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A Parametric Study on Ethanol Production from Xylose by *Pichia stipitis*

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Abstract Characteristics of ethanol production by a xylose-fermenting yeast, *Pichia stipitis* Y-7124, were studied. The sugar consumption rate and specific growth rate were higher in the glucose-containing medium than in the xylose-containing medium. Specific activities of xylose reductase and xylitol dehydrogenase were higher in the medium with xylose than glucose, suggesting their induction by xylose. Maximum specific growth rate and ethanol yield were achieved at 30 g xylose/L concentration without formation of by-products such as xylitol and acetic acid whereas a maximum ethanol concentration was obtained at 130 g/L xylose. Adding a respiratory inhibitor, rotenone, increased a maximum ethanol concentration by 10% compared with the control experiment. In order to evaluate the pattern of ethanol inhibition on specific growth rate, a kinetic model based on Luong's equations was applied. The relationship between ethanol concentration and specific growth rate was hyperbolic for glucose and parabolic for xylose. A maximum ethanol concentration at which cells did not grow was 33.6 g/L for glucose and 44.7 g/L for xylose.

Keywords: ethanol, xylose, *Pichia stipitis*, ethanol tolerance

INTRODUCTION

Cellulosic materials are one of the most abundant natural resources on earth and furthermore, continuously produced by photosynthesis. Many researchers are interested in economical production of fuel-grade ethanol from cellulosic materials. They generally consist of 40% cellulose, 30% hemicellulose and 20% lignin as main components and may be considered as a potential feedstock for the production of ethanol by microbial fermentations [1]. Hemicellulose constitutes up to 35% of hardwood species and other woody angiosperms by dry weight, with the aldopentose D-xylose as major constituent of xylan, amounting up to 25% of dry biomass. An economic analysis of xylose fermentation concluded that a fixed substrate cost, the yield and final concentration of ethanol are the most important factors in the cost of ethanol production [2]. For ethanol production from xylose to be commercially viable, it was suggested that a microorganism should be capable of producing 50 to 60 g/L ethanol within 36 h with a yield of at least 0.4 g ethanol/g sugar.

Fungi including *Fusarium oxysprum*, *Monilia* sp., *Mucor* sp., *Neurospora crassa* and *Paecilomyces* sp. can produce ethanol above 5% (v/v) but productivity is very low. In the case of bacteria, thermophilic *Clostridium thermosaccharolyticum* [3], *Thermoanaerobacter ethanolicus* [4] and mesophilic *Erwinia chrysanthemi, Klebsiella platicola* can utilize xylose to produce ethanol. But the final ethanol concentration and yield are very low in the fermentation processes by bacteria. For yeasts, *Cadida, Kluyveromyces, Pachysolen, Pichia* can produce ethanol. Among them, *P. stipitis* [5] can utilize glucose, mannose, galactose, cellulose and xylan as carbon source for ethanol production. It is generally known that *P. stipitis* is superior to all other yeast species for ethanol production from xylose. Furthermore, *P. stipitis* can convert xylose to ethanol at a high yield without xylitol formation and does not require vitamins. However, the low ethanol tolerance of this pentose-fermenting yeast is an impediment in the commercial production of fuel-grade ethanol from xylose. *P. stipitis* is known to produce ethanol up to 33-57 g/L, however 30 g/L is known as a critical concentration above which cells can not grow at 30°C [6,7]. While *Saccharomyces cerevisiae* strains generally have high ethanol tolerance, they are unable to convert xylose to ethanol efficiently owing to either the lack of, or the extremely low activities of key enzymes involved in xylose catabolism [8].

High initial xylose concentrations above 40 g/L exerted inhibitory effects on the growth of *P. stipitis* CBS 5773, while the ethanol yield of this strain remained constant at approximately 0.4 g ethanol/g xylose, with no xylitol accumulation up to initial xylose concentration of 110 g/L [9,10]. In our previous work [11] with *P.*

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stipitis CBS 5776, the maximum yield and concentration of ethanol was achieved at 100 g/L initial xylose concentration. Others reported that the volumetric ethanol productivity of *P. stipitis* was inhibited at initial xylose concentrations of between 76 and 99 g/L, whereas the ethanol yield was affected only above 145 g/L [12].

This research was undertaken to investigate effects of environmental factors including initial xylose concentrations and rotenone on ethanol production by *P. stipitis* NRRL Y-7124. An inhibitory effect of ethanol on cell growth was analyzed in order to determine an inhibition type and a maximum critical ethanol concentration.

MATERIALS AND METHODS

Strain and Media

P. stipitis NRRL Y-7124 (Northern Regional Research Center, Peoria, IL, U.S.A.) was used in this study. *P. stipitis* was incubated in YPD-agar medium (10 g yeast extract, 20 g peptone, 20 g glucose, 20 g agar per liter) and stored in a refrigerator. Seed- and pre-cultures were done in the YPD medium. Main cultures for ethanol production were carried out by using the medium containing 5.0 g yeast extract, 5.0 g peptone, 5.0 g KH_2PO_4, 0.2 g $(NH_4)_2SO_4$ and 0.4 g $MgSO_4 \cdot 7H_2O$ per liter. Xylose and glucose were used as carbon sources. Medium components were sterilized separately at 121°C for 15 min. Initial pH was set at 5.0 by addition of 2 N HCl and 2 N NaOH. Rotenone was purchased from Sigma Chemical Co. (St. Louis, U.S.A.).

Fermentations

A seed culture was grown at 30°C for 18 h in a rotary shaking incubator (Vision Science 8480SF; Incheon, Korea). A colony was picked up and transferred to 5 mL YPD medium and cultivated at 200 rpm until absorbance at 600 nm reached 20. The seed culture was transferred to a shake flasks for pre-culture or main shake flask cultures.

Analysis

Dry cell mass was estimated by measuring absorbance at 600 nm. Concentrations of glucose and xylose were determined by HPLC (Knauer 1000; Berlin, Germany) equipped with the HPX-87C or -87H column (300 mm × 7.8 mm, Bio-Rad, Hercules, U.S.A.). Ethanol concentration was measured by a gas chromatograph (YongLin 600D; Seoul, Korea) with the Carbowax 20 M column (Hewlett Packard, Boise, U.S.A.) and a flame ionization detector. Butanol (2%) was used as an internal standard for ethanol measurement. Nitrogen gas was utilized as a mobile phase at a flow rate of 30 mL/min. Temperatures at injector, detector and oven were 200°C, 200°C, and 150°C, respectively.

Enzyme Activity

Cells were separated from the culture broth by centrifugation at 8,000 × g for 15 min, washed twice in saline solution and resuspended in 50 mM phosphate buffer (pH 7.5). Glass beads (0.5 g, 0.5 mm diameter) were added to disrupt cells in a Dyno mill homogenizer (B. Braun MSK; Schwarzenberger, Germany). Cell debris was removed by centrifugation at 8,000 × g and the supernatant was defined as the crude enzyme solution. Total protein concentrations were determined by Sigma kit (Sigma Chemical Co.). The enzyme activity of xylose reductase (XR) was estimated by an absorbance change at 340 nm [13]. Crude enzyme (0.1 mL), 0.1 M phosphate buffer (pH 7.0, 2.7 mL), and 3.45 mM NADPH (0.1 mL) were mixed and incubated at 30 °C for 10 min. 1 M xylose (0.1 mL) was added and incubated at 30°C for 10 min. One unit of xylose reductase activity was defined as the amount of enzyme to oxidize 1 μmole of NADPH in 1 min under the given reaction conditions.

An absorbance change at 340 nm was also correlated with the activity of xylitol dehydrogenase (XDH) [13]. Tris buffer (0.1 M, pH 8.5, 0.6 mL), 0.1 M $MgCl_2$ (0.1 mL), 9 mM NAD (0.1 mL) and crude enzyme (0.1 mL) were mixed and incubated at 30°C for 10 min. 1 M xylitol (0.1 mL) was added and incubated at 30°C for 10 min. One unit of xylitol dehydrogenase activity was defined as the amount of the enzyme to reduce 1 μmole of NAD in 1 min at 30°C.

RESULTS AND DISCUSSION

Ethanol Production from Glucose and Xylose

The growth medium containing either 20 g/L of glucose or 20 g/L of xylose was used to investigate the pattern of cell growth and ethanol production by *P. stipitis* NRRL Y-7124 (Fig. 1). A maximum dry cell concentration was about 7 g/L for both carbon sources. The sugar consumption rate and specific growth rate were higher in the glucose-containing medium than xylose-containing medium. Ethanol concentrations were as low as 3 g/L for both cases, which could be attributed to aerobic cultivation conditions caused by high agitation speed. A theoretical ethanol yield in microaerobic conditions is identical for glucose and xylose, 0.51 g ethanol/g glucose or xylose [14]. It seemed that *P. stipitis* has no Crabtree effect which causes ethanol formation as in *S. cerevisiae* under aerobic culture conditions.

In the case of a mixture of glucose and xylose, glucose was utilized first by *P. stipitis* (Fig. 2). After glucose depletion, xylose was consumed with very low ethanol yield based on xylose. Glucose seemed to inhibit xylose utilization by *P. stipitis*. The specific activities of XR and XDH essential for xylose utilization were measured in both glucose and xylose media (Fig. 3). As shown in Fig. 3, the specific activities of XR and XDH were very low in the glucose medium, whereas much higher in the xylose medium. This suggested that the expression of

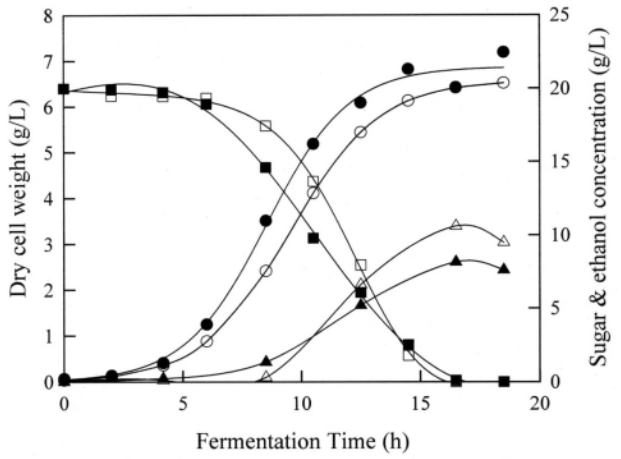

Fig. 1. Fermentation profiles of cell growth, sugar utilization and ethanol production by *P. stipitis* grown at 30°C and pH 5.0. Closed and open symbols represent the data for glucose and xylose, respectively. Circles, rectangles and triangles indicate dry cell weight, sugar and ethanol, respectively.

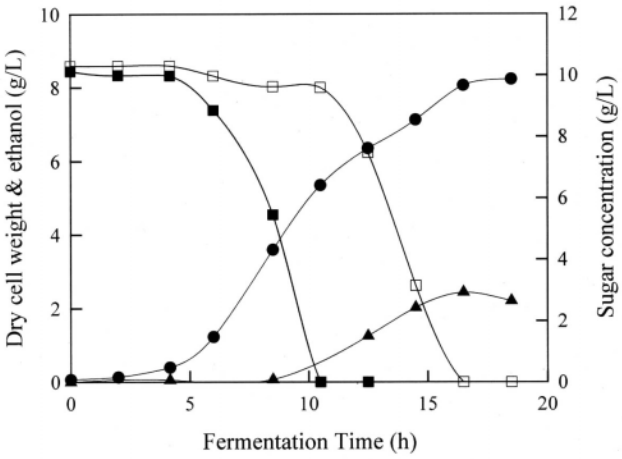

Fig. 2. Trajectories of cell growth, sugar utilization and ethanol production by *P. stipitis* grown in a mixture of glucose and xylose at 30°C and pH 5.0 (●: dry cell weight, ▲: ethanol, □: xylose,■: glucose).

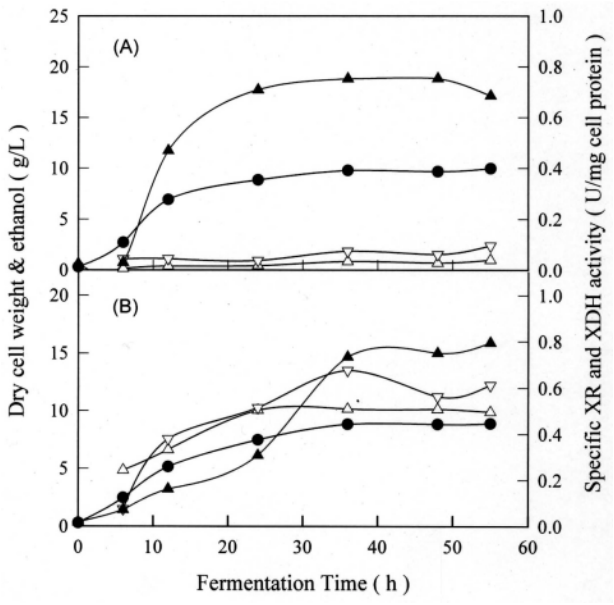

Fig. 3. Profiles of specific XR and XDH activities of *P. stipitis* grown in the YP media containing glucose (A) and xylose (B) as a sole carbon source at 30°C and pH 5.0 (●: dry cell weight,: ▲: ethanol, △: specific XR activity, ▽: specific XDH activity).

Table 1. Effects of initial xylose concentrations on fermentation characteristics of *P. stipitis* Y-7124 at 30°C and pH 5.0

Xylose concentration (g/L)	30	50	90	130	170
Maximum dry cell weight (g/L)	7.0	7.0	8.4	7.1	5.1
Maximum specific growth rate (h^{-1})	0.28	0.27	0.26	0.19	0.17
Maximum ethanol concentration (g/L)	12.3	17.7	29.1	31.8	26.4
Ethanol yield (g/g)	0.37	0.36	0.33	0.29	0.26
Xylitol (g/L)	ND[a]	0.81	4.8	11.8	5.7

[a] Not detected.

XR and XDH was repressed by glucose as reported elsewhere [15].

Effects of Initial Xylose Concentrations

Various concentrations of xylose were used to examine the effects of initial xylose concentrations on ethanol production. The experimental results were summarized in Table 1. Maximum specific growth rate and ethanol yield decreased with increasing initial xylose concentrations. Maximum dry cell weight of 8.36 g/L was obtained at 90 g/L xylose. Above 130 g/L, however, the yeast did not consume xylose completely and produced acetic acid instead (data not shown). It is very interesting to note that xylitol was also formed above 50 g/L xylose.

High ethanol concentration was obtained at xylose concentrations of 130 g/L and higher, whereas ethanol yield and specific rates of ethanol production were enhanced at low xylose concentrations.

Xylitol production in *P. stipitis* is known to increase with increasing xylose concentrations, but no detectable amounts of xylitol were produced under semi-aerobic culture conditions. Thus, the above experimental result could be attributed to severe oxygen-limited growth conditions because of higher cell densities or being a direct result of high xylose concentrations [16]. High ethanol concentrations could also result in increased xylitol production [14]. The xylitol yield of *P. stipitis* was especially sensitive to the ethanol level under anaerobic conditions, with a linear increase in xylitol yield and a concomitant decrease in ethanol yield as

Table 2. Effects of rotenone on xylose fermentation by *P. stipitis* at 30°C and pH 5.0.

Rotenone[a]	Maximum ethanol concentration (g/L)
Control	10.5
0.5 × addition at inoculation	8.5
0.5 × addition at exponential growth phase	11.5
1.0 × addition at inoculation	8.3

[a] The saturated concentration of rotenone in ethanol was defined as 1×.

an initial ethanol concentration increased [17].

Effects of a Respiration Inhibitor

Rotenone was added upon inoculation or during the exponential growth phase (Table 2). The saturated concentration of rotenone in ethanol was defined as 1×. Rotenone added upon inoculation inhibited cell growth. But 0.5× rotenone added during the exponential growth phase rate enhanced ethanol production by 10% compared with control experiment and a 35% increase compared with the case where rotenone was added at inoculation. Therefore, it can be concluded that maximum ethanol productivity could be obtained at microaerobic conditions than the given culture conditions. *P. stipitis* prefers a microaerobic condition for ethanol production, however, which is impractical in implementing pilot-scale operation. Thus, a respiration inhibitor, such as rotenone could be utilized to maintain a microaerobic culture condition.

Besides rotenone, potassium cyanide, antimycin A and sodium azide are known to prevent contamination, to block respiration and to repress by-product formation [18]. In case of *C. tropicalis*, azide increased the ethanol yield of per unit cell mass up to a four-fold by repressing xylitol production, but by acting as an uncoupler of oxidative phosphorylation, azide caused the loss of up to 60% of the carbon substrate as carbon dioxide [19,20].

Effects of Ethanol on Cell Growth

Ethanol tolerance as well as biomass productivity are very important factors in economical production of biofuel [16]. In order to describe and characterize the kinetic pattern of ethanol inhibition on cell growth, the kinetic model proposed by Luong [21] was applied:

$$\frac{\mu_i}{\mu_0} = 1 - \left(\frac{P}{P_{\max}}\right)^{\alpha}$$

where μ_0 and μ_i are the maximum specific growth rate and maximum specific growth rate in the presence of ethanol, respectively. P_{max} is the critical ethanol concentration above which cells cannot grow. The relationship between specific growth rate and critical ethanol concentration could be described by the ethanol tolerance index, α: a linear relationship for α=1, a hyperbolic relationship when α>1 and a parabolic relationship when α >1. The average specific growth rates were calculated and plotted against ethanol concentrations to estimate P_{max} and α (Fig. 4). P_{max} was 33.6 g/L for glucose and 44.7 g/L for xylose. α was estimated as 0.89 for glucose and 1.26 for xylose. Consequently, the relationship between ethanol concentration and specific growth rate is hyperbolic when glucose is utilized as a carbon source and parabolic for xylose. Our experimental results are compatible with those obtained by du Preeze *et al*. [6] who have grown the *P. stipitis* strain (CSIR Y-633) in a xylose medium. P_{max} of 42 g/L was determined experimentally and the strain was unable to ferment a mixture of sugars when an ethanol concentration exceeded 40 g/L. Ethanol levels completely inhibiting growth were 30 and 34 g/L for xylose and glucose, respectively. Ethanol tolerance of *P. stipitis* is quite low compared with *S. cerevisiae* which has been frequently used in ethanol fermentation. The maximum critical ethanol concentration of *S. cerevisiae* was known approximately 100 g/L [21].

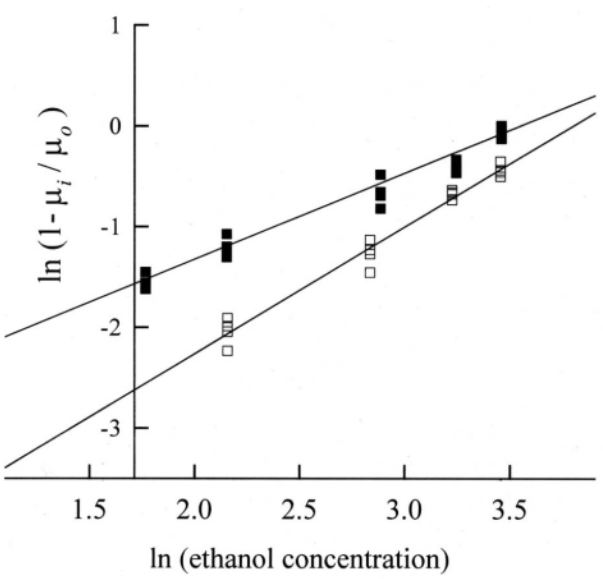

Fig. 4. Estimation of kinetic parameters for ethanol inhibition at 30°C and pH 5.0. Initial xylose concentrations were 0, 5.9, 8.6, 17.9, 25.7 and 31.8 g/L. Specific growth rates were estimated by the linear regression analysis (□: xylose, ■: glucose).

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