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Intensification of Bio-ethanol Fermentation by Recombinant Yeast with Xylose Isomerase Pathway

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Glucose and xylose are major component of the lignocellulosic biomass, and the efficient utilization of not only glucose but also xylose has been required for large-scale ethanol production. In order to establish a highly efficient bio-ethanol production process, this study tried to enhance xylose asimilation ability by introducing the metabolic pathway. Xylose isomerase (XI) pathway (called Pathway I in this study) was selected as a xylose metabolic pathway, and introduced into *Saccharomyces cerevisiae* genome by integration of XI expression cassette. The resulting recombinant yeast (MT8-1/XKδXI) attained successfully production of ethanol from not only xylose as the sole carbon source but also mixed sugar of xylose and glucose. The xylose consumption rate of the fermentation from xylose was estimated to be a second-order reaction model, and from mixed sugars was estimated to be a first-order reaction model, respectively. These results were expected to be a glucose effect on fermentation from xylose.

1. Introduction

Recently, the exhaustion of fossil fuels has been a global problem, and bio-ethanol has attracted international attention as one of the alternative fuels. The bio-ethanol is a renewable fuel produced from the biomass resources, and now mainly produced from edible biomass. Since a large amount of bio-ethanol production from edible biomass causes the food problem, the establishment of the efficient bio-ethanol production techniques from lignocellulosic biomass has been required. Glucose and xylose are major component of the lignocellulosic biomass, and the efficient utilization of not only glucose but also xylose is advantage for large-scale ethanol production. Since the yeast, *Saccharomyces cerevisiae,* which was usually used widely for ethanol fermentation, could not metabolize xylose, an introduction of the xylose metabolic pathway into the yeast had been widely studied. According to several research groups, two different kinds of xylose metabolic pathway have been proposed. The xylose reductase (XR)-xylitol-xylose dehydrogenase (XDH) pathway, called Pathway II in this study, had been widely used. This pathway requires two different kinds of coenzymes (NADPH, NAD+) for assimilating of the xylose. The difference of cofactor preference between XR and

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XDH frequently may be caused xylitol accumulation, limited the xylose metabolic flux, and disruption of metabolic balance in the long term fermentation (*Rizzi et al.*, 1989a, b, Toivari *et al.*, 2001). In addition, the glucose was up taken priority at initial stage of fermentation, the xylose consumption rate becomes slow in the ethanol production from mixed sugar by Pathway II.

On the other hand, the xylose isomerase (XI), called Pathway I in this study, does not need any coenzymes for metabolizing of the xylose. Recently, it became to be possible to introduce of Pathway I into yeast genome by introducing of XI encoding gene using δ integration method (Yamada *et al.*, 2009). This work tried to intensify bio-ethanol fermentation by recombinant yeast introduced the Pathway I and to elucidate kinetic characteristics of the fermentation aiming at developing large-scale ethanol production. It is generally known that fermentation conditions such as temperature, pH and the ratio of glucose and xylose in the mixed sugar affect ethanol production rate. From the aspect of practical application, the ratio of glucose and xylose in the mixed sugar could be varied by the selection of lignocellulosic biomass resource and its degradation methods. Therefore, the analysis of reaction kinetics focusing on the ratio of glucose and xylose in the mixed sugar is very important. In order to elucidate the ethanol production characteristics from xylose and/or mixed sugar, this study analyzed xylose consumption rate during the fermentation using the yeast introduced the Pathway I .

2. Materials and Methods

2.1 Strains and media

Escherichia coli NovaBlue (Novagen Inc., Madison, WI.) was used a host strain for recombinant DNA manipulation. *S. cerevisiae* MT8-1 (*MAT*a *ade his3 leu2 trp1 ura3*) (Tajima et al.,1985) was used as a host strain for introduction of XI based xylose metabolic pathway. *E. coli* was cultivated in Luria-Bertani (LB) medium (1 % (w/v) tryptone, 0.5 % yeast extract and 0.5 % sodium chloride) containing 100 μg/ml ampicillin. The yeast strain was cultivated in SD medium (0.67 % (w/v) yeast nitrogen base supplemented with appropriate amino acids and nucleotides and 2 % glucose). For solid media, 2 % (w/v) agar was added to the media described above liquid medium.

2.2 Construction of expression plasmids

The pLXKS for overexpression of *S. cerevisiae* xylulokinase (XK) gene *XKS1* was constructed as follows: A DNA fragment composed from glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter, *XKS1* and GAPDH terminator from *S. cerevisiae* was prepared by polymerase chain reaction (PCR) with the following one pair of primers: SacII-pGAP (5'-ATTACCGCGGACCAGTTCTCACACGGAACACC-3') and tGAP-XhoI (5'-GCCCGCCTCGAGTCAATCAATGAATCGAAAATGTC-3') with plasmid pWX1X2XK (Katahira et al.,2004) as template. The amplified fragment was digested *Sac* II and *Xho* I and inserted into *Sac* II and *Xho* I site of pRS405 (ATCC87516).

The pδWOXYLA1 for δ integration of XI from *Orpinomyces* sp. *OXYLA1* (Madhavan et al.,2009) overexpression cassette into the yeast genome was constructed as follows: A DNA fragment composed of the GAPDH promoter, *OXYLA1* and GAPDH terminator was prepared by PCR with following one pair of primers: pGAP-SacI-F (5'-

CGGCCGCGGACCAGTTCTCACACGGAACACCACTAATGGA-3') and tGAP-NotI-R (5'-ATTTTAATGACATTTTCGATTCATTGATTGAGCGGCCGCCGG-3') with plasmid pWOXYLA1 (Madhavan et al.,2009) as template. The amplified fragment was digested *Sac* I and *Not* I site and inserted into *Sac* I and *Not* I site of pδW (Yamada et al.,2009). The resulting plasmid vector was named pδWXI.

2.3 Yeast transformation

The expression plasmid pLXKS was digested with *Hpa* I at *LEU2* maker loci and transformed into *S. cerevisiae* MT8-1 using YEAST MAKER^{TM} (Clontech Laboratories Inc.). The resulting transformant was named MT8-1/XKS. Subsequently, the expression plasmid pδWXI was digested with *Asc* I and transformed into MT8-1/XKS using YEAST MAKER^{TM}. The resulting transformant was named MT8-1/XKδXI.

2.4 Cultivation of yeast transformants

Yeast transformants were pre-incubated in the SD medium at 30^{o}C for 16 h with shaking at 150 strokes/min and were inoculated into the SDC (0.67 % (w/v) yeast nitrogen base supplemented with appropriate amino acids and nucleotides, 2% glucose and 2 % Casamino acids) medium as seed to give an initial OD_{600} of 0.03. The cultivation was furthermore continued at 30^{o}C for 48 h with shaking at 150 strokes/min.

2.5 Fermentation

The cultivated yeast cells were collected by centrifugation for 5 min at 3,000 × g, and washed twice with distilled water. The collected cells corresponded 2.5 g wet cell weight was inoculated into 50 ml of fermentation medium, containing 1% (w/v) yeast extract, 2 % peptone, and various concentrations mixture of glucose and xylose. Ethanol fermentation was carried out in 100-ml closed bottles equipped with a bubbling CO_2 outlet. All fermentations were performed at 30^{o}C with mild agitation. Ethanol and sugars were measured by HPLC analysis described below.

2.6 HPLC analysis

HPLC analysis of fermentation medium was performed by using a refractive index detector (model RID-10A; Shimadzu, Kyoto, Japan). The column used for separation was a Shim-pack SPR-Pb column (Shimadzu). The HPLC apparatus was operated at 80^{o}C with water at a flow rate of 0.6 ml/min as the mobile phase.

2.7 Determination of reaction model

Xylose consumption rate was fitted with a first or a second order reaction model by using Curve Fitting Toolbox (MATLAB, Mathworks). The obtained models were evaluated the validity by approximation accuracy calculated from sum of square for error.

3. Results and Discussion

3.1 Ethanol fermentation from xylose or glucose

At first, the ethanol fermentation ability of xylose-assimilating yeast strain MT8-1XKδXI was confirmed by using glucose as a sole carbon source. As a result, this recombinant strain exhibited the high performance consumption rate of 59.3 g/l glucose and produced 19.9 g/l ethanol within 16 h of fermentation (Fig.1A). The carbon

utilization yield from glucose to ethanol was estimated to be 0.656, and it was well agreed with non-gene modified wild type *S. cerevisiae* strain. It was assumed that this strain has no negative effect on ethanol fermentation ability from glucose by introducing of exogenous genes. Then, the ethanol production from xylose was examined using approximately 50 g/l xylose as the sole carbon source using the MT8-1XKδXI. As a result, this strain consumed 45.4 g/l xylose and produced 13.6 g/l ethanol at 104 h of fermentation (Fig.1B). The carbon utilization yield from xylose to ethanol was estimated to be 0.586. Compared with previous reported several papers about the xylose assimilating yeast, this is the first report of xylose *S. cerevisiae* with theXI pathway by the integration of XI expression cassette into the genome. In addition, this strain did not need an adapt cultivation with xylose pier ethanol fermentation.

3.2 Co-fermentation of xylose and glucose sugar mixture

A mixture of 50 g/l each of glucose and xylose was prepared as the carbon source, and co-fermentation of glucose and xylose were carried out using recombinant yeast strain MT8-1XKδXI as a described above xylose fermentation. This strain consumed 58.5 g/l glucose and 34.8 g/l xylose, and produced 27.4 g/l ethanol at 104 h of fermentation (Fig.1C). MT8-1XKδXI successfully produced ethanol from not only xylose as the sole carbon source but also mixed sugar of xylose and glucose. By comparing with Fig.1B, the xylose consumption rate was different. This difference was also confirmed in other conditions of mixed sugar, and assumed that glucose assimilated pathway effect on the fermentation from xylose.

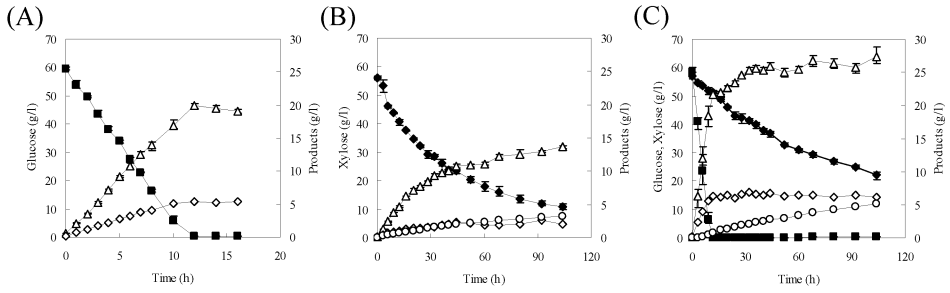

Fig.1. *Time courses of ethanol production from glucose, xylose, and xylose and glucose mixture using MT8-1XKδXI: (A) glucose 50 g/l, (B) xylose 50 g/l, and (C) mixture of xylose 50 g/l and glucose 50 g/l. Symbols show ethanol(open triangles), xylose(closed diamonds), glucose(closed square), glycerol(open diamonds) and xylitol(open circles). Data points are the average of three experiments.*

3.3 Reaction rate equation

The xylose consumption rate was assumed to depend on a first or a second-order to xylose concentration, and following two kinds of equation were set out for reaction models.

$$-\frac{dC_x}{dt} = k_1 C \Leftrightarrow \int_{C_0}^{C_t} \frac{dC}{C} = -k_1 \int_0^t dt \Leftrightarrow C_t = C_0 \times e^{-k_1 t} \qquad (1)$$

$$-\frac{dC_x}{dt} = k_2 C^2 \Leftrightarrow \int_{C_c}^{C_t} \frac{dC}{C^2} = -k_2 \int_0^t dt \Leftrightarrow C_t = \left(k_2 t + \frac{1}{C_0} \right)^{-1} \quad (2)$$

Equation (1) is a first-order reaction model. C_0 and C_t are concentration of xylose at time t=0 and time t, respectively. k is a reaction rate constant. Equation (2) is a second-order reaction model. As shown in Fig. 2, time courses of xylose concentration were fitted with the reaction models, and reaction rate constants were obtained by MATLAB. The results of approximation accuracy and reaction rate constants are summarized in Table 1. The xylose consumption rates from xylose as a sole carbon source was followed by a second-order reaction model, and those from mixture sugars was by a first-order reaction model.

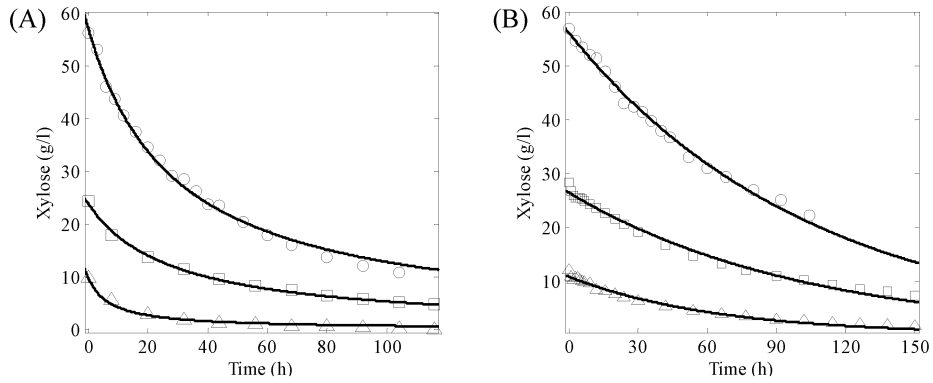

Fig.2. *The results of fitting between time courses of xylose concentration and reaction models: (A) second-order approximation of the fermentation from xylose, (B) first-order approximation of the fermentation from xylose and 50 g/l glucose. Symbols show initial xylose concentration, 10 g/l (open triangles), 25 g/l (open squares) and 50 g/l (open circles).*

Table 1 Approximation accuracy and reaction rate constants

Initial substrate concentration	Approximation accuracy		Reaction rate constant	
	First order	Second order	First order	Second order
10 g/l xylose	0.9834	0.9795	0.0542	0.0135
25 g/l xylose	0.9375	0.9958	0.0164	0.001466
50 g/l xylose	0.9794	0.9939	0.0186	0.000606
50 g/l glucose + 10 g/l xylose	0.9844	0.9911	0.0157	0.002685
50 g/l glucose + 25 g/l xylose	0.9948	0.9905	0.0098	0.000596
50 g/l glucose + 50 g/l xylose	0.9929	0.9916	0.0095	0.00024

4. Conclusion

MT8-1XKδXI successfully produced ethanol from not only xylose as the sole carbon source but also mixed sugar of xylose and glucose. To the best of our knowledge, this is the first report of xylose metabolic *S. cerevisiae* with the XI pathway by the integration of XI expression cassette into the genome. However, although the recombinant yeast was used, xylitol which is unpreferable by-product was produced. To achieve a higher ethanol yield in our XI integration system, genetic modifications not to produce xyilitol would be very important. Secondly, the reaction rate analysis by using MATLAB showed that glucose affects xylose consumption rate, but we should analyze the effect of other products such as ethanol, glycerol and xylitol. To set out and analyze reaction model which considered the effects of all products lead to improvement of ethanol productivity.

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References

Katahira S., Fujita Y., Mizuike A., Fukuda H., Kondo A., 2004, Construction of a Xylan-Fermenting Yeast Strain through Codisplay of Xylanolytic Enzymes on the Surface of Xylose-Utilizing *Saccharomyces cerevisiae* Cells, Appl. Environ. Microbiol. 70, 5407-5414.

Madhavan A., Tamalampudi S., Ushida K., Kanai D., Katahira S., Srivastava A., Fukuda H., Bisaria VS., Kondo A., 2009, Xylose isomerase from polycentric fungus Orpinomyces: gene sequencing, cloning, and expression in *Saccharomyces cerevisiae* for bioconversion of xylose to ethanol. Appl. Microbiol. Biotechnol. 82, 1067-78.

Rizzi M., Harwark K., Erlemann P., Buithanh N.A., Dellweg H., 1989a, Purification and properties of the NAD+-xylitol dehydrogenase from the yeast *Pichia stipitis*. J. Ferment. Bioeng. 67, 20-24.

Rizzi M., Harwart K., Buithanh N.A., Dellweg, H., 1989b, A kinetic-study of the NAD+-xylitol dehydrogenase from the yeast *Pichia pastoris*. J. Ferment. Bioeng. 67, 25-30.

Tajima M., Nogi Y., Fukasawa T., 1985, Primary structure of the *Saccharomyces cerevisiae GAL7* gene. Yeast 1, 67–77.

Toivari M.H., Aristidou A., Ruohonen L., Penttilä M., 2001, Conversion of xylose to ethanol by recombinant *Saccharomyces cerevisiae*: importance of xylulokinase (XKS1) and oxygen availability. Metab Eng. 3, 236-249.

Yamada R., Tanaka T., Ogino C., Fukuda H., Kondo A., 2009, Novel strategy for yeast construction using delta-integration and cell fusion to efficiently produce ethanol from raw starch. Appl. Microbiol. Biotechnol. In press.