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Isolation of Xylose Reductase Gene of *Pichia stipitis* and Its Expression in *Saccharomyces cerevisiae*

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ABSTRACT

A NADPH/NADH-dependent xylose reductase gene was isolated from the xylose-assimilating yeast, *Pichia stipitis*. DNA sequence analysis showed that the gene consists of 951 bp. The gene introduced in *Saccharomyces cerevisiae* was transcribed to mRNA, and a considerable amount of enzyme activity was observed constitutively, whereas transcription and translation in *P stipitis* were inducible. *S. cerevisiae* carrying the xylose reductase gene could not, however, grow on xylose medium, and could not produce ethanol from xylose. Since xylose uptake and accumulation of xylitol by *S. cerevisiae* were observed, the conversion of xylitol to xylulose seemed to be limited.

Index Entries: Xylose reductase; DNA sequence; gene expression; *Pichia stipitis; Saccharomyces cerevisiae*

INTRODUCTION

In yeasts, xylose is generally considered to be assimilated through xylitol and xylulose by the catalysis of xylose reductase (E.C.1.1.1.139) and xylitol dehydrogenase (xylulose reductase; E.C.1.1.1.9), as summarized in Fig. 1. However, *Saccharomyces cerevisiae,* commonly used for alcohol production, cannot utilize xylose for the production, but is able to

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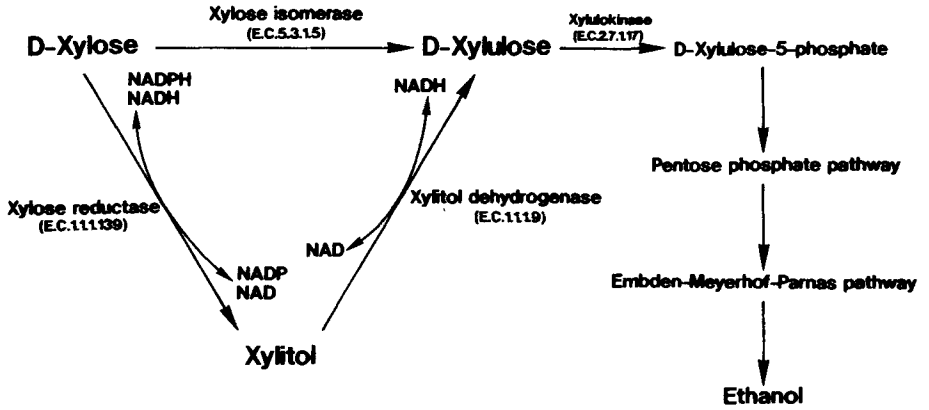

Fig. 1. Schematic illustration of the xylose-assimilation pathway.

ferment xylulose to ethanol (*1*). This fact suggests that the conversion of xylose to xylulose through xylitol is limited (*2*). Bruinenberg et al. suggested that the impossibility of the conversion is caused by the uncoupling of the NADPH and NAD regeneration circuit, as xylose reductase and xylitol dehydrogenase of *S. cerevisiae* are dependent on NADPH and NAD, respectively. On the other hand, *Pichia stipitis* can assimilate and ferment xylose, though the ethanol production is much less than that by *S. cerevisiae* (*3*). The xylose assimilation ability of *P. stipitis* is considered to be as a result of the cofactor dependency of its xylose reductase, which has affinity to both NADPH and NADH, and to the efficient circulation of NADH/NAD regeneration (*3*).

To make alcohol production possible from xylose, Sarthy et al. (*4*) introduced a bacterial xylose isomerase gene into *S. cerevisiae*. Xylose isomerase (E.C.5.3.1.5) from *E. coli* was cloned and its protein was synthesized in *S. cerevisiae*, but the enzyme protein did not show activity, owing to the unsuitable tertiary structure.

In this study, we isolated the xylose reductase gene from the chromosomal gene library of *P. stipitis*, using an oligonucleotide probe synthesized by referring the NH_2-terminal amino acid sequence of the enzyme protein. The cloned gene was sequenced, and the predicted amino acid sequence was compared with partial amino acid sequences determined by peptide sequencing. The xylose reductase gene introduced to *S. cerevisiae* was transcribed constitutively, and the substantial enzyme activity was detected. The transformants of *S. cerevisiae* could not, however, assimilate xylose; the conversion of xylitol to xylulose was considered to be inefficient.

MATERIALS AND METHODS

Microorganisms, Plasmids, and Phages

P. stipitis CBS5773 (*3*) was used for the isolation of xylose reductase and the source of its gene. *S. cerevisiae* NA87-11A (*ho MAT Leu2-3,112 trp1*

his3 pho5-1 [*cir*$^+$]), kindly provided by S. Harashima, Osaka University, and *S. cerevisiae* AM12 (wild type) (*5*) were used as host strains. *E. coli* JM109 (*6*) was used as host strain for the gene manipulations, and as an indicator strain of M13 phage (*6*). pUC18 and pUC19 (*6*) were used for vector plasmids in *E. coli*, and YEp13 (*7*) and YRpG1, kindly supplied through Y. Oshima, Osaka University, were for shuttle vectors in *S. cerevisiae* and *E. coli*. The λEMBL3 system (Toyobo Co. Ltd., Kyoto, Japan) (*8*) was applied for the cloning of chromosomal DNA of *P. stipitis*.

Media and Cultivations

A complete medium (YPD) composed of 2% glucose, 2% polypeptone (Difco Laboratories, Detroit, MI), and 1% yeast extract (Difco) (pH 5.5) was used for the cultivation of *P. stipitis* and *S. cerevisiae*. A medium composed of 2% carbon source (glucose, xylose, or xylulose) and 0.67% yeast nitrogen base (without amino acids, Difco) was used as a minimal medium for *S. cerevisiae*, with the addition of 1/20 vol of an amino acid–nucleic acid solution (H solution); 0.4 g of uracil, tryptophan, histidine, arginine, and methionine, 0.6 g of tyrosine, leucine, isoleucine, and lysine, 3.0 g of valine, and 1.2 g of phenylalanine/L.

LB (*9*) and M9 (*9*) media, supplemented with H solution, were used as a nutrient and a minimal medium, respectively, for *E. coli*. Forty microgram of ampicillin (viccillin; Meiji Seika Kaisha Co. Ltd., Osaka, Japan), 12.5 μg of tetracycline (Sigma Chemicals, St. Louis, MO), or 200 μg of G418/mL (Gibco Oriental, Osaka, Japan) was added in LB or YPD medium for the selection of antibiotic-resistants. *P. stipitis* and *S. cerevisiae* were cultivated at 30°C and *E. coli* was at 37°C.

Enzyme Assay

Xylose reductase and xylitol dehydrogenase were assayed at 30°C by the methods of Bruinenberg et al. (*2*). The activity of xylose reductase or xylitol dehydrogenase, which oxidized 1 μ*M* of NADPH and NADH or reduced 1 μ*M* of NAD/min, respectively, was defined as one unit. The concentration of protein was measured by the Lowry-Folin method (*10*), using the calf serum albumin as a standard.

Enzyme Purification and Amino Acid Sequencing

Cells of *P. stipitis* suspended in 50 m*M* potassium phosphate buffer, pH 7.0, containing 10% glycerol and 0.1% β-mercaptoethanol (PK buffer) were disrupted at 4°C by Dyno-Mill (Willy A Bachofen Mashinenfabrik Basel Schweiz, Wien, West Germany). The crude extract was prepared by centrifugation at 12,000 rpm for 10 min. Enzymes were purified through DEAE-cellulose (Pharmacia Fine Chemicals, Uppsala, Sweden) column chromatography and affinity column chromatography, using Cibacron Brilliant Yellow GE (Ciba-Geigy)-Sephacryl S200 (Toyo Roshi Co. Ltd.,

Tokyo, Japan) prepared by the method of Verduyn et al. (3). High performance liquid chromatography (HPLC) was also applied for further purification, using a Cosmosil 5-C4 300 column (Nacalai Tesque, Kyoto, Japan), and samples were eluted under a gradient of acetonitrile (1%/min). The amino acid sequence of xylose reductase was determined by protein/peptide sequencer (model 477A; Applied Biosystems, Foster, CA).

DNA Manipulation and Hybridizations

DNA isolation and general DNA manipulations in *E. coli* followed the method of Maniatis et al. (9). Chromosomal DNA of *P. stipitis* was extracted according to the method of Hereford et al. (11). Enzymes for DNA manipulations were purchased from Takara Shuzo Co. Ltd. (Kyoto, Japan) and Toyobo Co. Ltd. The transformations of *E. coli* and *S. cerevisiae* were performed by the methods of Morrison (12) and Ito et al. (13), respectively.

Southern and Northern hybridization were carried out by the methods of Southern (14) and Alwine et al. (15), respectively, using Hybond-N membrane (Amersham, Buckinghamshire, UK). Total RNAs of *S. cerevisiae* were prepared according to the method of Jensen et al. (16). An oligonucleotide for use as a probe was synthesized by DNA synthesizer (Applied Biosystems). The probe DNA was labeled with [γ-^{32}P] ATP (Amersham), using T4 polynucleotide kinase or with [α-^{32}P] dCTP (Amersham) by using the random primed DNA labeling kit (Boehringer Mannheim Gmbh, Biochemica, Mannheim, West Germany).

Cloning of Chromosomal DNA of *P. stipitis* and DNA Sequencing

The gene library of chromosomal DNA of *P. stipitis* was prepared using the λEMBL3 phage system (Toyobo) (8). Chromosomal DNA was digested partially with *Sau*3AI and ligated with *Bam*HI-digested λEMBL3 arms, and packaged using the packaging system (Packagene; Promega, Madison, WI), and the mixture was subjected to transfection in *E. coli*. Positive clones carrying the xylose reductase gene were selected by plaque hybridization (17). For DNA sequencing, fragmented DNAs were subcloned in M13-mp19 or pBluescript (Stratagene, La Jolla, CA). The DNA sequence was determined by using a Sequenase kit (Toyobo), and analyzed using GENETYX programs (Nippon Software Kaihatsu, Tokyo, Japan).

RESULTS

Purification of Xylose Reductase

NADPH/NADH-dependent xylose reductase of *P. stipitis* was partially purified and characterized by Verduyn et al. (3), but the amino acid se-

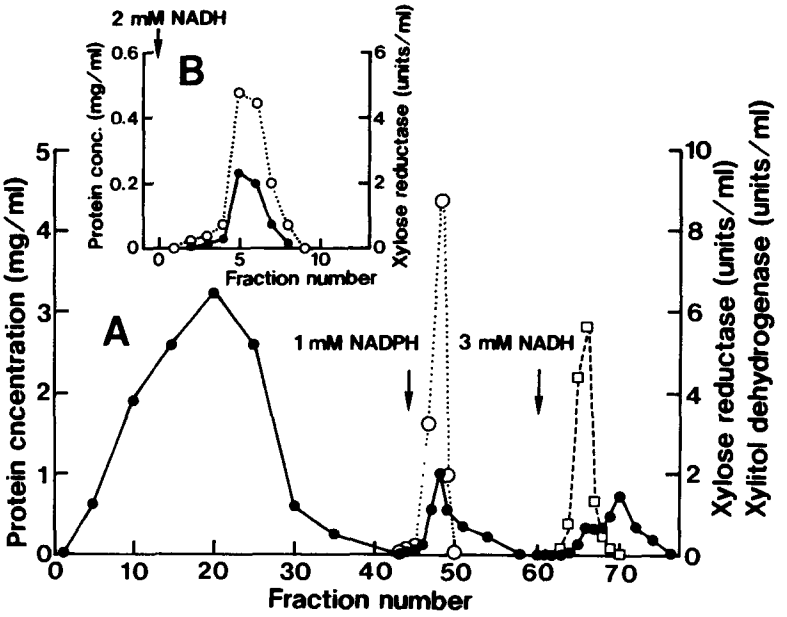

Fig. 2. Purification of xylose reductase by affinity chromatography, using Cibacron brilliant yellow GE-Sephacryl S-200. (A) the crude enzyme purified by DEAE-cellulose chromatography was applied, and eluted by NADPH and NADH. (B) the enzyme fractions having xylose reductase activity in the chromatograph shown in (A) were used for further purification. The protein concentration, and the activities of xylose reductase and xylitol dehydrogenase were indicated by ●, ○, and □, respectively.

quence was not deduced. To synthesize a probe DNA for gene isolation, we purified the enzyme and determined the NH_2-terminal amino acid sequence. Cells of *P. stipitis* were cultivated on the complete medium using xylose as a carbon source, since the enzyme production was induced by xylose about 50-fold, compared with the production in the complete medium using glucose. Cells were harvested at the late-logarithmic phase, and disrupted by Dyno-Mill.

The enzyme was partially purified through DEAE-cellulose ion exchange column chromatography with 0–1 *M* KCl gradient. Since the fractions containing xylose reductase activity contained xylitol dehydrogenase activity (data not shown), the sample was then applied on affinity column for chromatography, using Cibacron brilliant yellow GE-Sephacryl S-200, and eluted by 1 m*M* NADPH and 0–3 m*M* gradient of NADH, successively, as shown in Fig. 2A. Xylose reductase (NADPH/NADH-dependent) and xylitol dehydrogenase (NADH-dependent) were completely separated from each other by NADPH and NADH elution. For further purification, the fractions having xylose reductase activity were subjected again to affinity chromatography and eluted by 2 m*M* NADH (Fig. 2B). The profiles of the enzyme activity and protein concentration correlated completely.

The purity was confirmed to be more than 95% by SDS polyacrylamide gel electrophoresis (data not shown), and the mol wt of the monomer was estimated to be 35,000 dalton. The specific activity dependent on NADH was calculated as 19.8 U/mg protein, which was 100 times that of the crude extract, and the ratio of activity dependent on NADH to that dependent on NADPH was 0.73. These results accorded well with those determined by Verduyn et al. (*3*).

Amino Acid Sequence and Synthesis of Probe Oligonucleotide

The purified enzyme was also fragmented with lysil-endopeptidase, and the generated peptides were separated by HPLC. The whole enzyme and one of the peptides fractionated were subjected to amino acid sequencing by the peptide sequencer. The 23 amino acids of the NH_2-terminus (except for the 18th amino acid) of the enzyme was determined as *N*-Pro-Ser-Ile-Lys-Leu-Asn-Ser-Gly-Tyr-Asp-Met-Pro-Ala-Val-Gly-Phe-Gly-(?)-Trp-Lys-Val-Asp-Val-Asp. Another peptide fragment generated by lysil-endopeptidase digestion was also deduced as (Lys;cutting site)-Ser-Pro-Ala-Gln-Val-Leu-Leu-Arg-Trp-Ser-Ser-Gln-Arg-Gly-Ieu-Ala-Ile-Ile-Pro-Lys.

According to the amino acid sequence of the NH_2-terminus of the enzyme peptide, an oligonucleotide as a probe DNA was synthesized to hybridize with its mRNA, referring to the codon usage observed in *S. cerevisiae* (*18*). The probe DNA was a mixture of 29mers corresponding to the 8th of glycine, to the 17th of glycine from the terminus; 3'-CCAATGCTAT(A/G)CGGTCG(A/G)CA(A/G)-CCAAAACC-5'. The probe was hybridized efficiently with the 2.3 kbp *Eco*RI-digested chromosomal DNA. The probe also hybridized well with a specific mRNA, induced in the medium containing xylose as a sole carbon source, as well as the enzyme synthesis induced by xylose (Fig. 3A). The mol size of the specific mRNA was estimated to be 1.0 kb, by referring to the size of the rRNAs as standards (estimated to be 1.8 kb and 3.4 kb for 18S and 25S rRNA, respectively; *19*).

Cloning of Xylose Reductase Gene and Its DNA Sequence

The gene library was made by the λEMBL3 vector system. The chromosomal DNA of *P. stipitis* was digested partially with *Sau*3A. The 10–20 kb DNA fragments recovered by sucrose-gradient centrifugation were ligated with the *Bam*HI-digested DNA of λEMBL3, and packaged in coat protein by the in vitro packaging system. Following the transfection of *E. coli* with packaged phages, the possible positive clones carrying the xylose reductase gene were screened by the plaque hybridization method, using the probe.

Three positive clones were obtained among 5000 plaques. From one of them, the 7.5 kbp *Hin*dIII–*Hin*dII fragment of *P. stipitis* chromosome,

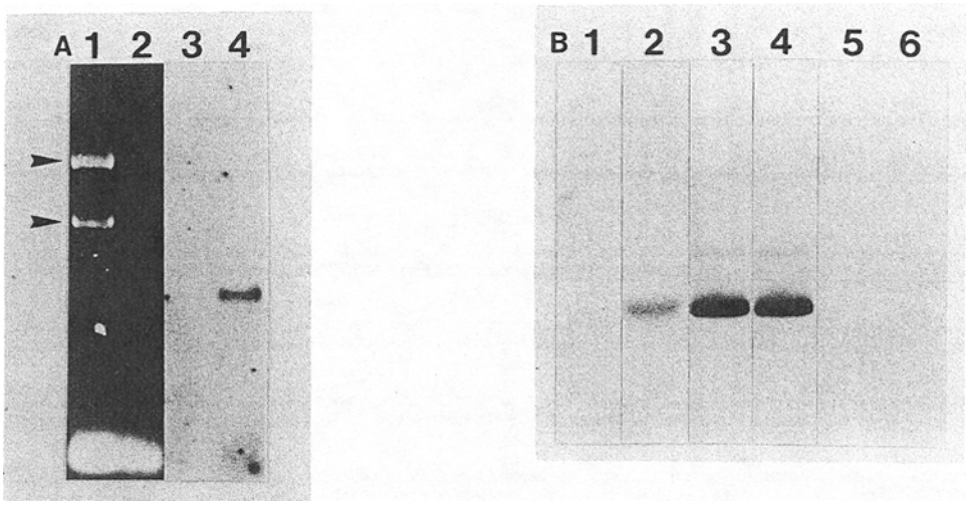

Fig. 3. Northern hybridization of total mRNAs from *P. stipitis*, *S. cerevisiae*, and transformants with a synthetic probe and a probe of the partial fragment of the xylose reductase gene. (A) lanes 1 and 2; agarose electrophoresis of total mRNAs from *P. stipitis* cultivated under induced and uninduced conditions, respectively, and lanes 3 and 4; northern hybridization of the mRNAs obtained under induced and uninduced conditions, respectively, with a synthetic probe. Arrows show the positions of 18S and 25S RNA. (B) Northern hybridization of total mRNAs with the probe derived from chromosomal DNA. Lanes 1, 3, and 5; mRNAs from *P. stipitis*, transformants of *S. cerevisiae*, and a host strain, NA87-11A, of *S. cerevisiae*, respectively, cultivated under uninduced condition, respectively. Lanes 2, 4, and 6; mRNAs from these, respectively, cultivated under induced condition.

hybridizing strongly with the probe, was cut out and subcloned on a vector plasmid pUC18. The recombinant plasmid was designated as pTN1. As the region hybridizing with the probe was limited to ca. 300 bps of the *Pst*I–*Sal*I region (Fig. 4; 115-148 nucleotide position) by Southern hybridization analysis, the *Bam*HI–*Eco*RV fragment of pTN1 was subcloned on pUC19, and subjected to the determination of the DNA sequence.

According to the DNA sequence shown in Fig. 4, we observed a long open reading frame consisting of 954 bp, and also found identical sequences to one of the probe sequences (corresponding to the nucleotide positions from 379 to 407). The NH_2-terminal sequence of 24 amino acids predicted from the DNA sequence (except for the first amino acid, Met, corresponding to the 358th–429th base) and the 20 amino acid sequence (corresponding to the 1102th–1165th base in the DNA sequence) were completely identical to those determined by the amino acid sequencing of the enzyme protein and the fragmented peptide. The 23rd amino acid of the NH_2-terminus that was unable to be determined by amino acid sequencing was deduced to be cysteine. These results indicate that the open reading frame corresponds to the xylose reductase gene.

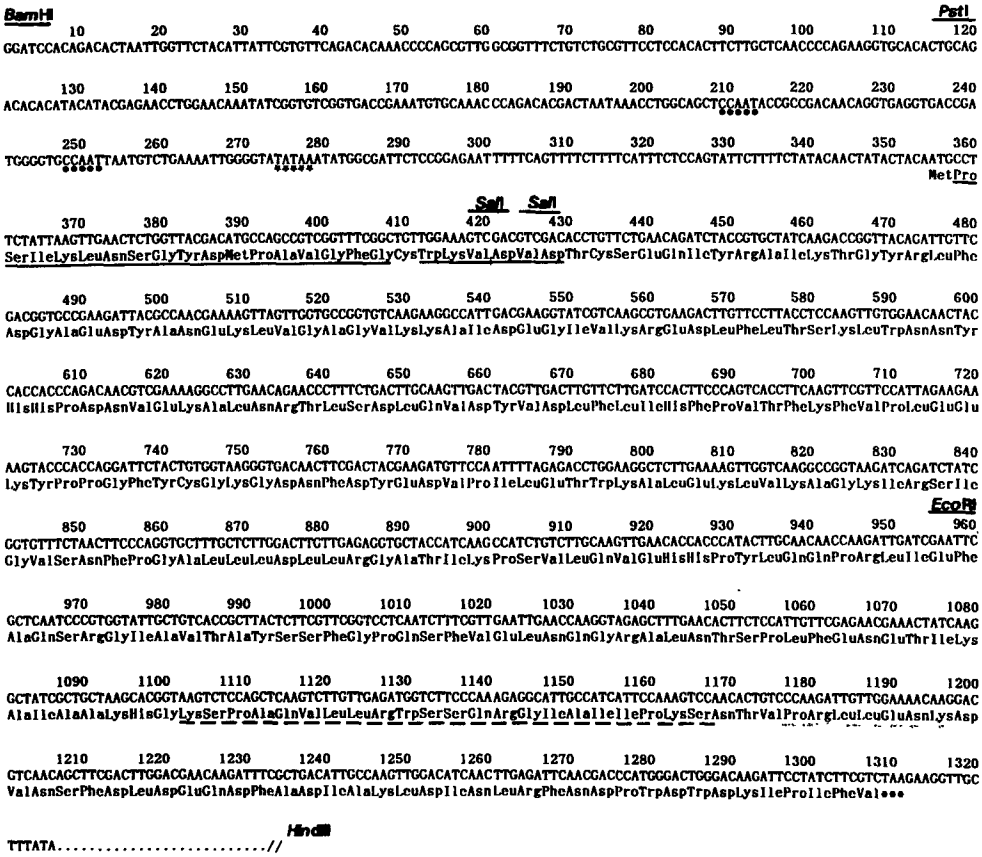

Fig. 4. DNA sequence of the gene for xylose reductase and its upstream region. The closed and dotted lines under the amino acid sequence show the parts of the amino acid sequence identical to that determined by peptide sequencer. The regions marked with closed circles and stars correspond to the consensus sequences for the yeast promoter.

The mol wt of xylose reductase calculated from the DNA sequence was 35,773, which accords well with that estimated by SDS gel electrophoresis of the monomer protein. Upstream of the gene, consensus sequences, i.e., the TATA and CAAT boxes, for the promoters found in the genes of *S. cerevisiae*, were also observed. The codon usages for the amino acids predicted from the cloned gene were similar to those used in *S. cerevisiae*.

Transfer of Cloned Xylose Reductase Gene in *S. cerevisiae*

The *Bam*HI-*Bam*HI fragment (*Bam*HI at the 5'end was in the *Hind*III-*Hind*III region carrying xylose reductase and another *Bam*HI at the 3'end was in pUC18) of pTN1 was subcloned on a YEp13 (Fig.5). The resultant plasmid, pTN 131, was transformed in *S. cerevisiae* NA87-11A. The transformant (designated as NA87-11A/pTN131) selected by the leucine proto-

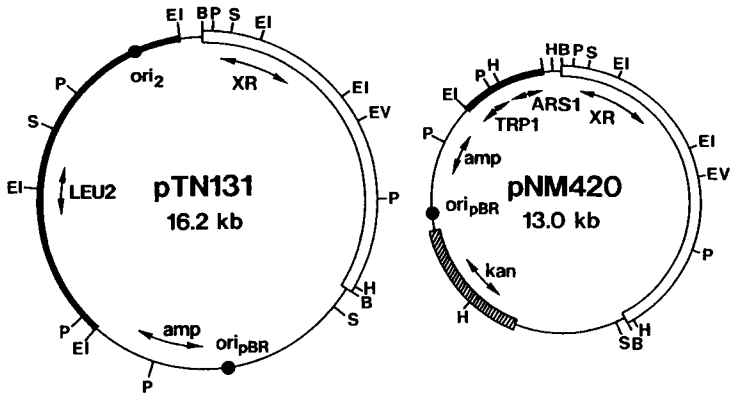

Fig. 5. Physical maps of pTN131 and pNM420 carrying xylose reductase gene of *P. stipitis*. Xylose reductase gene of *P. stipitis* was shown by XR. Abbreviations for restriction sites were as follows: B; *Bam*HI, EI; *Eco*RI, EV; *Eco*RV, H; *Hin*dIII, S; *Sal*I, and P; *Pst*I.

troph could not, however, grow on the medium containing xylose as a sole carbon source (xylose medium).

We considered conceivable reasons for the inability of the transformant to grow on xylose medium: (1) the cloned gene for xylose reductase is not transcribed, (2) mRNA specific for xylose reductase is not translated, (3) the protein of xylose reductase is inactive, (4) the transportation of xylose is missing, (5) xylitol dehydrogenase is insufficient for the conversion of xylitol to xylulose, and (6) xylulose cannot be assimilated to ethanol.

To confirm transcription of the cloned xylose reductase gene, Northern hybridization of mRNA was carried out. Total RNAs were extracted from *P. stipitis* and the transformant, NA87-11A/pTN131, cultivated in the medium containing 2% glucose (glucose medium) or the medium containing the xylose (7.5%)-xylulose (2.5%) mixture (xylose–xylulose medium), respectively, and used for hybridization with the *Sal*I–*Eco*RI fragment of the xylose reductase gene (425th–955th base, Fig. 4). The hybridized band for a specific mRNA was clearly observed in the mRNA preparations from strain NA87-11A/pTN131 under induced and uninduced conditions (Fig. 3B, lanes 3 and 4), whereas in the case of *P. stipitis*, the specific hybridization was observed only under the induced condition (lanes 1 and 2). The results suggest that the cloned gene was transcribed constitutively in the transformant, though minor expression of large RNAs (estimated to be 1.3 kb) was observed.

To confirm xylose reductase activity in the transformant, crude enzyme solutions were prepared from the transformants, *S. cerevisiae* NA87-11A/pTN131 and AM12/pNM420 (Fig. 5). pNM420 was constructed by subcloning of the *Bam*HI–*Bam*HI fragment of pTN131 containing the xylose reductase gene in a G418 resistant plasmid, YRpG1, that was able to select the transformants in wild type strain, and transformed in an

Table 1
Xylose Reductase and Xylitol Dehydrogenase Activity of P. Stipitis, S. Cerevisiae, and Transformants

Strain	Medium[a]	Xylose reductase activity (10^{-2} units/mg)			Xylitol dehydrogenase activity (10^{-2} units/mg)
		NADH-depedent (A)	NADPH-dependent (B)	Ratio (A/B)	
P. stipitis CBS5773	G	1.4	1.5	0.93	2.4
	X	15.5	25.6	0.61	20.8
S. cerevisiae					
NA87-11A	G	0	1.1	0	1.1
	XX	0	1.1	0	1.3
AM12	G	-[b]	-	-	0.4
	XX	0	1.6	0	9.7
Transformants					
NA87-11/pTN131[c]	G	8.6	9.8	0.89	0.7
	XX	2.1	3.6	0.58	1.6
AM12/pNM420[d]	G	-	-	-	-
	XX	11.9	14.4	0.83	10.2
Purified Enzyme from P. stipitis		19.8 x 10^2	27.1 x 10^2	0.73	24.9 x 10^2

Enzyme activities are shown as units per mg protein in the crude enzyme preparations except for the purified enzymes. a: G; glucose medium, X; xylose medium, XX; xylose-xylulose medium. b: not detemined. c:transformant harboring a plasmid, pTN131. d: transformant harboring pNM420.

alcohol-fermenting strain, AM12 (wild type). Strain AM12 has also higher activity of xylitol dehydrogenase than a laboratory strain, NA87-11A, but lower activity than *P. stipitis* has. As shown in Table 1, the transformants produce NADPH/NADH-dependent xylose reductase constitutively, whereas host strains of *S. cerevisiae* produced little NADPH-dependent xylose reductase. The results indicated that the mRNA was translated, and that the enzyme protein has the normal activity.

To confirm the permiability of xylose in *S. cerevisiae*, the xylose uptake was measured by using ^{14}C-xylose as tracer. *P. stipitis* was cultivated in the xylose medium, and *S. cerevisiae* was in the xylose-xylulose medium

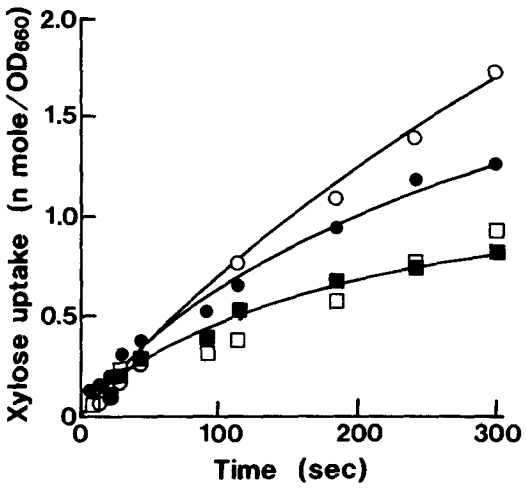

Fig. 6. Xylose uptake by *P. stipitis*, *S. cerevisiae* NA87-11A and AM12, and transformant NA87-11A/pTN131. The uptakes were measured using ^{14}C-xylose. Symbols, ○, *P. stipitis*; □, NA87-11A; ●, AM12; ■, NA87-11A/pNT131.

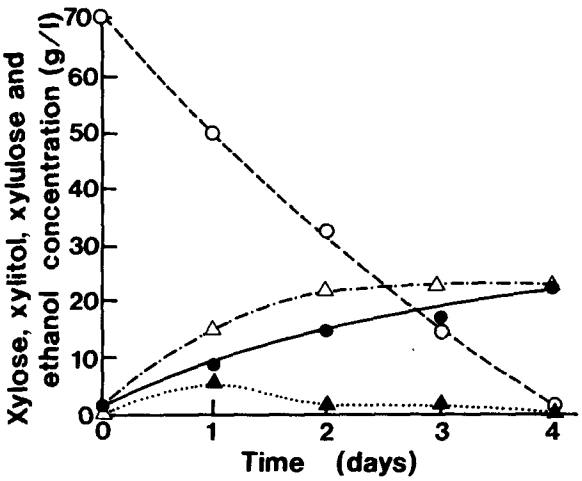

Fig. 7. Cultivation of *S. cerevisiae* AM12 in the xylose medium coexisting with immobilized xylose isomerase. The initial concentration of xylose was 7%. The pH was controlled at 6.0, and aeration was carried out at 0.5 vvm during the fermentation. Xylose, xylitol, xylulose, and ethanol concentrations are shown with ○, ●, ▲, and △, respectively.

because it was unable to grow in the xylose medium. The initial rates of xylose uptake by the host strains of *S. cerevisiae* and the transformant seemed to be almost the same as *P. stipitis* (Fig. 6), whereas the uptake was not observed at 0°C (data not shown). The result suggests that the xylose uptake was considered not to be a limiting step for assimilation. On the other hand, *S. cerevisiae* AM12 was able to grow and produce ethanol on the xylulose medium, in which xylose was transformed to xylulose by immobilized bacterial xylose isomerase (Fig. 7). This result

suggests that *S. cerevisiae* would be able to assimilate xylose if xylose were to be converted effectively to xylulose through xylitol. However, it was expected that an equilibrium of xylitol dehydrogenase inclined toward xylitol more than to xylulose in *S. cerevisiae*, since xylitol was accumulated during the fermentation.

The result of the thin layer chromatography of intermediates showed that xylose and xylitol were mainly present in the cells of *S. cerevisiae* (data not shown). Nevertheless, one of the hosts, AM12, seemed to have enough xylitol dehydrogenase activity (Table 1), though the transformant, AM12/pNM420, still could not grow on the xylose medium. This inconsistency might be the reason for the inability of xylose assimilation by the transformants, though the gene for the xylose reductase gene are expressed effectively.

DISCUSSION

We isolated the gene for NADPH/NADH-dependent xylose reductase of *P. stipitis* from the gene library by using a probe specific for the NH_2-terminal amino acid sequence. The gene was successively introduced in *S. cerevisiae*, and the specific enzyme of NADPH/NADH-dependent xylose reductase was synthesized. According to the analysis of DNA and amino acid sequences, we have found no similarity between the cloned xylose reductase and other dehydrogenases that require NAD/NADH as a cofactor (*20*). And the NH_2-terminal protein sequence of xylose reductase of *P. stipitis* had no significant similarity to those of *Pachysolen tannophilus* (*21*) and *Candida shehatae* (*22*). The expression of the gene in *S. cerevisiae* was considered to be directed by the consensus promoter (*23*) found in the upstream region of the gene. Since the gene was expressed constitutively in *S. cerevisiae*, the original regulation mechanism relating to induction in *P. stipitis* was not available in *S. cerevisiae*.

S. cerevisiae has a strong alcohol fermentation ability, but cannot produce alcohol from xylose, a component of hemicellulose and one of the unutilized natural resources. Generally, xylose is considered to be assimilated through xylitol and xylulose in yeast strains, as shown in Fig. 1 (*2*), and no presence of xylose isomerase as seen in bacteria has been confirmed in yeasts. The inability of xylose assimilation by *S. cerevisiae* is suggested to be resulting from a lack of NADH-dependent xylose reductase for the effective circulation of NAD/NADH reproduction coupling with NAD-dependent xylitol dehydrogenase (*3*). In this study, we introduced the NADPH/NADH-dependent xylose reductase gene of *P. stipitis* into *S. cerevisiae*. As a result, the transformants could not assimilate xylose, though all of the enzymes required for xylose assimilation and the xylose transport system seemed to be available in the transformant. This inconsistency might be related to the equilibration between xylitol and xylulose

by xylitol dehydrogenase. Generally, an equilibrium reaction catalyzed by xylitol dehydrogenae (xylulose reductase) favors the accumulation of xylitol (*1,2*). To test the fermentation of xylulose (Fig. 7), *S. cerevisiae* was cultivated under semiaerobic condition, because the NAD/NADH circuit requires oxygen, and the accumulation of xylitol was observed as a main byproduct. *P. stipitis,* however, did not accumulate a noticeable amount of xylitol during semiaerobic cultivation (data not shown). This fact suggests that the equilibrium in xylitol–xylulose conversion in *S. cerevisiae* inclined toward xylitol more than that in *P. stipitis*. As another possibility, it was considered that xylulokinase activity might have an effect on the assimilation of xylose.

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