent on NADPH produced by the oxidative pentose phosphate cycle and could be more active with NADH produced through fermentation.

Therefore, in the present study, isolation of the *CbXYL1* gene was attempted and CbXYL1p activity was investigated in recombinant *S. cerevisiae* that was transformed with *CbXYL1*.

## Materials and Methods

### Yeasts, Media, Enzymes, and Chemicals

*C. boidinii* NRRL Y-17213 was maintained on agar plates at 4°C *(15)*. *S. cerevisiae* L2612 (*Matα, trp-, ura-, leu-*) *(17)* was used for transformation and

expression of aldose reductase (AR) gene from *C. boidinii*. *Escherichia coli* DH5α was used for all of DNA manipulations.

*E. coli* was grown in Luria-Bertani medium. Ampicillin (50 µg/mL) was added to the medium when required. Yeast strains were grown in yeast peptone medium (10 g/L of yeast extract, 20 g/L of bacto peptone), or yeast synthetic (YS) medium containing 6.7 g/L of yeast nitrogen base (YNB) without amino acids (Difco, Grayson, GA), plus 20 g/L of casamino acid (Difco). Glucose (20 g/L) or xylose (20 g/L) was used as a carbon source. Yeast cells were cultivated at 30 °C in 50 mLof medium in a 125-mLErlenmeyer flask.

Restriction enzymes, DNA-modifying enzymes, and other molecular reagents were obtained from New England Biolabs (Beverly, MA), Promega (Madison, WI), Stratagene (La Jolla, Ca), Invitrogen (Carlsbad, CA), Clontech (Palo Alto, CA), Roche (Indianapolis, IN), and Applied Biosystems (Foster City, CA). Reaction conditions were as recommended by the suppliers. All general chemicals were purchased from Sigma (St. Louis, MO). Primers for polymerase chain reaction (PCR) amplification and sequencing were synthesized by Sigma-Genosys (The Woodlands, TX).

## *Cloning of* CbXYL1 *Gene*

To isolate the *CbXYL1* gene, a set of closely related AR proteins from the yeasts *Candida tenuis (18)*, *P. stipitis (19)*, *Candida tropicalis (20)*, *Candida guilliermondii (21)* and *Pachysolen tannophilus (22)* were identified using the Blast search program on the NCBI server (*see* Website: http://www.ncbi.nlm.nih.gov/blast/). Conserved regions of the various aldose (xylose) reductase amino acid sequences were then aligned using ClustalW with default settings at the EBI Website (*see* Website: http://www.ebi.ac.uk/clustalw). Degenerate primers were designed against the most conserved segments and PCR amplification was used to obtain a partial *CbXYL1* gene from *C. boidinii* genomic DNA. The 5'-terminal degenerate primer was 5'-CCCGGGATCCGGNTAYAGRTTRTT-YGAY-3' and the 3'-terminal degenerate primer was 5'-CCCGGGATCCYTGYA-ARTANGGRTGRTGYTC-3'. PCR amplification was carried out in 50 µL of a reaction mixture containing 500 ng of *C. boidinii* genomic DNA, 100 ng of each primer, 1X reaction buffer, 1.5 m*M* $MgCl_2$, 0.2 m*M* dNTP, and 2.5 U of AmpliTaq-gold DNA polymerase (Applied Biosystems. It was then run for 30 cycles, denatured at 95 °C for 1 min, annealed at 45°C for 1 min, and polymerized at 72°C for 2 min. About 465 bp of PCR products was subcloned into cloning vector, pCR2.1-Topo (Invitrogen), and then sequenced by the University of Wisconsin Biotechnology Center using a 371 ABI automated sequencer (Perkin Elmer, Foster City, CA).

To obtain the full *CbXYL1* sequence, a mini–genomic library was constructed using the Genome Walker™ kit (Clontech) with the following modification: After genomic DNA of *C. boidinii* was digested with *Msc* I to generate blunt-ended fragments, adapters (Clontech) were ligated onto the 5'- and 3'-terminals of digested DNA, respectively. PCR amplification was then attempted with gene-specific primers and with the adapter primers

(AP1 and AP2, from the genome walker kit from Clontech). Gene-specific primers were synthesized on the base of the partial *CbXYL1* gene. For primary PCR amplification, 5XRSEQ (5'-TTCTCTTTTAACTAAACCATC-3') and 3XRSEQ2 (5'-GGATCCATGGAAGAATTGGTTGAAGAA-3') were used, and then, for secondary PCR amplification, 5XRSEQ2 (5'-CGGGATCCTTTGATCGCTTTGTTAACACC-3') and 3XRSEQ (5'-GGTTTAGTTAAATCAATT-GGT-3') were used. A single PCR product from the *Msc* I minilibrary was obtained, subcloned into pCR2.1-Topo (Invitrogen), and sequenced.

Restriction enzyme digestion, electrophoresis, DNA ligation, transformation, and DNA preparation from *E. coli* were performed by using standard methods *(23)*, as was Southern blot analysis for *CbXYL1*. Genomic DNA was digested with *Msc* I, *Pst* I, and *Xba* I. After separation on an agarose gel, DNA was transferred onto a nylon membrane, hybridized with probe DNA that was synthesized by random primer extension. The signal was then developed according to the protocol provided by Roche. DNASIS software was used for sequence analysis *(24)*. A phylogenetic tree was generated from the alignments using the neighbor-joining method *(25)*. By throwing out the sequences in the gap, ambiguous parts of the alignment were excluded from the phylogenetic analysis.

### *Expression and Enzyme Assays*

DNA fragments (1.4 kb) containing the *CbXYL1* open reading frame (ORF) (1.0 kb), upstream region (0.4 kb), and terminator (0.1 kb) were amplified by using two synthetic primers from *C. boidinii* genomic DNA. 5'-CTGCAGTGGCCACAATGGCATGGC-TTC-3' was used for the 5'-terminal primer and 5'-CTGCAGATGTATTGAAAAGCGTGATTAATT-3' was used for the 3'-terminal primer. These primers have *Pst* I-restriction enzyme sites on their ends. The amplified *CbXYL1* gene was subcloned into the *Pst* I site of a yeast shuttle vector, pRS424 *(26)*, and then pRS424-*CbXYL1* was introduced into *S. cerevisiae* L2612 (*Matα, trp-, ura-, leu-*) *(17)* by using a Yeast EZ-transformation kit (BIO 101, Vista, CA). Transformants were selected on YS medium containing 20 g/L of glucose (YSD) and 20 g/L of agar.

Wild-type and recombinant *S. cerevisiae* (S4α) were grown on YS–2% xylose (YSX) and 2% glucose medium for 2 d at 30°C and 200 rpm. Culture medium was then collected to analyze the accumulation of xylitol by using high-performance liquid therapy (HPLC) (HP, Wilmington, DE) with an ION300 column (Interaction Chromatography, San Jose, CA).

Enzyme activity for CbXYL1p expressed in *S. cerevisiae* was assayed by measuring the decrease of absorbance of NADH or NADPH at $A_{340}$ *(27)* using a photodiode array spectrophotometer (Hewlett Packard). The 1-mL reaction mixture contained 0.1 *M* Na phosphate buffer (pH 6.5), 0.4 *M* D-xylose, various concentration of NAD(P)H, and cell homogenate at 30°C. Reactions were started by addition of NAD(P)H. The initial reaction rate was determined as a function of NAD(P)H concentration and expressed as units/milligram of protein. One unit of enzyme activity refers to 1 μmol of

NAD(P)H consumed/min. All rates were corrected for the appropriate blank readings to account for the nonspecific oxidation of NAD(P)H such as autooxidation mediated by aldoses *(28)*. Affinity of the AR for other aldoses also was assayed. The amounts of total proteins in crude extracts were determined by bicinchonic acid assay (Pierce, Rockford, IL).

### *Effect of Aeration on NADH- and NADPH-/Linked CbXYL1p Activities*

*C. boidinii* was cultured in YSX (6.7 g/L of YNB, 2% casamino acids, and 2% xylose). Recombinant *S. cerevisiae* (S4α) was cultured in YSX plus 2% glucose. For aerobic conditions, cells were cultured in 2 mL of YSX or YSX plus 2% glucose, overnight, and used to inoculate into 50 mL of medium in 125-mL flasks on the same media at 30°C. For low oxygen transfer conditions, the cell suspensions were adjusted to an initial cell density of 1.25 mg/mL and cultured at 50 rpm. For high oxygen transfer conditions, cell suspensions were adjusted to an initial cell density of 0.125 mg/mL and cultured at 200 rpm. After 18 h, cells were harvested and resuspended in 0.1 *M* Na-phosphate buffer (pH 6.5). Vigorous mixing with glass beads disrupted the cells, and the homogenates were clarified by centrifuging at 10,000*g* for 10 min. Supernatant solutions were then assayed.

## Results

### *Cloning of* CbXYL1

We successfully amplified a DNA fragment of approx 400 bp from genomic DNA of *C. boidinii* using degenerate primers designed against conserved regions GYRLFD and EHHPYLQ found in xylose (aldose) reductases *(18–22)* (Fig. 1), and the amplicon sequence was identified as a partial gene for *CbXYL1* (data not shown). Gene-specific primers were designed from this fragment. The 5'-upstream, ORF and 3'-downstream sequences for *CbXYL1* were obtained by genome walking and deposited into GeneBank (accession no. AF451326). This sequence consists of 387 bp 5'-upstream, an ORF of 966 bp that encode putative 321 amino acids with a calculated molecular weight of 36 kDa, and 52 bp 3'-downstream. The calculated molecular weight is in good agreement with the protein size of 36 kDa mentioned in a previous review *(29)*. Sequence analysis showed a TATAAA and three CAAT boxes located –54, and –120, –159 and –201, respectively, in the 5'-upstream region. As shown in Fig. 2, a phylogenetic analysis showed that the CbXYL1p is significantly different from three *Candida* XYL1 proteins and from *P. stipitis* XYL1p, and it is most closely related to the XR of *K. lactis* and *S. cerevisiae*, with which it shows 63 and 62 % identity and 78 and 76% similarity, respectively. IPKS, which is the coenzyme-binding motif, was highly conserved in the *C. boidinii* protein in other yeast aldose (xylose) reductases, and in mammalian aldo-keto reductase family enzymes *(30)*.

Southern blot analysis was performed with the *CbXYL1* ORF as a molecular probe to determine whether other related sequences were present in the *C. boidinii* genome. As shown in Fig. 3A, only a single band

```
C. boidinii              29 CAETIYEAIKVGYRLFDGAMDY 50
  Conserved sequence                 GYRLFD
C. tenuis                31 AGEQVYQAIKAGYRLFDGAEDY 52
P. stipitis              27 CSEQIYRAIKTGYRLFDGAEDY 48
C. tropicalis            33 AADQIYNAIKTGYRLFDGAEDY 54
C. guilliermondii        26 CADTIYNAIKVGYRLFDGAEDY 47
P. tannophilus           27 AADMVYAAIKEGYRLFDCACDY 48


C. boidinii             180 GCKIRPAVLEIEHHPYLVQPRL 201
  Conserved sequence                EHHPYLQ
C. tenuis               182 GATIKPAVLQVEHHPYLQQPKL 203
P. stipitis             178 GATIKPSVLQVEHHPYLQQPRL 199
C. tropicalis           184 GATIKPAVLQIEHHPYLQQPKL 205
C. guilliermondii       177 SAKIKPAVLQIEHHPYLQQPRL 198
P. tannophilus          178 AARIKPASLQIEHHPYLQQNKL 199
```

Fig. 1 Alignment of amino acid sequences between CbXYL1p and known XRs or ARs. Amino acid sequences were aligned by using FASTA (*see* Website: http://www.ncbi.nlm.nih.gov). Highly conserved sequences in bold were used to design degenerate primers.

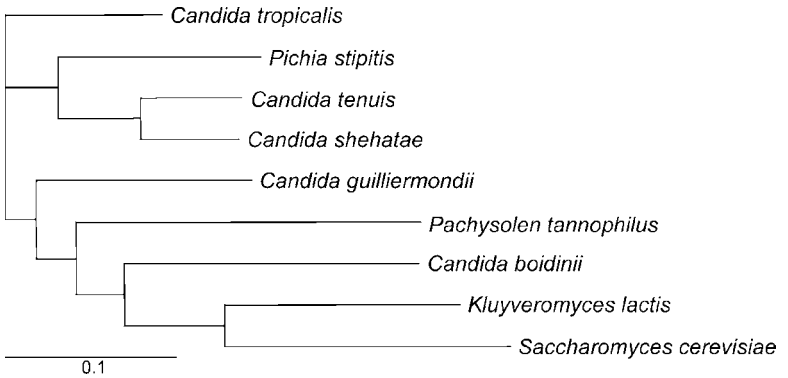


Fig. 2. Phylogenetic tree of XRs or ARs found in yeasts. Amino acid sequences were identified by BLAST, and the resulting protein sequences were then aligned. A phylogenetic tree was generated from the aligned regions after excluding sequences creating gaps.

hybridized to the probe under low-stringency conditions, which implies that only a single-copy gene of *CbXYL1* exists in *C. boidinii*.

## Expression and Activity

To express *CbXYL1* in *S. cerevisiae*, a DNA fragment of 1405 bp was amplified and cloned from genomic DNA of *C. boidinii* by using the 5'-primer (5'-CTGCAGTGGCCACAATGGCATGGCTTC-3') and the 3'-primer (5'-CTGCAGATGTATTGAAAAGCGTGATTAATT-3') for the full sequences. This fragment was then subcloned into the pRS424 vector *(26)*

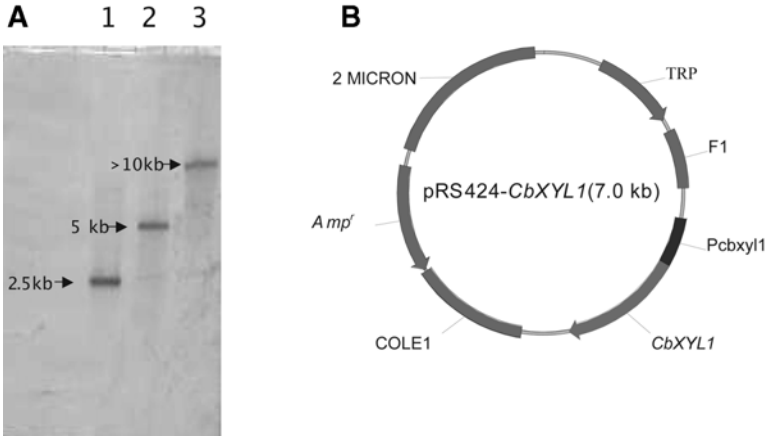


Fig. 3. Genomic Southern blot analysis of *CbXYL1* and construction map for expression in *S. cerevisiae.* **(A)** Genomic DNA from *C. boindinii* was digested with *Msc* I, *Pst* I, and *Xba* I. After running on an agarose gel, DNA was transferred onto a nylon membrane, hybridized with probe DNA that was synthesized by random primer extension. The signal was then developed according to a protocol given by Roche. Arrows indicate the approx size of hybridized DNA fragments. **(B)** Construction map of pRS424-*CbXYL1* for expression in *S. cerevisiae.* The *CbXYL1* gene was constructed under the control of its own promoter. PRS424-*CbXYL1* was introduced into *S. cerevisiae* L2612 (Matα, trp-, ura-, leu-) *(12)*. Pcbxyl1 and *CbXYL1* indicate promoter and coding region, respectively.

Table 1
Specific Activity of CbXYL1p Expressed in *S. cerevisiae*[a]

| | NADH (U/mg) | | NADPH (U/mg) | |
|---|---|---|---|---|
| | Control | CbXYL1p | Control | CbXYL1p |
| D-Arabinose | 0.009 | 0.010 | 0.009 | 0.017 |
| L- Arabinose | 0.019 | 0.032 | 0.020 | 0.494 |
| D- Galactose | 0.006 | 0.006 | 0.012 | 0.094 |
| D- Glucose | 0.008 | 0.008 | 0.008 | 0.047 |
| D- Ribose | 0.007 | 0.007 | 0.015 | 0.159 |
| D- Xylose | 0.009 | 0.016 | 0.020 | 0.417 |

[a]AR activity was not detected in *S. cerevisiae* L2612 (wild-type).

(Fig. 3B). After selection for growth on YSD medium, the transformant was confirmed by PCR amplification (data not shown) and named S4α. To identify CbXYL1p enzyme activity, S4α was cultured on YS-2% glucose and 2% xylose medium. After culturing for 2 days, xylitol production was analyzed by HPLC. The transformant produced significant (5.8 g) xylitol from 20 g of xylose whereas the wild-type strain produced only trace amounts (0.71 g). Glucose was not detected because it was consumed completely for cell growth within 2 d. This experiment demonstrated that *CbXYL1* could be expressed under control of its own promoter in *S. cerevisiae*.

Table 2
Effect of Aeration on CbXYL1p Activity in *C. boidinii* and *S. cerevisiae* (S4α)[a]

| | *C. boidinii* | | | | *S. cerevisiae* (S4α) | | | |
|---|---|---|---|---|---|---|---|---|
| | 50 rpm | | 200 rpm | | 50 rpm | | 200 rpm | |
| | NADH | NADPH | NADH | NADPH | NADH | NADPH | NADH | NADPH |
| $V_{max}$ (U/mg) | 0.19 | 0.09 | 0.2 | 0.26 | ND | 0.7 | ND | 0.22 |
| $K_m$ (μ*M*) | 3.38 | 7.36 | 2.57 | 5.39 | ND | 3.08 | ND | 3.66 |
| NADH/NADPH ratio for specific activity | 2.13 | | 0.76 | | NA | | NA | |

[a]ND, not detected; NA, not available.

To investigate the relative affinities of CbXYL1p for NADPH and NADH and the substrate specificity, an enzyme assay of the CbXYL1p was performed with several carbohydrates: D-arabinose, L-arabinose, D-galactose, D-glucose, D-ribose, and D-xylose. As shown in Table 1, CbXYL1p had higher activity with 5-carbon than with 6-carbon sugars, and it also had much higher affinity for NADPH than for NADH. The results were in good agreement with a previous report on the activity of the same enzyme purified from *C. boidinii (31)*. CbXYL1p showed even higher activity for L-arabinose than D-xylose, thereby demonstrating that the cloned gene coded for an NADPH-linked AR.

### *Effect of Aeration on AR Activity Against NAD(P)H*

When we cultivated *C. boidinii* at two different agitation levels, we noted a change in the relative XYL1p activities of the crude cell homogenates against NADH and NADPH. As the aeration rate was increased, the activity ratio for NADH/NADPH decreased from 2.13 to 0.76. However, when *CbXYL1* was expressed in *S. cerevisiae* under the same aeration rates and tested for activity with NADH and NADPH, cell homogenates only showed the activity for NADPH (Table 2). When the endogenous *S. cerevisiae* AR activity was tested as control, no significant activity was detected (data not shown). A decrease in the NADH/NADPH ratio with increasing aeration was in good agreement with a previous report *(15)*.

## Discussion

The *XYL1* gene coding for XYL1p was isolated from *C. boidinii* through PCR amplification and by genome walking. The *CbXYL1* clone was identified as XYL1p by comparing its implied protein sequence with other aldose (xylose) reductases. The catalytic tetrad of XYL1p, Tyr48/Asp43/Lys77/His110 *(32–34)* is completely conserved as Tyr50/Asp45/Lys79/His112 in CbXYL1p. According to these reports *(32–34)*, Tyr50 is proposed to be the proton donor of the aldehyde reaction by CbXYL1p. The residue pair Asp45/Lys79 is expected to interact with the hydroxy group of Tyr50. His112 may facilitate proton donation by Tyr50. Therefore, CbXYL1p has the conserved amino acids for AR function. Jez et al. *(32)* have reviewed the cofactor-binding domain and residues. These consensus sequence and amino acids were well conserved in CbXYL1p.

When this gene was expressed in *S. cerevisiae* under its native promoter, xylitol accumulation was observed in the medium. This result showed that the *CbXYL1* promoter is recognized in *S. cerevisiae*. When the cell homogenate from recombinant *S. cerevisiae* S4α was used for enzyme assay AR activity was detected, but no AR activity was detected in homogenates from the nonrecombinant parent. XR from *P. stipitis* can be expressed in *S. cerevisiae* with its native promoter *(19,35)*. Other aldose (xylose) reductases were also successfully expressed in *E. coli (18,19)* or *Pichia pastoris (21)*.

CbXYL1p could reduce several aldose sugars when it was expressed in *S. cerevisiae*. It showed higher activity for 5-carbon than 6-carbon sug-

ars. This CbXYL1p showed even higher activity for L-arabinose than for D-xylose. However, CbXYL1p showed cofactor affinity only for NADPH. XR from *P. stipitis* with dual activity for NAD(P)H can also assimilate other aldoses *(36)*. Even though crude cell homogenates were used for enzyme assays rather than purified activities, AR activity was detected only in recombinant cells, so the cofactor and substrate activities are indicative of the recombinant protein.

Cell homogenates from *C. boidinii* cells grown under different aeration rates showed different affinities with NADH and NADPH. As the aeration rate was increased, the NADH:NADPH affinity ratio decreased. This was in good agreement with the previous report in which NADH/NADPH activity ratio for CbXYL1p varied when xylitol production was examined at various aeration rates *(15)*.

We infer from these findings that other aldose (xylose) reductases with significant activity for NADH are induced in *C. boidinii* under oxygen-limited conditions. Previous reports have shown that AR activities with different affinities for NADH and NADPH could exist in the cells. Some yeasts possess aldose (xylose) reductases that are specific for NADPH *(21,37)*. Other yeasts have enzymes with dual activity for NADH and NADPH *(7,38,39)*, and still other organisms have multiple forms *(40,41)* that show different activity against NADH and NADPH, respectively. However, genomic Southern blot analysis in the present work revealed only a single gene for AR in *C. boidinii*. The cloned *CbXYL1* also had highly specific activity for NADPH when expressed in *S. cerevisiae*. The NADH-linked enzyme activity in cell homogenates may be due to other cellular enzymes capable of utilizing NADH in *C. boidinii (21)*. Because the consumption of NADH at high aeration would be greater than at low aeration, it could cause the NADH/NADPH ratio to decrease. Therefore, we conclude that the *CbXYL1* of *C. boidinii* is a single-copy gene for AR that is specifically linked to NADPH.

We also infer from these results that at least two NADH- and NADPH-linked reductases are present in *C. boidinii*. The gene that we have cloned only shows activity for NADPH—such as the NADPH-linked XYL1p of *K. lactis*, which was most closely related to CbXYL1p. The second gene is not closely related to *CbXYL1* because no additional bands were observed in Southern hybridization.

## Acknowledgments

Y.-S. Jin and J. Laplaza provided helpful comments and discussion. We acknowledge financial support from the National Renewable Energy Laboratory under subcontract no. ZCG-9-29009-01 and from Iogen.

## References

1. Aristidou, A. and Penttila, M. (2000), *Curr. Opin. Biotechnol.* **11,** 187–198.
2. Maleszka, R. and Schneider, H. (1982), *Can. J. Microbiol.* **28(3),** 360–363.

3. Slininger, P. J., Bothast, R. J., van Cawenberge, J. E., and Kurtzman, C. P. (1982), *Biotechnol. Bioeng.* **24,** 371–384.
4. Skoog, K. and Hähn-Hagerdal, B. (1988), *Enzyme Microb. Technol.* **10,** 66–80.
5. Schneider, H. (1989), *CRC Crit. Rev. Biotechnol.* **9,** 1–40.
6. Mishra, P. and Singh, A. (1993), *Adv. Appl. Microbiol.* **39,** 91–152.
7. Verduyn, C., van Kleef, R., Frank, J., Schneider, H., van Dijken, J. P., and Scheffers, W. A. (1985), *Biochem. J.* **226,** 669–677.
8. Bolen, P., L. and Detroy, R. W. (1985), *Biotechnol. Bioeng.* **27(3),** 302–307.
9. Jin, Y. S., Jones, S., Shi, N.-Q., and Jeffries, T. W. (2002), *Appl. Environ. Microbiol.* **68,** 1232–1239.
10. Richard, P., Tiovari, M. H., and Penttila, M. (2000), *FEMS Microbiol. Lett.* **190,** 39–43.
11. Hagedorn, J. and Ciriacy, M. (1989), *Curr. Genet.* **16,** 27–33.
12. Bruinenberg, P. M., de Bot, P. H. M., van Dijken, J. P., and Sheffers, W. A. (1984), *Appl. Microbiol. Biotechnol.* **19,** 256–260.
13. Verduyn, C., Frank, J., van Dijken, J. P., and Scheffers, W., A. (1985), *FEMS Microbiol. Lett.* **30,** 313–317.
14. Vandeska, E., Kuzmanova S., and Jeffries, T. W. (1995), *J. Ferment. Bioeng.* **80,** 513–516.
15. Jeffries, T. W. and Shi, N.-Q. (1999), *Adv. Biochem. Eng. Biotechnol.* **65,** 117–161.
16. Flores, C.-L., Rodríguez, C., Petit, T., and Gancedo, C. (2000), *FEMS Microbiol. Rev.* **24,** 507–529.
17. Cho, K. M., Yoo, Y. J., and Kang, H. S. (1999), *Enzyme Microb. Technol.* **25,** 23–30.
18. Hacker, B., Habenicht, A., Kiess, M., and Mattes, R. (1999), *J. Biol. Chem.* **380,** 1395–1403.
19. Amore, R., Kotter, P., Kuster, C., Ciriacy, M., and Hollenberg, C. P. (1991), *Gene* **109,** 89–97.
20. Yokoyama, S., Kinoshita, Y., Suzuki, T., Kawai, K., Horitsu, H., and Takamizawa, K. J. (1995), *J. Ferment. Bioeng.* **80,** 603–605.
21. Handumrongkul, C., Ma, D. P., and Silva J. L. (1998), *Appl. Microbiol. Biotechnol.* **49,** 399–404.
22. Bolen, P. L., Hayman, G. T., and Shepherd, H. S. (1996), *Yeast* **12,** 1367–1375.
23. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989), *Molecular Cloning: A Laboratory Manual,* 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
24. Mandler, J. and Ludwig, S. (1990), SUBSIS: DNASIS/PROSIS subdirectory management. *Comp. Appl. Biosci.* **6,** 291–292.
25. Saitou, N. and Nei, M. (1987), *Mol. Biol. Evol.* **4,** 406–425.
26. Christianson, T. W., Sikorski, R. S., Dante, M., Shero, J. H., and Hieter, P. (1992), *Gene* **110,** 119–122.
27. Bruinenbur, P. M., van Dijken, J. P., and Alexander-Scheffers, W. (1983), *J. Gen. Microbiol.* **129,** 965–971.
28. Wolff, S. P. and Crabbe, M. J. C. (1985), *Biochem. J.* **226,** 625–630.
29. Lee, H. (1998), *Yeast* **14,** 977–984.
30. Kostrzynska, M., Sopher, C. R., and Lee, H. (1998), *FEMS Microbiol. Lett.* **159,** 107–112.
31. Bolen, P. L. and McCracken, D. A. (1990), *J. Ferment. Bioeng.* **69,** 211–214.
32. Jez, J. M., Bennett, M. J., Schlegel, B. P., Lewis, M., and Penning, T. M. (1997), *Biochem. J.* **326,** 625–636.
33. Bohren, K. M., Grimshaw, C. E., Lai, C. J., Harrison, D. H., Ringe, D., Petsko, G. A., and Gabbay, K. H. (1994), *Biochemistry* **33,** 2021–2032.
34. Tarle, I., Borhani, D. W., Wilson, D. K., Quiocho, F. A., and Petrash, J. M. (1993), *J. Biol. Chem.* **268,** 25,687–25,693.
35. Takuma, S., Nakashima, N., Tantirungkij, M., Kinoshita, S., Okada, H., Seki, T., and Yoshida, T. (1991), *Appl. Biochem. Biotechnol.* **28/29,** 327–340.
36. Shi, N.-Q., Prahl, K., Hendrick, J., Cruz, J., Lu, P., Cho, J.-Y., Jones, S., and Jeffries, T. (2000), *Appl. Biochem. Biotechnol.* **84/86,** 201–216.
37. Billard, P., Menart, S., Fleer, R., and Bolotin-Fukuhara, M. (1995), *Gene* **162,** 93–97.
38. Neuhauser, W., Haltrich, D., Kulbe, K. D., and Nidetzky, B. (1997), *Biochem. J.* **326,** 683–692.

*39.* Ho, N. W. Y., Lin, F. P., Huang, S., Andrew, P. C., and Tsao, G. T. (1990), *Enz. Microb. Technol.* **12,** 33–39.
*40.* Bolen, P. L., Bietz, J. A., and Detroy, R. W. (1985), *Biotechnol. Bioeng. Symp.* **15,** 129–148.
*41.* Mayr, P., Bruggler, K., Kulbe, K. D., and Nidetzky, B. (2000), *J. Chromatogr. B* **737,** 195–202.