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Environmental Effects on D-Xylose Fermentation by *Schizosaccharomyces pombe*

Scientific Note

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ABSTRACT

Many efforts have been made to convert D-xylose to ethanol. Studies include a search in nature for xylose-fermenting yeasts; mutagenesis of xylose-utilized yeasts such as *Pachysolen trannophilus* (*1*), *Candida sp.* (*2*), and gene transformation of well-characterized strongly fermenting yeasts to introduce a xylose utilization pathway (*3,4*).

Most yeast that are capable of D-xylose catabolism, use two enzymes for D-xylose conversion to D-xylulose (*5*). Xylose reductase couples NADPH to reduce D-xylose to xylitol, which is then oxidized to D-xylulose by xylitol dehydrogenase and NAD^+. Once D-xylulose is formed, this intermediate flows through Pentoshunt and cooperates with glycolytic pathway to produce ethanol. However, the regulation of cofactor such as NADPH or NAD^+ in the yeast cells often causes an inefficient D-xylose metabolism (*6*). This limitation can be overcome by introducing a xylose isomerase gene into the yeast cells for direct, cofactor-free conversion of D-xylose to D-xylulose, then it results in a high ethanol production from D-xylose fermentation.

Previously, we reported the production of ethanol from D-xylose by the fission yeast *Schizosaccharomyces pombe* transformed with the xylose isomerase plasmid (*3,7*). The genetically modified *S. pombe* has the same physiological behaviors and morphology as that of the non-genetically modified one (unpublished data). Furthermore, the subsequent integration of the plasmid into the chromosomal DNA of the host *S. pombe* has been verified by using the dot blot and southern

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blot techniques (*8*). The expressed xylose isomerase has shown activity by a technique of nondenaturing polyacrylamide gel electrophoresis (*8*). In this study, the effects of environmental conditions on direct D-xylose fermentation by this yeast were described.

Index Entries: D-xylose; ethanol; yeast; fermentation.

MATERIALS AND METHODS

Organism and Chemicals

The fission yeast *Schizosaccharomyces pombe* (ATCC 38399) cloned with a xylose isomerase gene was isolated by Ueng et al. (*3*). Yeast culture was maintained in 0.67% yeast nitrogen base (YNB) containing 2% D-xylose. Cultures were incubated at 30°C with constant shaking in an environmental shaker (New Brunswick Scientific Co.). In fermentation experiments, yeast cells were inoculated in 0.67% yeast nitrogen based medium containing appropriate sugars. YMP and YNB were purchased from Difco (Detroit, MI). D-glucose and D-xylose were obtained from Sigma Chemical Company (St. Louis, MO). D-xylulose was prepared by using immobilized glucose isomerase (Sweetzyme; type Q, 220 IU/g; Novo Industries, Inc.). The detailed D-xylulose preparation was described by Chiang et al. (*9*).

Fermentation

Fermentations were performed in batch. Cells were previously grown in 1 L YMP medium that contained 0.3% yeast extract, 0.3% malt extract, 0.5% peptone, and 2% D-glucose. After cells were harvested during the mid-logarithmic phase and washed twice with phosphate buffer (0.05 *M*, pH 7), cells were inoculated into 50 mL Erlenmeyer flasks containing 20 mL yeast nitrogen base (YNB) broth and suitable sugars. Fermentation was operated with constant shaking at 200 rpm and 30°C.

The effects of environmental conditions such as pH, yeast cell density, substrate concentration, and temperature on the ethanol production was then studied. D-xylose at a concentration of 100 g/L was used for studying the effects of pH, temperature, and yeast cell density. For studying the effect of pH, YNB (0.67%), phosphate buffer (0.05 *M*) media were used and adjusted to pH4, 5, 6, and 7, respectively. The different aerated conditions for the D-xylose fermentation were performed by filling 10, 20, and 40 mL YMP medium containing 8% D-xylose in 125 mL flasks. The initial cell density was adjusted to 2×10^8 cells/mL in each flask, and cultures were incubated at 30°C with 200 rpm shaking.

Analysis

During fermentation experiments, samples were withdrawn and cell density was measured by hemocytometer. After samples were centri-

fuged, the supernatants were collected, frozen, and later analyzed. Ethanol concentration was measured by gas chromatography (Varian 1700, 60/80 Tenax), and sugar concentrations were analyzed by high performance liquid chromatography (Waters Assoc.) with IBM carbohydrate column and 80% acetonitrile/water solvent system. Dissolved air in the fermentation broth was monitored by YSI 5331 oxygen probe (Yellow Springs Instrument).

RESULTS AND DISCUSSION

Preliminary Investigation on D-Xylose Fermentation

The nontransformed *S. pombe* (ATCC 38399) was used as a control in the experiments of D-xylose fermentation under various environmental conditions to compare the ability of D-xylose utilization with the transformed *S. pombe*. The results showed that the previously D-glucose grown nontransformed yeast cells could not utilize D-xylose in the medium containing yeast nitrogen base (YNB) or in a rich medium containing yeast extract, malt extract, and bacto peptone (YMP). On the other hand, by using the transformed *S. pombe* culture D-xylose in the YNB or YMP medium was consumed and ethanol was produced. Due to this reason the transformed *S. pombe* culture was used in the D-xylose fermentation.

Results also showed that both the transformed and nontransformed yeasts could grow in the medium containing YMP without any sugar and trace amount of ethanol was produced. This is because the YMP contains trace amount of fermentable sugar such as maltose. Therefore, in the following fermentation experiments the ethanol production was calibrated by subtracting the amount of ethanol that was fermented from YMP.

Effect of Cell Density on Ethanol and Xylitol Production

The maximal ethanol productivity increased from 0.063 to 0.177 g/L/h with an increase in cell density from 1×10^8 to 3×10^8 cells/mL in the YNB medium containing 100 g/L D-xylose (Table 1). In this cell density range, the specific ethanol productivity was approximately 0.06 g/L/h/10^{11} cells and ethanol yield was approximately 0.22 g/g, regardless of the difference in cell densities in the fermentation medium. Figure 1 shows that the ethanol production rate increased with the increase of cell densities from 1 to 3×10^8 cells/mL. However, when the cell density increased to 5×10^8 cells/mL, the ethanol production decreased. Furthermore, when the cell density exceeded 10^9 cells/mL, the ethanol productivity dropped to 0.004 g/L/h and ethanol yield decreased to 0.04 g/g.

The transformed yeast needs nutrients, especially nitrogen, to amplify the expression of the xylose isomerase gene (*8*). Also, yeast cells need an

Table 1
Ethanol Production by the Transformed *S. pombe* Under Various Fermentation Conditions

Fermentation conditions				Maximal ethanol productivity, g/L/h	Yield, (g/g xylose)	
Xylose, g/L	Temp, °C	pH	Cell density, x10^8 cells/mL		ethanol	xylitol
100	30	4	2	0.14	0.2	0.1
100	30	5	2	0.13	0.19	0.15
100	30	6	2	0.08	0.15	0.01
100	30	7	2	0.01	0.01	0.04
20	30	NC[a]	2	0.063	0.23	0.0
50	30	NC	2	0.076	0.22	0.36
150	30	NC	2	0.16	0.23	0.28
200	30	NC	2	0.073	0.2	0.2
100	40	NC	2	0.03	0.3	0.4
100	20	NC	2	0.04	0.31	0.46
100	30	NC	1	0.063	0.25	0.34
100	30	NC	2	0.135	0.21	0.23
100	30	NC	3	0.177	0.22	0.17
100	30	NC	5	0.156	0.21	0.15

[a]NC = *not* controlled.

energy source in order to facilitate D-xylose transport across a membrane. A minimal medium contains a limited nutrient and nitrogen source, which can not sustain the large number of yeast cells for D-xylose metabolism. Therefore, in D-xylose fermentation, the decrease of the ethanol productivity by high cell density in minimal medium is expected. On the other hand, the low ethanol yield might be caused by the yeast cells reconsuming ethanol as a carbon or energy source for D-xylose metabolism in a limited nutrient medium (*8*).

The formation of the byproduct, xylitol, in D-xylose fermentation is dependent on the availability of oxygen in the medium (*6*). Table 1 shows that a higher xylitol yield of 0.34 g/g was reached when 1×10^8 cells/mL was inoculated, compared to the higher inoculated cell density. This indicates that the fewer the cells in the medium, the less oxygen utilized, and the medium has a higher dissolved oxygen level. Then the xylitol formation was enhanced by the oxygen left in the medium. Therefore, the optimal cell density in the D-xylose fermentation process should be determined with the consideration of other fermentation parameters such as nutrient and oxygen concentration in the fermentation broth and the formation of xylitol.

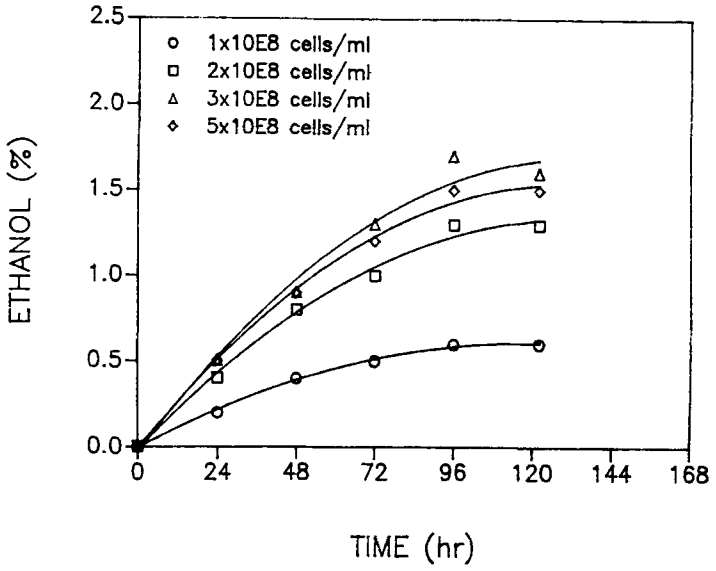

Fig. 1. Effect of yeast concentration on the production of ethanol from D-xylose by transformed *S. pombe*. The fermentations were operated at 30°C and constant shaking at 200 rpm. The media contained 100 g/L D-xylose, 0.67% YNB, and pH was not controlled. The cell density was 2×10^8 cells/mL.

Effect of Aeration on D-Xylose Fermentation

Different aeration conditions affect the D-xylose utilization and ethanol production of the transformed yeast. In this study, aeration conditions of the fermentation medium were defined in term of different media volumes in a constant volume vessel that was shaked at a constant rate of 200 rpm.

It is interesting to note that oxygen availability for the D-xylose fermentation has a similar pattern as the nutrient effect: the more oxygen available for yeast in D-xylose fermentation, the faster D-xylose is consumed. Figure 2A shows that under a highly aerobic condition (8% vessel vol), D-xylose was rapidly utilized. However, in this condition, the ethanol yield only reached 0.14 g/g. This is owing to ethanol being consumed by the yeast when air was still present in the medium. When the degree of aeration was reduced by increasing the volume of the medium (16% vessel vol), the rate of D-xylose utilization decreased (Fig. 2B). However, the ethanol yield (0.25 g/g) was higher compared to that under a highly aerobic condition, and ethanol was not consumed later because no oxygen was available at this level of reduced aeration for ethanol assimilation. When aeration was further decreased, the D-xylose utilization was also decreased, but the ethanol yield was maintained the same as that in the

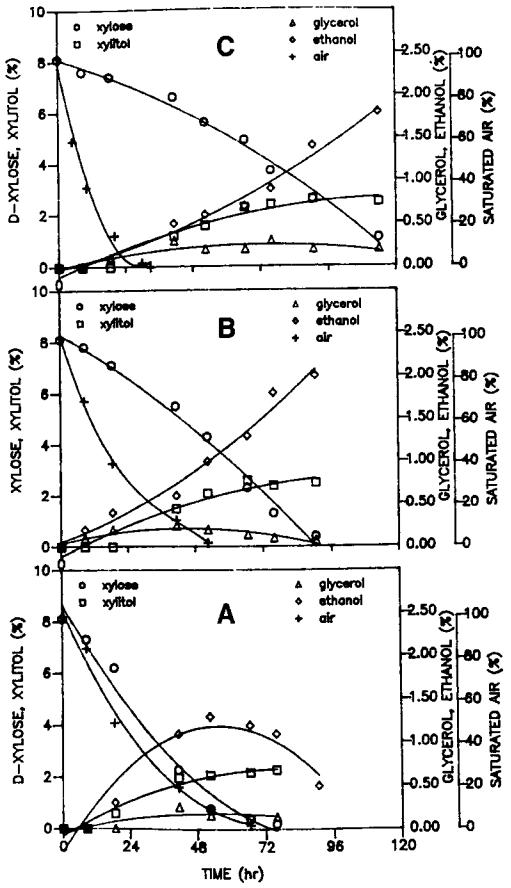

Fig. 2. Effect of aeration on ethanol fermentation from D-xylose. A: 32% vessel vol.; B: 16% vessel vol.; C: 8% vessel vol. The cell density was controlled to 2×10^8 and culture was incubated in the YMP medium containing 8% D-xylose at 30°C with 200 rpm shaking.

16% vessel vol aeration condition (Fig. 2A). When fermentations were performed under anaerobic conditions, latter D-xylose was almost not utilized by yeast, and no ethanol was produced.

The enhancement of D-xylose utilization by the availability of oxygen implies that D-xylose transport system in the transformed yeast is energy dependent. The more oxygen is supplied, the faster D-xylose is transport into the cells. However, providing oxygen to the medium causes yeast cells to consume the ethanol and thus decrease the ethanol yield. Therefore, the oxygen level in the fermentation medium seems to have a contrary effect on D-xylose utilization and ethanol yield. In the previous study, the D-xylose transport rate was found not to be a primary limiting step for ethanol production, but the low xylose isomerase activity expressed in the transformed yeast was the limiting step (Chan et al., paper submitted). However, even if the problem of amplifying the expression of xylose iso-

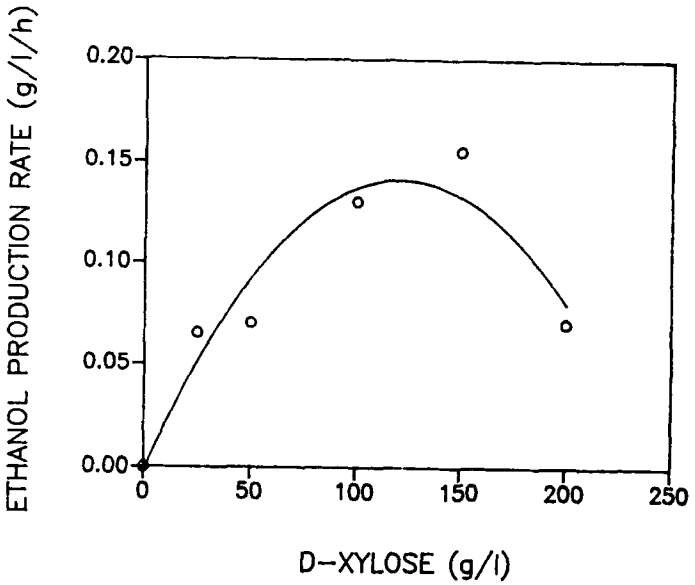

Fig. 3. Effect of D-xylose concentration on ethanol production rate. The fermentations were operated at 30°C and constant shaking at 200 rpm. The media contained 0.67% YNB and various concentrations of D-xylose and pH was not controlled. The cell density was 2×10^8 cells/mL.

merase gene in the transformed yeast is solved, the oxygen level in the fermentation medium must be kept as minimal as possible for yeast cells to utilize D-xylose but not ethanol.

Effect of D-Xylose Concentration

In order to determine the optimal D-xylose concentration for ethanol production, different D-xylose concentrations were used in fermentation and ethanol productivities and yields were measured. Results showed that the ethanol productivity increased with increasing concentration of D-xylose (Fig. 3). The optimal ethanol production rate of D-xylose fermentation was in term of Vmax and Km, which were 0.17 g/L/h and 40 g/L, respectively. However, when the D-xylose concentration was at 20% (w/v), the rate of ethanol production was reduced. An unexpected result showed that the final ethanol yields from different concentrations of D-xylose had similar values (about 0.22 g/g xylose). One of the reasons for this phenomenon was a result of consumption of ethanol by transformed yeast during the fermentation process in a minimal nutrient medium. Ethanol was thought to be used as an advantageous energy source for D-xylose uptake when a trace amount of oxygen was still present in the medium (*10*). However, the time at which ethanol consumption started and the rate of ethanol utilization by yeast cells during the D-xylose fermentation still remained to be investigated. Data in this study showed that after most of the D-xylose was exhausted, the ethanol was consumed by the

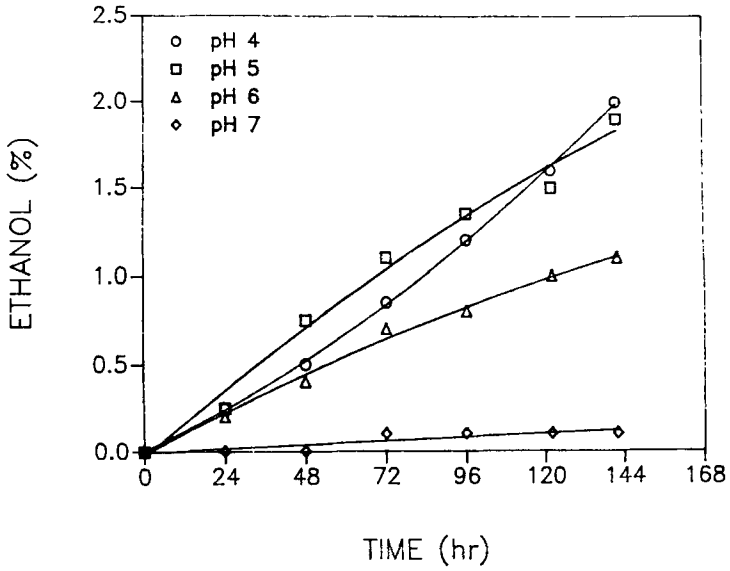

Fig. 4. Effect of pH on the production of ethanol from D-xylose by transformed *S. pombe*. The temperature was 30°C, cell density was 2×10^8 cells/mL. pH was controlled by phosphate buffer (0.05 *M*).

yeast with a rate of 0.21 g/L/h. Thus, the ethanol yield can be increased, if a mutant that does not utilize ethanol can be isolated.

An increased rate of ethanol production with increasing sugar concentration was indirect evidence of the "Crabtree Effect" that might exist in the D-xylose fermentation of the transformed yeast. Although we did not demonstrate the cell respiration rate, the increased rate of ethanol production implied the repression of respiratory activity in the cells. Barford (*11*) has reported that *S. pombe* does not have respiratory repression under glucose-limited chemostat conditions. In order to determine whether the transformed yeast has respiratory repression by high D-xylose concentration or just a transient response, respiratory enzymes and other related factors should be investigated in the xylose-limited chemostat condition.

Effect of pH and Temperature on D-Xylose Fermentation

Figure 4 shows the effect of pH on D-xylose fermentation. When the medium contained 100 g/L D-xylose and fermentation was operated at 30°C and constant shaking at 200 rpm, the rate of ethanol production and D-xylose consumption at pH 4 and 5 had a similar pattern. At pH 7, the fermentation rate and the final ethanol yield were much lower than the value of pH 4 (Table 1). On the other hand, at low pH, the xylitol production was enhanced. Xylitol yield reached 0.16 g/g xylose at pH 4. The rate

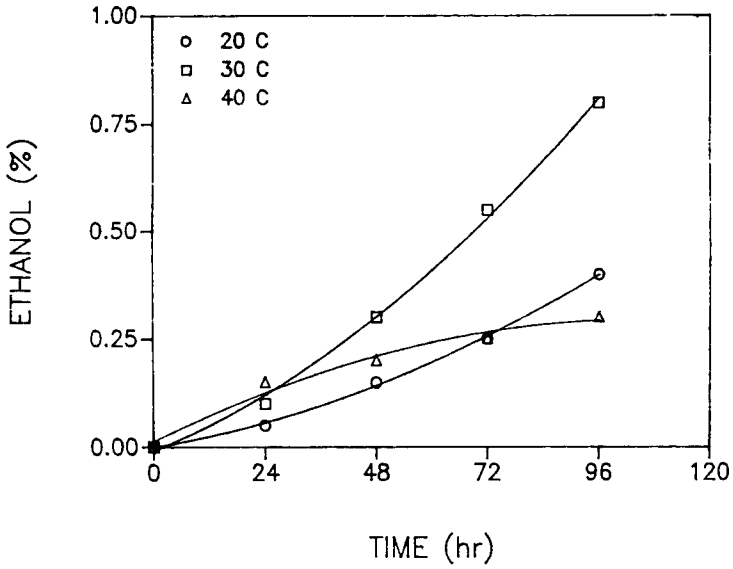

Fig. 5. Effect of temperature on the production of ethanol from D-xylose by transformed *S. pombe*. The cell density was 2×10^8 cells/mL. The media contained 100 g/L D-xylose on 0.67% YNB and pH was not controlled.

of D-xylose consumption by transformed yeast was 0.7, 0.68, 0.5, and 0.32 g/L/h at pH 4, 5, 6, and 7, respectively.

The experiment of pH effect indicated that the optimal pH range for ethanol production from D-xylose fermentation was between 4 and 5. This result could be taken as an advantage for simplifying other fermentation experiments by not controlling the pH value of the media. Because once D-xylose was fermented by the transformed *S. pombe* that could produce organic acids to maintain the pH values of media between 4 and 5.

Nassar et al. (*12*) reported that the uptake of sugars by *S. pombe* was classified into glucose transport and nonglucose transport systems; D-xylose uptaken by *S. pombe* is a nonglucose transport system that is energy independent and not accompanied by a stoichiometric cotransport of H^+. From the viewpoints of oxygen requirement and pH dependence of D-xylose utilization by *S. pombe*, it seems that D-xylose uptake is an active transport but not a facilitated-diffusion system independent of metabolic energy.

At temperatures of 20 and 40°C, the rates of ethanol production were slower than at 30°C (Fig. 5). Although the ethanol yields from D-xylose at 40°C were higher than at 30°C. The xylitol production also increased when the fermentation condition was not at 30°C (Table 1). The optimal temperature for fermentation is usually higher than the temperature of optimal growth (*13*). However, it is more difficult to maintain the viability of a yeast at higher temperatures (*14*).

Table 2
Substrate Specificity for *S. pombe* Fermentation

Substrate	Ethanol yield, g/g sugar	Ethanol productivity, g/L/h
D-glucose	0.37	0.58
D-xylulose	0.31	0.3
D-xylose	0.21	0.13

Carbon Source and Nitrogen Source Effect

The transformed yeast could ferment different sugars to ethanol with different ethanol yield in the medium containing a limited nitrogen source (yeast nitrogen based medium). As Table 2 shows, the rate of ethanol production was 0.58, 0.3, and 0.13 g/L/h for D-glucose, D-xylulose, and D-xylose, respectively. The ethanol yields from D-glucose, D-xylulose, and D-xylose fermentation were 0.37, 0.31, and 0.21 g/g, respectively. In previous work (*7*), it was shown that the ethanol yield could reach 0.37 g/g from D-xylose fermentation and 0.43 g/g from D-glucose fermentation by the transformed yeast in a rich medium (YMP) containing sufficient nitrogen source. The importance of the nitrogen source for ethanol production by yeast fermentation has been discussed in many studies (*15–18*); the more nitrogen that was available for yeast in fermentation, the more ethanol could be produced.

This phenomenon can be explained the sufficient nitrogen but not ethanol was supplied in the medium as the energy source for the D-xylose transport into the yeast cell. Therefore, the less ethanol utilized, the greater the ethanol yield that would be obtained. The transformed yeast at a density of 10^8 cells/mL inoculated in 3% yeast extract-malt extract-peptone medium containing 5% D-xylose could obtain 0.42 g/g ethanol yield. Although the nitrogen amount can affect the ethanol yield from D-xylose fermentation, the maximal ethanol productivity is not influenced by it. In spite of the amount of nitrogen in the fermentation medium, the maximal ethanol productivity maintains approximately constant at 0.19 g/L/h. This is because the xylose isomerase in the yeast cells has low activity for converting D-xylose to D-xylulose, which makes it become the limiting step for ethanol production (*7*). Thus, no matter how much nitrogen facilitates D-xylose transport and accumulation in the cells, the rate of ethanol production is maintained at the same level.

The utilization rates of D-glucose, D-xylulose, and D-xylose were 1.58, 0.97, and 0.68 g/L/h, respectively. One can suspect that the different rates between D-glucose and D-xylose utilization are caused by the different

transport system in the transformed yeast. However, the different utilization of D-xylose and D-xylulose by the transformed yeast was not caused by the different transport systems, since the D-xylose and D-xylulose had the same uptake rate by this yeast in the previous study. Thus, the low activity of xylose isomerase in the transformed yeast was the reason for the low D-xylose utilization (Chan et al., paper submitted).

CONCLUSIONS

From the viewpoint of conversion of D-xylose in biomass to ethanol, genetic engineering techniques are of interest in introducing appropriate genes from other organisms into yeasts to ferment D-xylose. After we transformed the xylose isomerase gene from *E. coli* to *S. pombe*, the ethanol production from D-xylose fermentation reached a reasonable level. The results reported here show the physiological effects on D-xylose conversion to ethanol.

The ethanol productivity was increased from 0.063 to 0.177 g/L/h by increasing the yeast cell density in the fermentation broth containing 100 g/L D-xylose from 1×10^8 to 3×10^8 cells/mL. Aerobic conditions were required for D-xylose utilization by the yeast cells. However, a highly aerated condition caused a low ethanol yield. The optimal temperature for D-xylose fermentation was approximately 30°C, and the optimal pH range was 4–5. The formation of the byproduct, xylitol, was enhanced in a low pH, low temperature, or low cell density condition. Different D-xylose concentrations below 200 g/L in the fermentation medium affected the ethanol productivity but not the ethanol yield. The transformed yeast could ferment D-glucose, D-xylulose, and D-xylose to ethanol in a limited nitrogen source medium; the ethanol yield was 0.37, 0.31, and 0.21 g/g, respectively. In D-xylose fermentation, a maximal ethanol yield could reach 0.42 g/g in an enriched nitrogen medium.

After the environmental effects on the transformed yeast are understood, the next strategy should study the genetic level of transformed yeast to improve the efficiency of ethanol production from D-xylose fermentation. One approach is to use mutagensis to eliminate or evade the ethanol utilization pathway in the transformed yeast. Another general approach is to introduce gene promoters into yeast cells to increase the xylose isomerase activity and more efficiently use D-xylose to produce ethanol.

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