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Integration and expression of the *Escherichia coli* xylose isomerase gene in *Schizosaccharomyces pombe*

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SUMMARY

The xyclose isomerase gene in *Escherichia coli* was cloned complementarily into a Leu2-negative *Schizosaccharomyces pombe* mutant (ATCC 38399). The subsequent integration of the plasmid into the chromosomal DNA of the host yeast was verified by using the dot blot and southern blot techniques. The expressed xylose isomerase showed activity on a nondenaturing polyacrylamide gel. The expression of xylose isomerase gene was influenced by the concentration of nutrients in the fermentation broth. The yeast possessed a xylose isomerase activity of 20 nmol/min/mg by growing in an enriched medium containing yeast extract-malt extract-peptone (YMP) and D-xylose. The conversion of D-xylose to D-xylulose catalyzed by xylose isomerase in the transformed yeast cells makes it possible to ferment D-xylose with ethanol as a major product. When the fermentation broth contained YMP and 5% (w/v) D-xylose, the maximal ethanol yield and productivity reached 0.42 g/g and 0.19 g/l/h, respectively.

INTRODUCTION

D-xylose, which is prevalent in hemicellulose, can be utilized to produce ethanol by several yeast species [11,14,19]. These organisms have been improved to ferment D-xylose more efficiently, which include mutagenesis [14,19] and gene cloning of yeasts such as *Schizosaccharomyces pombe* [22] and *Saccharomyces cerevisiae* [18], to introduce a D-xylose metabolic pathway.

Most yeasts metabolize D-xylose mainly through reduction of D-xylose to xylitol catalyzed by NADPH-linked reductase, followed by the oxidation of xylitol to D-xylulose catalyzed by NAD^+-linked dehydrogenase [6,10,23]. Subsequently, D-xylulose is phosphorylated to D-xylulose-5-phosphate, which is then channelled into the pentose

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phosphate pathway. Gong et al. [10] postulated that yeasts employ a combination of the pentose phosphate and Embeden-Meyerhof pathway to convert D-xylulose-5-phosphate to pyruvate and then ultimately to ethanol and CO_2.

Although D-xylose metabolic pathway exist in some yeasts, the D-xylose utilization is not efficient [2,4]. These yeasts were found to utilize D-xylulose as efficiently as D-glucose [8]. Therefore, the inefficient utilization of D-xylose by yeasts can be caused by the enzyme regulations involving in the pathways between D-xylose and D-xylulose. Recently, Batt et al. [2] showed that the yeasts do not efficiently utilize D-xylose because of the cofactor regulation (NADPH for xylose reductase or NAD_+ for xylitol dehydrogenase), but not because of the absence of enzymes necessary for catalysis. In order to improve the D-xylose utilization by yeasts, a new D-xylose isomerization reaction can be introduced to the yeast for converting D-xylose to D-xylulose without any cofactor regulation such as NADPH or NAD^+. Ueng et al. [22] have transformed the hybrid plasmid pDB248-XI containing a 2.4 Kb *Escherichia coli* xylose isomerase gene (Fig. 1) into *Schizosaccharomyces pombe*. The presence of this gene results in *S. pombe* being able to utilize D-xylose as a sole carbon source. Chan et al. [4] have demonstrated the ability of the transformed *S. pombe* to ferment D-xylose in the yeast nitrogen based medium.

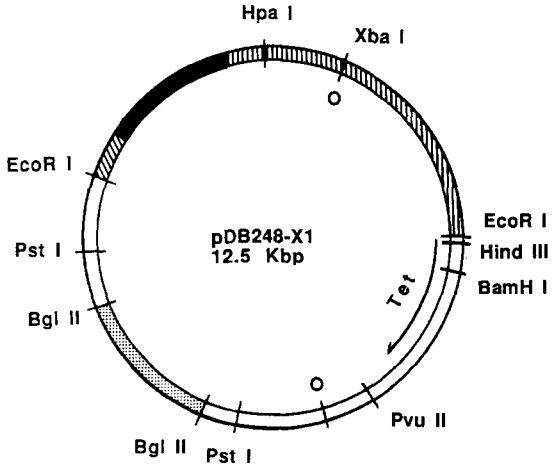

Fig. 1. Construction of Plasmid pD248-XI. pB322 ( ), 2-u DNA ( ), *LEU* 2 gene ( ), *Pst*-I fragment of pLC32-9 carrying the isomerase gene ( ).

Since this plasmid seems relatively stable and since recent attempts to isolate it from the transformed *S. pombe* strain have failed, it is believed that the plasmid might have integrated into the chromosome of the host yeast. In order to confirm the event of chromosomal integration, southern and dot blots were performed. The nutrient effect, especially the nitrogen source, on the expression of xylose isomerase gene and D-xylose fermentation were also studied.

MATERIALS AND METHODS

Strains and maintenance

Schizosaccharomyces pombe (ATCC 38399), a xy^- and $leu2^-$ strain, was obtained from the American Type Culture Collection. The transformed *Schizosaccharomyces pombe* was obtained from Ueng et al. [22] procedure. *Escherichia coli* C600 and *E. coli* K12 were purchased from the American Type Culture Collection. The yeast strain was maintained in the yeast nitrogen base medium (Difco) containing 2% (w/v) D-xylose. The *E. coli* cells were grown in Luria broth.

Chromosomal DNA extraction

The yeast chromosomal DNA was extracted as previously described [6] with a modification of the protoplasting technique using Novozyme 234 [1]. Yeast cells to be extracted were grown to the late exponential phase (1×10^8 cells/ml) in a YMP medium containing 2% D-xylose (w/v). They were harvested by centrifugation, washed twice with 20 mM citrate-phosphate buffer (prepared from 0.2 M solution of citric acid and 0.2 M solution of dibasic sodium phosphate), pH 5.6, and resuspended in the same buffer containing 40 mM EDTA, 0.14 mM mercaptoethanol for 30 min. The cellls were resuspended in 20 mM citrate-phosphate buffer, pH 5.6, containing 0.8 M sorbitol and 5 mb/ml Novozyme 234 (Novo Lab.) at 30°C; after 45 min protoplasts had formed. The *E. coli* C600 DNA was extracted as previously described [24].

Radioactive probes

The 1.6 Kb fragment, containing the *E. coli* 2.4 Kb xylose isomerase gene, was isolated by restricting pDB248-XI with *Bgl*-II and recovering the fragments from the low melting agar [16]. The fragment was then labeled with P^{32} using the Amersham Nich Translation Kit (Arlington Heights, IL) according to the suppliers' instructions.

Southern and dot blot

The DNA samples of pDB248-XI, *E. coli* C600, *S. pombe* and transformed *S. pombe* were restricted independently with *Bgl*-II, *Pst*-I and *Ecor* RI for the Southern blotting experiment. Samples were then run on a 0.6% agarose gel, blotted onto nitrocellulose and hybridized to the 1.6 Kb probe as Maniatis et al. [16] described. Unrestricted DNA samples of pDB248-XI, *E. coli*, *S. pombe* and transformed *S. pombe* were bound to the nitrocellulose using a dot blot apparatus [16].

Enzyme assays

Approximately 10 g of yeast cells were harvested and washed twice with distilled water. In order to break the yeast cells to the release the isomerase, the cell walls were partially hydrolyzed by 0.5 g of Novozyme 234 (Novo Lab.). After being hydrolyzed for 50 min at 30°C in a citrate-phosphate buffer (20 mM. pH 7.5) containing 1.2 M sorbitol, the cells were washed three times with the same buffer and then resuspended in 5 ml of 0.1 M glycerol phosphate buffer (prepared from 0.2 M solution of glycerol and 0.2 M solution of dibasic sodium phosphate) (pH 7.5) containing 0.1% cysteine. The cells were sonified and centrifuged at 35 000 × *g* for 20 min. The supernatant was then used for enzyme assay.

Xylose isomerase activity was determined by the formation of D-xylulose from D-xylose, and xylulokinase activity was monitored by the disappearance of D-xylulose during the reaction [21]. The D-xylulose concentration was measured by the cysteine–carbazole method [9] with pure D-xylulose as the standard [5]. In order to assure the D-xylulose formation and disappearance was caused by the xylose isomerase and xylulokinase, the crude extract solution was added cold acetone to precipitate the protein; then NADPH and NAD^+, the cofactors for xylose reductase and xylitol dehydrogenase were left in the supernatant. The protein residue was dried in vacuum desiccator and stored at 4°C. A suspension of the protein residue in distilled water was used for all enzyme assays. The assay solution was incubated at 25°C and contained 300 μg substrate, 40 mg protein residue, 0.03 M phosphate buffer (pH 7.0), and 0.003 M $MgCl_2$. The protein concentration was measured by the Lowry method [15] with bovine serum albumin as the standard.

The xylose reductase activity as determined by measurement of the decrease in optical density at 340 nm cause by the oxidation of NADPH in the presence of D-xylose. The xylitol dehydrogenase activity was determined by measuring the increase in optical density at 340 mu caused by the reduction of NAD^+ in the presence of xylitol. One unit of enzyme was defined as that amount which would cause a decrease or increase in μmol of NADPH or NADH per min. Specific activity was expressed in terms of units of enzyme per milligram of protein.

Electrophoretic and staining procedures

Nondenaturing polyacrylamide gel electrophoresis was performed at 4°C using the buffer system of Ornstein [17] and Davis [8]. The stacking gel was 5% polyacrylamide and the running gel was 10% polyacrylamide. The stacking gel was photopolymerized using riboflavin (FMN) as a catalyst. The sample and upper reservoir buffer contained 1 mM thioglycolate. The voltage was maintained at 100 V during sample stacking after which it was increased to 150 V.

After completion of electrophoresis, the gel was immediately stained by covering with a piece of filter paper (Whatman no. 1) which was previously soaked with the xylose isomerase detecting solution. The detecting solution was made by modifying Scopes' preparation [20]. The solution was buffered at pH 7.5 with tris (50 mM) which contained 5 mM $MgSO_4$, 5 mM $MnCl_2$, 3 mg/ml nitro-blue tetrazolium, 0.5 mg/ml phenazine methosulphate, 10 mM NAD^+, 10 mM D-xylulose, and 10 IU/ml glucose dehydrogenase (EC 1.1.1.47) (Sigma Chemical).

With this procedure, xylose isomerase converted the xylulose to xylose which was then oxidized to xylonic acid and coupled the NADH formation. The NADH could reduce nitro-blue tetrazolium and appeared purple color at the sites of xylose isomerase on the gel.

D-xylose fermentation

Fermentations were performed in batch. Cells were previously grown in the 0.3% YMP (yeast extract-malt extract-bacto peptone) containing 2% D-xylose. At mid-logarithmic phase, one milliliter of cells was inoculated into 100 ml medium which contained 5% D-xylose and various nitrogen sources (YMP, corn steep, and NH_4Cl). The fermentation samples were taken at various intervals and their ethanol and D-xylose concentrations were analyzed. Ethanol was detected by gas chromatography (Varian 1700, 60/80 Tenax column, oven temp., 150°C), and D-xylose concentrations were analyzed by HPLC (Waters Asso.) with and IBM carbohydrate column (80% acetonitrile/water solvent system, 1.2 ml/min flow rate) at room temperature.

RESULTS AND DISCUSSION

Southern and dot blot

Schizosaccharomyces pombe is a fission yeast which can maintain and independently propagate the hybrid plasmid pDB248 [1]. The introduction of a 2.4 Kb *E. coli* fragment containing the xylose isomerase gene by this plasmid into *S. pombe* results in a low level of isomerase activity which was not present prior to transformation. The presence of this gene also results in *S. pombe* being able to utilize D-xylose as its sole carbon and energy source [4], thereby enabling *S. pombe* to ferment D-xylose to ethanol.

The presence of the xylose isomerase gene in *S. pombe* has now been confirmed by hybridizing a probe containing the *E. coli* isomerase gene to the chromosomal DNA of both *S. pombe* and pDB248-XI transformed *S. pombe* (Figs. 2 and 3). In these blots all of the samples except the nontransformed yeast show hybridization upon autoradiography. In

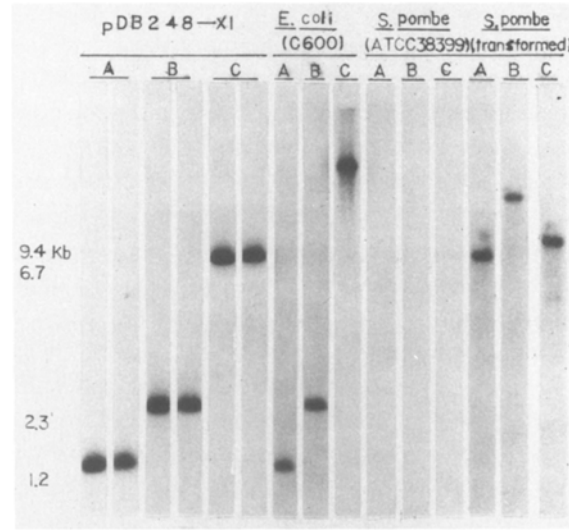

Fig. 2. Southern blot of genomic DNA from *E. coli*, *S. pombe*, and the transformed *S. pombe*. Photography shows the hybridization pattern of the 1.6 Kb fragment probe from pDB248-XI. Lanes labeled A were restricted with *Bgl*-II, lanes labeldd B were restricted with *Pst*-I and lanes labelled C were restricted with *Eco*RI. The pDB248-XI standard was loaded at concentrations of 1.8 ng and 0.9 ng (2 and 1 copies fragment). *E. coli* DNA was loaded at a concentration of 300 ng (1 copy of fragment), and *S. pombe* was loaded at a concentration of 1000 ng (1copy of fragment).

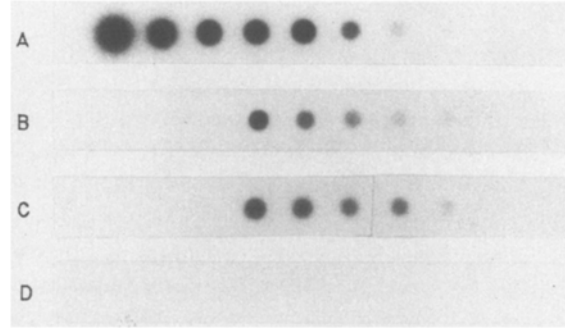

Fig. 3. Dot blot showing the hybridization pattern of the 1.6 kb fragment probe from pDB248-XI. Lane A, pDB24-XI standard with concentrations of 900, 720, 540, 360, 180, 90, 45, and 22.5 pg. Lane B, transformed *S. pombe* with concentrations of 1000, 800, 600, 400, and 200 ng. Lane C, *E. coli* with concentrations of 300, 240, 180, 120, and 60 ng. Lane D, nontransformed *S. pombe* with the same concentrations as transformed one.

addition, the southern blot (Fig. 2) shows that the size of the DNA fragment in the transformed *S. pombe* is larger than the fragments in either *E. coli* C600 or pDB248-XI. This indicates that the xylose isomerase gene is not extrachromosomal any longer but that it has now been integrated into the chromosome of *S. pombe*.

Further investigation is needed to verify the actual integration site of the xylose isomerase gene. However, the likely target site of integration is the area surrounding the leu-1 gene of *S. pombe*. This assumption was based on two observations; first, Beach at al.'s [1] report that using the pDB248 containing a leu-2 gene could repair a leu-1$^-$ strain of *S. pombe*; second, the growth of transformed *S. pombe* no longer requires leucine which is required for the nontransformed *S. pombe* growth.

Expression of the xylose isomerase gene

Fig. 4 shows the results of nondenaturing polyacrylamide gel electrophoresis of the crude extract samples from *E. coli*, transformed *S. pombe*, and nontransformed *S. pombe*. By using the xylose isomerase detecting solution two major bands in each sample except the nontransformed yeast extract sample were observed, which showed the xylose isomerase activities existing in these microorganisms. This indicates that the activity of xylose isomerase of the transformed *S. pombe* is caused by the expression of xylose isomerase gene which is of *E. coli* origing. The reason for the xylose isomerase possessing a two-band pattern on the nondenaturing polyacrylamide gel is unclear. The most likely explanation is that the subunits of xylose isomerase also have activities, during the electrophoresis the subunit and nondenatured xylose isomerase were separated, thus, two bands were observed. Another possibility is that xylose isomerase may have two different structural conformations possessing the same activity and each conformation shows different migration [20], so two bands can be localized on the gel after electrophoresis. It is interesting to note that the xylose isomerase of *E. coli* has a different gel migration pattern from that of the transformed *S. pombe*. This might be caused by the modification of enzyme synthesis in the yeast cells.

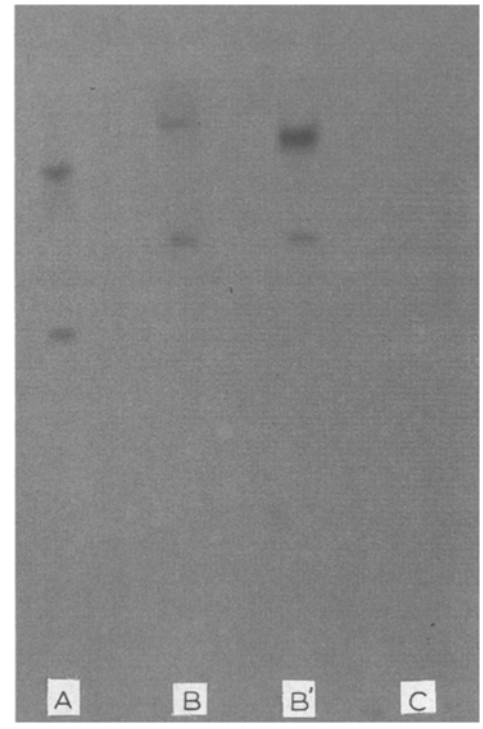

Fig. 4. Nondenaturing polyacrylamide gel electrophoresis of xylose isomerase. Lane labeled A, 100 μg *E. coli* crude extract, Lanes labeled B, 100 μg and 500 μg transformed *S. pombe* crude extract, and Lane labeled C, 500 μg nontransformed *S. pombe* crude extract. After electrophoresis, the gel was stained by a piece of filter paper which was previously soaked in the staining solution. The principle of stain is coupling the xylose isomerase, glucose dehydrogenase and NAD for D-xylulose conversion to xylonic acid with NADH. Thus, NADH can reduce nitro-blue tetrazolium and produce purple bands at the sites of xylose isomerase.

Further investigation on the mechanisms of post-transcription and post-translation of the xylose isomerase gene in the yeast is needed to verify this as the yeast regulation system.

Sarthy et al. [18] transformed a xylose isomerase plasmid to *Saccharomyces cerevisiae* and found that the gene was expressed, but the activity was a 1000-fold less than that in *Escherichia coli*. Ueng et al. have done the transformation of the *E. coli* xylose isomerase gene to both *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. The result showed that the D-xylose metabolic pathway of *Schizosaccharomyces pombe* could be strongly induced by the

Table 1

Induction of xylose isomerase in the transformed *S. pombe*[a]

Time(h)	Cell mass (g wet weight)	Xylose (%)	protein (mg/ml)	Specific activity of xylose isomerase (nmol/min/mg)
0	–[b]	5	–	–
24	–	5	–	–
48	0.55	4.9	16	0.0
60	0.65	4.9	25	0.0
72	0.78	4.2	34	4.0
84	0.90	3.7	40	3.8
96	1.0	3.3	45	4.3
108	1.3	2.4	43	4.0
144	1.7	1.0	50	3.5

[a] The yeast cells were grown in YMP containing 2% D-xylose. At the mid-log phase 10 ml of the culture were inoculated into a 1 liter YMP medium containing 5% D-xylose and this was shaken at 200 rpm at 30°C. At various intervals, 100 ml cell culture were withdrawn and analyzed for xylose isomerase.

[b] Data were not available because the cell mass was not enough for measuring.

transformation event and the expression of xylose isomerase gene was much greater in *Schizosaccharomyces pombe* (unpublished data). It seems that the transformation of the prokaryotic gene to eukaryotic cells requires a proper plasmid shuttle and a proper recipient.

The xylose isomerase of the transformed *S. pombe* was induced by D-xylose as low as 0.66 mM (0.01%). The activity induction of the transformed yeast xylose isomerase by D-xylose is shown in Table. 1. This low isomerase activity in the transformed *S. pombe* could result from low transcription initiation from the promoter region. It has been shown that the natural promoter for the *E. coli* xylose isomerase gene cannot express the gene when cloned into *Saccharomyces cerevisiae*. A very low level of expression can be obtained when the *E. coli* promoter is replaced with the yeast Trp-5 promoter [13]. Therefore, continued studies are being conducted on possible promoters to increase the transcription of the gene.

The results show that 12 h after the enzyme activity was induced, the cell mass and D-xylose consumption were increased but the enzyme activity kept relatively constant during this time. This indicated that the time for reaching the steady state of xylose isomerase synthesis and degradation was shorter than 12 h. In order to increase the activity of xylose isomerase in the transformed yeast cells, studies on the decreasing xylose isomerase degradation rate or avoiding post-translation modification by the yeast are essential.

Nutrient efect on the xylose isomerase activity

When the transformed yeast cells were grown in different concentrations of YMP media containing a constant concentration of D-xylose, the xylose isomerase activity increased with increasing YMP concentration (Table 2). One explanation might be that the nutrient component in the medium could increase the rate of xylose isomerase synthesis. The data shown in Table 2 are insufficient to support the conclusion that the nutrient concentration factor is important for expression of a foreign gene in the host yeast. However, the nutrient effect on increasing the expression of a foreign gene seems to be an interesting subject to study.

The in vitro enzyme assay also provided evidence

Table 2

The activities of D-xylose metabolism involving enzymes of the transformed *S. pombe* in de different YMP concentration media.

YMP[a] conc.	Extracted protein conc. (mg/ml)	Specific activity (nmol/min/mg/protein)			
		Xylose isomerase	Xylulose kinase	Xylose reductase	Xylitol dehydrogenase
0×	4.0	0.5	42	403	560
1×	9.6	4.4	40	410	550
3×	11.5	10.4	43	412	654
5×	14.2	16.4	45	415	655
10×	17.0	20.0	44	413	665

[a] 1× YMP concentration consisted of 63% yeast extract, 0.3% malt extract, and 0.5% bacto peptone. The yeast cells were grown in 1 liter YMP medium containing 2% D-xylose. At midlog phase, the cells were harvested and sonified. The crude extracts from the cells were used for enzyme assays.

that xylose reductase and xylitol dehydrogenase exist in the *S. pombe* cells. However, the in vivo cofactor regulation, such as low levels of NADPH and NAD^+, might dramatically decrease these enzymes abilities to metabolize D-xylose. This result is similar to the conclusion of Batt et al. [2] who investigated the factors for inefficient D-xylose metabolism in *Saccharomyces spp.*

Nutrient effect on D-xylose fermentation

Figs. 5 and 6 show that different YMP concentrations would affect the patterns of substrate utilization and ethanol production of the transformed *S. pombe* in D-xylose fermentation. The fermentation parameters affected by the YMP concentrations are shown in Table 3. Because the YMP medium contains maltose which can be fermented to ethanol by yeast, ethanol production from D-xylose fermentation was calibrated by subtracting the amount of ethanol fermented from YMP. When yeast cells fermented D-xylose in the 10-fold YMP medium, the ethanol productivity and yield reached 0.13 g/l/h and 0.42 g/g, respectively. By comparing Table 2 and Table 3, the xylose isomerase seems to be a

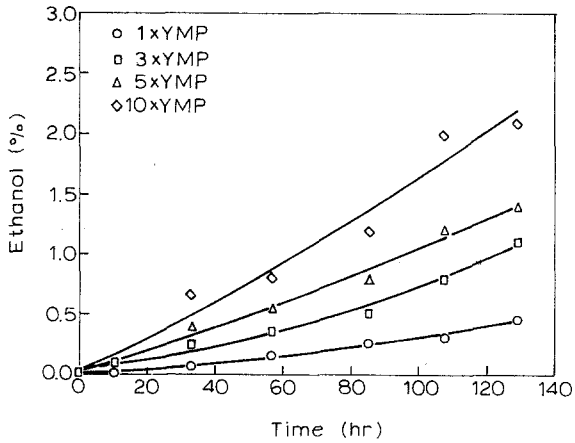

Fig. 5. Effect of Different YMP Concentrations on Ethanol Production from D-xylose Fermentation. The fermentation was operated at 30°C and constant shaking at 200 rpm. The cell density was $2x10^8$ cells/ml.

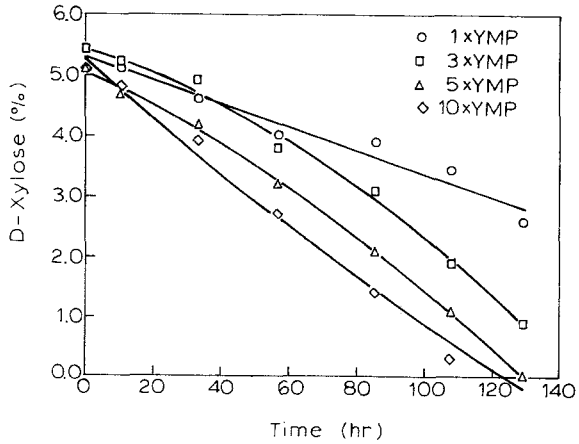

Fig. 6. Effect of different YMP concentration on D-xylose utilization by the transformed *S. pombe*. The fermentation condition was the same as in Fig. 5.

Table 3

Effect of Different YMP Concentrations on D-xylose Fermentation

YMP conc.	Initial ethanol productivity (g/l/h)	Ethanol[a] yield (g/g)	Initial rate of xylose utilization (g/l/h)	Final cell density[b] ($\times 10^8$ cells/ml)
0×	0.02	0.15	0.05	0.3
1×	0.05	0.3	0.17	0.5
3×	0.06	0.35	0.18	0.6
5×	0.08	0.4	0.20	0.8
10×	0.13	0.42	0.24	1.0

[a] The ethanol production from D-xylose fermentation was calibrated by subtracting the ethanol production from the medium containing YMP only (controlled).

[b] In each batch study, one ml of yeast culture, which was previously grown in YMP medium containing 1% D-xylose, was inoculated to 100 ml medium containing various amount of YMP and 5% D-xylose.

primary factor which influences the ethanol yield and productivity from D-xylose fermentation by this transformed yeast. This result verified the previous work of Chan et al. [4], which indicated that the low activity of xylose isomerase was the limiting step for the ethanol production from the D-xylose fermentation by the transformed *S. pombe*.

Although adding a large number of nutrients to the fermentation broth can increase the ability of the transformed *S. pombe* to ferment D-xylose, using this process in industrial ethanol production is not practical and economical. Therefore, finding an alternative to supply the nutrient in the fermentation broth is essential. Fig. 7 shows the effect of nitrogen from different sources on the D-xylose fermentation. The results indicate that using a cheap nitrogen source such as NH_4Cl could give a comparable ethanol yield and productivity as other nitrogen sources.

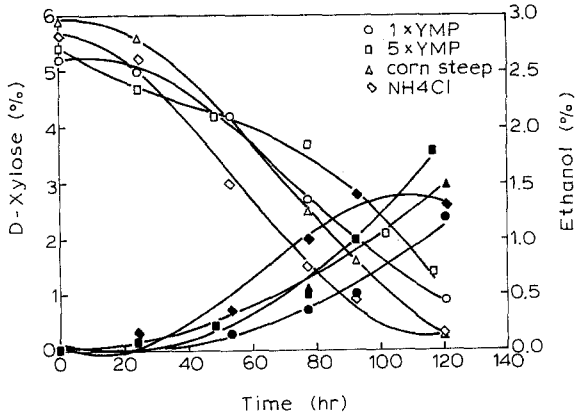

Fig. 7. Effect of nitrogen from different sources on D-xylose fermentation by the transformed *S. pombe*. The fermentation condition was the same as in Fig. 5.

CONCLUSION

Through subsequent studies it has now been demonstrated that the *E. coli* xylose isomerase gene has been integrated into the chromosome of *S. pombe*. The results of the DNA dot bolt and the detection of low isomerase activity in the transformed strain confirmed the presence of the gene. The southern blot indicated that integration into the chromosomal DNA had occurred. The nutrient effect on the expression of the xylose isomerase gene has been demonstrated. The more nutrients that were supplied to the transformed *S. pombe*, the higher the activity of xylose isomerase could be obtained. This higher activity of xylose isomerase in the yeast cells resulted in an efficient D-xylose fermentation. The availability of a nitrogen source in the nutrient content for the transformed yeast seems to be a key factor for the expression of the

xylose isomerase gene, and simple ammonium salts can be used for nitrogen source.

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