Comparative Study on Ethanol Production with Pentose and/or Hexose by *Saccharomyces cerevisiae* and/or *Pichia stipitis*

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Glucose and xylose are the most abundant materials in nature which can be used to produce ethanol by yeast fermentation. Three combinations of cultivation with glucose and xylose were carried out: separated, co-culture, and sequential fermentation with *Saccharomyces cerevisiae* and *Pichia stipitis*. In the separated fermentation, *S. cerevisiae* fermented glucose to produce 14.5 g/l ethanol from 29.4 g/l glucose but hardly used xylose. However, *P. stipitis* utilized not only glucose but also xylose to produce ethanol 11.9 g/l and 11.6 g/l from 29.4 g/l glucose and 29.0 g/l xylose, respectively. In the mixture of glucose and xylose, *P. stipitis* fermented both sugars, producing 21.1 g/l ethanol while *S. cerevisiae* fermented only glucose, producing 13.4 g/l ethanol. In the co-culture and sequential fermentation, the co-culture showed more efficient ethanol productivity with 18.6 g/l ethanol than the sequential fermentation with 12.4 g/l ethanol. To investigate the effect of nutrients in the growth of microorganisms and ethanol production, yeast nitrogen base (YNB) was used in the sequential fermentation with *S. cerevisiae* and *P. stipitis*. YNB supplemented some nutrients which *S. cerevisiae* used up in the broth and the culture showed increased growth rate, increased consumption of xylose, and increased ethanol productivity producing 22.5 g/l ethanol from 54.6 g/l sugar with a yield of 0.41 g/g.

Key words : Ethanol, *Saccharomyces cerevisiae*, *Pichia stipitis*, glucose, xylose

Introduction

The production and use of ethanol for fuel are increasing worldwide in response to economic, security, and environmental concerns. Because ethanol can be easily applied to the internal combustion engine vehicles, it is used as an oxygenate to reduce automotive emissions [19]. In the usage of ethanol, the by-products derived from fossil energy are strongly reduced or even absent with biofuels and it could help avoid accumulation of carbon dioxide causing environmental problems in the atmosphere [6,8,20]. Moreover, other grounds for use of ethanol include the benefits of producing ethanol for farmers and rural economies [19].

Ethanol has been produced mainly by fermenting sugars with yeasts. These sugars were obtained from grains and sugarcane. The United States and Brazil are the world's largest ethanol producers and consumers using corn grain and sugar cane. However, ethanol production using grains and sugarcane has faced a conflict of a food shortage. Fortunately, there are plenty of sugars in nature in the form

of lignocelluloses which is the most abundant material in the wood and consists of cellulose (40%), hemicelluloses (30%), and lignin (20%) in average [21]. Glucose is the major component of cellulose and xylose is a main constituent of hemicelluloses [7,17,21]. Because pretreatment and saccharification of lignocellulosic biomass yields mainly glucose and xylose, it is a big challenging task to ferment two sugars.

Saccharomyces cerevisiae is used widely and traditionally for industrial ethanol production because it is highly effective on the production of ethanol from hexose sugars and has high tolerance to ethanol and other inhibitory compounds [10-12]. Although xylose is the second major fermentable sugars present in hard woods and herbs, xylose cannot be fermented by *S. cerevisiae*. For the efficient conversion of pentose sugars, many kinds of microorganisms have been investigated to ferment xylose to ethanol. *P. stipitis* and *Candida shehatae* were reported to use xylose efficiently than other yeasts [5]. Moreover, *P. stipitis* was reported to produce more ethanol from xylose than *C. shehatae*. Among the xylose fermenting yeasts, *P. stipitis* was reported to be the most effective strain because of its high ethanol yield [3,5].

In an attempt to produce ethanol from lignocellulosic bio-

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mass, we studied the way of efficient fermentation with glucose and/or xylose. Considering the characteristics of two yeast strains, different culture conditions such as separated, co-culture and sequential fermentation of different yeasts, and the effect of yeast nitrogen base (YNB) were investigated.

Materials and Methods

Yeast strains and media

S. cerevisiae strain was obtained from an industrial source, baker's dry yeast (Cho Heung, Ansan, Kyungkido, Korea) and *P. stipitis* was obtained from Korea Biological Resource Center (KCTC 7228; ATCC 58784). *S. cerevisiae* was grown and maintained on 1.5 % agar plate containing YPD (yeast peptone dextrose; 10 g/l yeast extract, 20 g/l peptone, 30 g/l glucose and 20 g/l agar) media at 37°C and *P. stipitis* was grown at 30°C and maintained on YPX (yeast peptone xylose) media agar plate which has same ingredients with YPD media except 30 g/l xylose instead of glucose. Glucose (30 g/l or 60 g/l) and xylose media (30 g/l or 60 g/l) for main culture were prepared separately and 60 g/l of glucose and xylose media were mixed to make 30 g/l glucose-30 g/l xylose mixed medium (60 g/l total sugar concentration). The concentrations of glucose, xylose and the mixture in medium were measured with high performance liquid chromatography (HPLC) and the values were 29.4 g/l glucose, 29.0 g/l xylose and 54.6 g/l mixed sugars. When glucose was depleted, the yeast nitrogen base (YNB, 5.0 g/l (NH₄)₂SO₄, 0.1 g/l KH₂PO₄, 0.5 g/l MgSO₄, 0.1 g/l NaCl, 0.1 g/l CaCl₂, amino acids, vitamins and trace elements, Difco, Detroit, MI, USA) was added to the medium to supply nutrients in fermentation at the concentration of 1.7 g/l.

Fermentation conditions

For pre-culture, colonies of *S. cerevisiae* from a maintained plate were grown in YPD (30 g/l of glucose) media overnight in a 50 ml Erlenmeyer flask at 37°C, 180 rpm and *P. stipitis* were grown in YPX (30 g/l of xylose) media at 30°C, 180 rpm. Both yeasts were pre-cultivated overnight above OD₆₀₀=5 and the cultured broth was took out with desired volume and centrifuged at 3,000 rpm for 10 min. The centrifuged cell pellet was resuspended with fresh media and then inoculated to a main fermentation medium to be 0.5 of OD₆₀₀ at initial point. Fermentation was performed in sterile 100 ml Erlenmeyer flasks with 50 ml working volume. *S. cer-*

visiae was grown at 37°C under anaerobic condition (with air trap filled with 0.1 N HCl), whereas *P. stipitis* was grown at 30°C with an agitation speed of 100 rpm in a shaking incubator. Fermentation profiles shown in figures are from one representative fermentation.

Analytical methods

Fermentation was monitored for 6 days by taking 1 ml of samples and the samples were centrifuged at 12,000 rpm for 10 min then the supernatants were filtered with a 0.2 µm syringe filter (Sartorius stedim biotech, Minisart RC 15). The concentrations of sugars and ethanol were determined using a HPLC (Young Lin CO., Korea) system equipped with Aminex HPX-87H column (BIO-RAD, CA, USA), and a BIO-RAD Cation H refill guard column and a refractive index detector (RID; Model LY9170, Young Lin CO., Korea). The mobile phase was 0.0017 N H₂SO₄ and its flow rate and injection volume were 0.6 ml/min and 20 µl at 35 °C of oven temperature. The serial concentration of standards for glucose and xylose were prepared with a five point calibration (0, 1.25, 2.5, 5, and 10 g/l) and ethanol was prepared with four point (0, 12.5, 25, and 50 g/l) with R²>0.999. The cell concentrations were determined by optical density with a UV-Vis spectrophotometer (Amersham, ultrospec 2100pro) at 600 nm. Yield was calculated as produced ethanol concentration divided by initial sugar concentration.

Results

Fermentation of glucose or xylose by *S. cerevisiae* or *P. stipitis*

To see whether *S. cerevisiae* and *P. stipitis* can use glucose or xylose to produce ethanol, batch cultures were carried out using *S. cerevisiae* or *P. stipitis* alone. The glucose fermentation by *S. cerevisiae* proceeds by a reduction of oxygen availability and *P. stipitis* has best rate of ethanol production under oxygen-limited conditions [9]. Anaerobic cultivations for *S. cerevisiae* were conducted 100 ml Erlenmeyer flasks with air trap filled with 0.1 N HCl and microaerobic cultivation for *P. stipitis* were preformed with siliistoppers.

All glucose was consumed by *S. cerevisiae* after 24 hr of fermentation, and the ethanol concentration was 14.5 g/l with a yield of 0.49 g/g (Fig. 1A). However, xylose was hardly fermented by *S. cerevisiae* producing little amount of ethanol and the growth of cell was not significantly increased (Fig. 1B). *P. stipitis* consumed not only glucose but

Fig. 1. Glucose or xylose fermentation by *S. cerevisiae*. A: *S. cerevisiae* was cultured in YPD (29.4 g/l glucose) media at 37°C under anaerobic condition with air trap filled with 0.1 N HCl. B: *S. cerevisiae* was cultured in YPX (29.0 g/l xylose) media at 37°C under anaerobic condition with air trap filled with 0.1 N HCl. Symbols: glucose (—■—), ethanol (—x—), and OD₆₀₀ for *S. cerevisiae* (—■— with OD value).

also xylose, producing ethanol. The glucose was slowly consumed by *P. stipitis* until 24 hr, and after then was rapidly consumed and depleted at 72 hr, producing 11.9 g/l ethanol (Fig. 2A). The yield was low (0.40 g/g), compared to *S. cerevisiae* (0.49 g/g). *P. stipitis* slowly utilized xylose until 24 hr and after then utilized it at higher speed. All the xylose was consumed by 96 hr. The maximum production of ethanol (11.6 g/l ethanol, yield 0.40 g/g) did not show differences with that of glucose by *P. stipitis* (Fig. 2B).

Fermentation of mixture of glucose and xylose by *S. cerevisiae* and/or *P. stipitis*

As shown in xylose fermentation by *S. cerevisiae* (Fig. 1B), *S. cerevisiae* hardly used xylose in mixture with glucose as well but consumed glucose rapidly by 24 hr and produced 13.4 g/l ethanol at 72 hr at its maximum with a yield of 0.25 g/g (Fig. 3A). *P. stipitis*, on the contrary, consumed the glucose completely at 48 hr, and then xylose fermentation was started. *P. stipitis* produced 21.0 g/l ethanol with a yield

Fig. 2. Glucose or xylose fermentation by *P. stipitis*. A: *P. stipitis* was cultured in YPD (29.4 g/l glucose) media at 30°C, 100 rpm in a shaking incubator under microaerobic condition. B: *P. stipitis* was cultured in YPX (29.0 g/l xylose) media at 30°C, 100 rpm in a shaking incubator under microaerobic condition. Symbols: xylose (—■—), ethanol (—x—), and OD₆₀₀ for *P. stipitis* (—■— with OD value).

of 0.38 g/g (Fig. 3B). The utilization of glucose and xylose showed same trends with the previous results (Fig. 2). *P. stipitis* produced 11.9 g/l ethanol in glucose only (Fig. 2A) and 11.4 g/l ethanol in the mixture of glucose and xylose at 48 hr when it consumed glucose completely. The ethanol concentration went up to 21.0 g/l at 120 hr fermentation. Considering the ethanol yield from xylose by *P. stipitis* (11.6 g/l at 120 hr), the reasonable amount (11.4g/l ethanol from glucose and 9.6 g/l ethanol from xylose) of ethanol was produced.

The co-culture fermentation with simultaneous inoculation of both yeasts produced 18.6 g/l ethanol at its maximum at 144 hr with a yield of 0.34 g/g and the time reaching to the maximum ethanol concentration was delayed compared to previous results (Fig. 4). The yield was higher compared to the fermentation with single *S. cerevisiae* (0.24 g/g) but slightly lower compared to that with single *P. stipitis* (0.38 g/g) (Fig. 3).

Fig. 3. Fermentation of mixture of glucose and xylose by *S. cerevisiae* or *P. stipitis* in separated fermentation. A: *S. cerevisiae* was cultured in the mixture of YPD (29.4 g/l glucose) and YPX (29.0 g/l xylose) media at 37°C under anaerobic condition with air trap filled with 0.1 N HCl. B: *P. stipitis* was cultured in the mixture of media (29.4 g/l) and YPX (29.0 g/l) media at 30°C, 100 rpm in a shaking incubator under microaerobic condition. Symbols: glucose (—■—), xylose (—▲—), ethanol (—×—), OD₆₀₀ for *S. cerevisiae* (■ with OD value) and OD₆₀₀ for *P. stipitis* (▲ with OD value).

Sequential fermentation and effects of nutrients

Sequential fermentation was carried out to compare the production of ethanol with that of co-culture with both yeasts. Because glucose decreased to zero around 24 hr, *P. stipitis* inoculated at 24 hr after removing *S. cerevisiae* by centrifugation. First of all, glucose seemed to be consumed by *S. cerevisiae* before 24 hr, producing 12.4 g/l ethanol at its maximum with a yield of 0.23 g/g but xylose was not utilized. After reaching high concentration of ethanol production, it decreased slowly to 10.4 g/l ethanol until 144 hr (Fig. 5A). Compared to co-culture, concentration of ethanol product was lower (18.58 g/l ethanol; yield of 0.35 g/g). We suspected that *S. cerevisiae* depleted essential nutrients during making ethanol from glucose so the nutrients for *P. stipitis* was not enough to make ethanol from xylose.

To improve the abilities of xylose fermentation by *P. stip-*

Fig. 4. Fermentation of mixture of glucose and xylose by *S. cerevisiae* and *P. stipitis* in co-culture fermentation. Both *S. cerevisiae* and *P. stipitis* were cultured in the mixture of YPD (29.4 g/l glucose) and YPX (29.0 g/l xylose) media at 37°C under anaerobic condition with air trap filled with 0.1 N HCl. After 24 hr, the air trap was replaced by a sillistopper to make microaerobic condition at 30°C, 100 rpm in a shaking incubator. Symbols: glucose (—■—), xylose (—▲—), ethanol (—×—), OD₆₀₀ for *S. cerevisiae* and *P. stipitis* (■ with OD value).

itis, the yeast nitrogen base (YNB) was added to the mixed media as nutrients after removing *S. cerevisiae* at 24 hr. The growth of cell was increasing up to OD₆₀₀ of 13.2 after adding YNB. In contrast to the result without nutrients (YNB), the concentration of xylose reached zero at 120 hr and ethanol was produced as much as 22.5 g/l with a yield of 0.41 g/g (Fig. 5B).

Discussion

S. cerevisiae is used for industrial ethanol production because it is highly effective on the production of ethanol from hexose sugars and has high tolerance to ethanol and other inhibitory compounds [10–12]. However, it is unable to convert xylose to ethanol efficiently, because of the absence or the low activity of key enzymes involved in xylose catabolism [3,18]. Moreover, when cultivated on glucose, the productivity of ethanol by *S. cerevisiae* is 5 times higher than that of xylose-fermenting yeast on glucose or xylose [4]. Our results showed that glucose was easily fermented by *S. cerevisiae*, because glucose is the most preferred substrate (Fig. 1) as reported previously [15]. Glucose was completely consumed after 24 hr of fermentation producing 14.5 g/l ethanol while xylose was hardly utilized by *S. cerevisiae*.

Some microorganisms can utilize pentose sugars efficiently [1]. Among them, *P. stipitis* is more suitable microorganism, having great potential and broad substrate specif-

Fig. 5. Sequential fermentation by *S. cerevisiae* and *P. stipitis*. *S. cerevisiae* was cultured in the mixture of YPD (29.4 g/l glucose) and YPX (29.0 g/l xylose) media at 37°C under anaerobic condition with air trap filled with 0.1 N HCl. After 24 hr, the medium was centrifuged to remove *S. cerevisiae* and then *P. stipitis* was inoculated to the supernatant without yeast nitrogen base (YNB) or with YNB and cultured at 30°C, 100 rpm in a shaking incubator under microaerobic condition. A: without YNB. B: with YNB. Symbols: glucose (—■—), xylose (—▲—), ethanol (—×—), OD₆₀₀ for *S. cerevisiae* (■ with OD value) and OD₆₀₀ for *P. stipitis* (▲ with OD value).

icity for pentose utilization [16]. In glucose medium, *P. stipitis* completed fermentation by 48 hr producing 11.9 g/l ethanol with yield 0.40 g/g while it completed fermentation by 96 hr in xylose medium, producing 11.6 g/l ethanol with yield 0.40 g/g. The yields were similar to results of Agbogbo *et al* [2]. In the growth of cell, the growth of *P. stipitis* was slightly higher in glucose medium than in xylose medium (Fig. 2) as reported by Meyrial *et al* [13].

Because of the repression of xylose uptake by glucose, glucose is the most preferred substrate and consumed before xylose fermentation started [15]. *S. cerevisiae* can utilize the glucose but not xylose to make ethanol while *P. stipitis* consumed xylose after using glucose. Taniguchi *et al* reported that co-culture system is useful with sugar mixture where potential glucose-fermenting yeast is cultivated with xy-

lose-fermenting yeast. In our experiment, ethanol concentration at the end of fermentation was 18.6 g/l (yield 0.34 g/g) in the co-culture fermentation and 12.4 g/l (yield 0.23 g/g) in the sequential fermentation. It seems that the co-culture fermentation produced ethanol more efficiently than sequential fermentation. Surely in this study, co-culture was better than the sequential fermentation in ethanol production if YNB was not added. However, the sequential fermentation was better to make more ethanol when YNB was added, although YNB never contained any sugar to be converted into ethanol. Even though *P. stipitis* does not need vitamin requirements for xylose fermentation and can utilize a wide range of sugars [14,16], when YNB was added to the mixture fermentation, the cell growth rate, consumption of each sugar and ethanol productivity were increased compared to co-culture fermentation. Because YNB is expensive nutrient for the industrial usage, the component which supplemented the nutrient for *P. stipitis* needs to be elucidated. In addition, lignocellulosic biomass has many additional nutrients which are different from YPD or YPX media, the culture systems developed in this study needs to be applied in a real lignocellulose hydrolysate.

In the present study, *S. cerevisiae* did not ferment xylose but *P. stipitis* fermented both glucose and xylose to produce ethanol. *P. stipitis* fermented the mixture of glucose and xylose while *S. cerevisiae* fermented only glucose. The co-culture fermentation had more efficient ethanol productivity than the sequential fermentation without YNB. However, the sequential fermentation with YNB was the most efficient way of ferment both sugars to ethanol. After depleting glucose, *P. stipitis* seemed to utilize xylose continuously with addition of YNB instead of using ethanol as carbon resource, producing more ethanol. In application of ethanol production using lignocelluloses biomass, it is desirable to convert glucose to ethanol by *S. cerevisiae* then inoculate *P. stipitis* with additional nutrients to convert xylose to ethanol.

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초록 : *Saccharomyces cerevisiae*와 *Pichia stipitis*를 이용한 오탄당과 육탄당으로부터 에탄올 생산에 관한 비교연구

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포도당과 자일로스는 자연계에서 가장 풍부한 물질이며 이들은 효모에 의해 에탄올로 전환될 수 있다. 본 연구에서는 *Saccharomyces cerevisiae*와 *Pichia stipitis*를 이용하여 분리배양, 공동배양 그리고 순차배양 등의 조합을 통해 가장 효과적인 발효의 방법을 찾고자 하였다. 분리배양에서 *S. cerevisiae*은 29.4 g/l의 포도당을 발효하여 14.5 g/l의 에탄올을 생산한 반면에 자일로를 이용하지 못했다. 그렇지만 *P. stipitis*은 포도당뿐만 아니라 자일로스로도 분해하여 각각 포도당 29.4 g/l로부터 11.9 g/l의 에탄올을, 자일로스 29.0 g/l로부터 11.6 g/l의 에탄올을 생산하였다. 포도당과 자일로스 혼합배양에서, *S. cerevisiae*은 13.4 g/l의 에탄올을 생산한 반면에 *P. stipitis*은 21.1 g/l의 에탄올을 생산하였다. 공동배양과 순차배양에서, 공동배양이 18.6 g/l, 순차배양이 12.4 g/l의 에탄올을 생산하여 공동배양이 더 효과적인 것으로 나타났다. 두 효모의 생장에서 영양분의 효과를 보기 위해 yeast nitrogen base (YNB)을 *S. cerevisiae*가 포도당을 소모한 시점에 첨가하니 자일로의 소비량과 미생물의 성장이 증가하였고 54.6 g/l의 당 혼합배양액에서 22.5 g/l의 에탄올을 생산하여 0.41 g/g의 수득율을 나타내었다.