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# Two-Step Process Using Immobilized *Saccharomyces cerevisiae* and *Pichia stipitis* for Ethanol Production from *Ulva pertusa* Kjellman Hydrolysate

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We established a two-step production process using immobilized *S. cerevisiae* and *P. stipitis* yeast to produce ethanol from seaweed (*U. pertusa* Kjellman) hydrolysate. The process was designed to completely consume both glucose and xylose. In particular, the yeasts were immobilized using DEAE-corncob and DEAE-cotton, respectively. The first step of the process included a continuous column reactor using immobilized *S. cerevisiae*, and the second step included a repeated-batch reactor using immobilized *P. stipitis*. It was verified that the glucose and xylose in 20 L of medium containing the *U. pertusa* Kjellman hydrolysate was converted completely to about 5.0 g/l ethanol through the two-step process, in which the overall ethanol yield from total reducing sugar was 0.37 and the volumetric ethanol productivity was 0.126 g/l/h. The volumetric ethanol productivity of the two-step process was about 2.7 times greater than that when *P. stipitis* was used alone for ethanol production from *U. pertusa* Kjellman hydrolysate. In addition, the overall ethanol yield from glucose and xylose was superior to that when *P. stipitis* was used alone for ethanol production. This two-step process will not only contribute to the development of an integrated process for ethanol production from glucose- and xylose-containing biomass hydrolysates, but could also be used as an alternative method for ethanol production.

**Keywords:** *Saccharomyces cerevisiae*, *Pichia stipitis*, *Ulva pertusa* Kjellman, immobilization, DEAE-corncob, DEAE-cotton

## Introduction

Many studies on ethanol production from the hydrolysates of lignocellulosic and seaweed biomass containing glucose and xylose have been conducted and comprehensively reviewed [9, 16, 29, 36]. Various attempts have used yeasts in order to utilize both glucose and xylose for ethanol production, including the fermentation of glucose and xylose using *Pichia*, *Pachysolen*, or *Candida* yeast [1, 28, 39], co-fermentation of glucose and xylose using *Saccharomyces* and *Pichia* [5, 11, 18, 34], and co-immobilization of *Saccharomyces*/*Pichia* or *Saccharomyces*/*Candida* yeast [12, 19, 21]. In addition, genetically-engineered yeast and bacteria have been studied to produce ethanol from glucose and xylose, in which the metabolic pathways for ethanol production were rebuilt [8, 10, 17, 30, 35].

In particular, because yeasts utilize xylose through the pentose phosphate pathway *via* xylitol and xylulose, reducing power must be supplied from the tricarboxylic acid cycle to utilize xylose for ethanol production [15]. Therefore, fine-tuning and controlling the aeration during fermentation are essential for maximum ethanol production from xylose-containing media [7, 13, 14, 27, 32]. Until now, various operational strategies for ethanol production from

glucose- and xylose-containing hydrolysates such as batch, repeated-batch, fed-batch, and continuous operations have been reported based on these ethanol production methods [3].

However, when all of the above-mentioned studies on ethanol production from glucose- and xylose-containing hydrolysates are summarized, more studies were generally conducted in which *Pichia/Pachysolen* utilized xylose and *Saccharomyces* utilized glucose for ethanol production, respectively. *Pichia/Pachysolen* yeasts are rarely used alone for ethanol production from glucose- and xylose-containing hydrolysates. In addition, the reports relevant to co-fermentation of *Saccharomyces* and *Pichia/Pachysolen* and their co-immobilization for ethanol production from glucose and xylose are numerous. Many studies chose the operational strategy, in which *Saccharomyces* and *Pichia/Pachysolen* utilized glucose and xylose, respectively, for ethanol production. *Saccharomyces* is actually able to produce the ethanol from glucose under anaerobic conditions; however, *Pichia/Pachysolen* can produce ethanol from xylose under microaerobic conditions. Consequently, it is difficult to culture these two yeasts simultaneously under optimal ethanol production conditions from a glucose- and xylose-containing hydrolysate. We frequently found incomplete consumption of xylose in co-culture/co-fermentation and co-immobilization systems of *Saccharomyces/Pichia* [12, 18] or *Saccharomyces/Candida*, respectively [19–21]. Some reports showed that these problems were overcome by changing the operational strategy [11] and using mutant yeast [33, 34].

In our previous studies, we accomplished batch and repeated-batch ethanol production processes from the hydrolysates of rape stem [37] and seaweed (*Sargassum sagamianum* and *Ulva pertusa* Kjellman) hydrolysates using *Pichia stipitis* [24, 25, 38], in which the hydrolysates contained both glucose and xylose. Xylose was subsequently utilized after *Pichia* utilized the glucose. In particular, we used the microaeration method, such as surface aeration, to efficiently produce ethanol from xylose. In addition, we have recently developed a carrier to immobilize yeast cells. Interestingly, corncob and cotton show great performance as carriers for immobilization, although they are derivatized with 2-(diethylamino)ethyl chloride hydrochloride (DEAE·HCl) [22, 23].

Thus, in this study, we developed a process using immobilized *Sacchromyces cerevisiae* (*S. cerevisiae*) and *Pichia stipitis* (*P. stipitis*) to produce ethanol from *Ulva pertusa* Kjellman (*U. pertusa* Kjellman) hydrolysate. In addition, the process was designed to completely consume both glucose and xylose to overcome the incomplete utilization of xylose during the ethanol production process. In particular, *S. cerevisiae* and *P. stipitis* were immobilized using DEAE-corncob and DEAE-cotton, respectively. Based on previous reports, we designed a two-step process for ethanol production from *U. pertusa* Kjellman hydrolysates using two immobilized yeasts. The first step was conducted with a continuous column reactor using immobilized *S. cerevisiae*, and the second step was conducted with a repeated-batch reactor using immobilized *P. stipitis*. Then, the performances of these two-step operations were analyzed based on volumetric ethanol productivity and ethanol yield, and were compared with those when immobilized *P. stipitis* was used alone for ethanol production using glucose- and xylose-containing medium.

## Materials and Methods

### Chemicals, *Ulva pertusa* Kjellman Hydrolysate, and Yeasts

DEAE·HCl and dinitrosalicylic acid were purchased from Acros (Rockford, IL, USA) and Lancaster (Lancaster, UK), respectively. Yeast extract and peptone were obtained from Becton Dickinson (Franklin Park, NJ, USA). Industrial-grade yeast extract was obtained from Choheung Chemical Co. (Gyeonggi-Do, Republic of Korea). Corn-steep liquor (CSL) was purchased from Corn Product Korea (Gyeonggi-Do, Republic of Korea). Corncob grits were obtained from corncob chips (JT Co., Seoul, Republic of Korea). All other chemicals were reagent-grade.

The detailed protocol on the hydrolysis of *U. pertusa* Kjellman was described in our previous report [6]. *S. cerevisiae* ATCC 24858 and *P. stipitis* CBS 7125 were used for ethanol production in this study.

### Flask Co-Culture of *S. cerevisiae* and *P. stipitis*

After preparing 100 ml of YPDX medium (10 g/l yeast extract, 20 g/l peptone, 20 g/l glucose, and 10 g/l xylose) in a 250 ml Erlenmeyer flask, *S. cerevisiae* was inoculated and cultivated. Next, after the glucose was consumed completely, *P. stipitis* was subsequently inoculated at 6, 12, and 14 h and then cultivated. The culture was conducted in a shaking incubator at 30°C and 150 rpm.

### Surface-Aerated Fermentor Culture

After preparing 800 ml of YPEX medium (10 g/l yeast extract, 20 g/l peptone, 10 g/l xylose, 7.0 g/l ethanol), *P. stipitis* was inoculated and cultivated in a surface-aerated fermentor, in which the surface aeration rates were 30, 100, 600, and 1,000 ml/min. The surface-aerated fermentor culture was performed in a 2.5 L jar fermentor (KoBiotech, Gyeonggi-Do, Republic of Korea) with an initial working volume of 880 ml. The temperature, pH, and agitation speed were maintained at 30°C, 5.0, and 200 rpm, respectively. The surface aeration protocol used has been described previously [24, 26, 37].

**DEAE·HCl Derivatization**

The derivatization protocol of Ladisch *et al.* (United States Patent 5,808,010) was used. After the corncob grits were delignified according to a method described previously [2], they were soaked in 18% NaOH at 4°C overnight and then derivatized with various concentrations of DEAE·HCl at room temperature for 1 h. The ratio of delignified corncob grits to 0.5 M DEAE·HCl solution was 1:50 (w:v). The DEAE-derivatized corncob grits (DEAE-corncobs) were soaked in distilled water for 1 h, and then the distilled water was removed for cell immobilization. Defatted cotton was also derivatized with 0.5 M DEAE·HCl using the same method as described for the delignified corncob grits. The more detailed protocol for preparing the DEAE-corncob and DEAE-cotton has been described previously [22, 23].

**Immobilization of *S. cerevisiae* on DEAE-Corncob**

Three grams of DEAE-corncobs was added to the column reactor (polypropylene column (1.5 cm ID × 14 cm H; Bio-Rad, Hercules, CA, USA), and then a 10 ml culture of *S. cerevisiae* prepared in YPD medium (10 g/l yeast extract, 20 g/l peptone, and 20 g/l glucose) (optical density (OD) at 600 nm = 3.0) was passed through the column reactor twice [22]. When 6.0, 16.0, and 64.0 g DEAE-corncobs were used for immobilizing *S. cerevisiae*, the volumes of *S. cerevisiae* culture (OD at 600 nm = 3.0) increased proportionally as the amount of DEAE-corncobs increased.

**Immobilization of *P. stipitis* on DEAE-Cotton**

Six grams of DEAE-cotton was placed in a 3.0 L beaker, and 200 ml of *P. stipitis* culture (OD at 600 nm = 7.0) was poured into the beaker. Next, the DEAE-cotton and cell suspension were mixed well. The DEAE-cotton adsorbed by the *P. stipitis* was packed into a glass column (2.5 cm ID × 10 cm H; Bio-Rad), and the remaining cell suspension was passed through the column.

**Flask Culture of Immobilized *P. stipitis***

After *P. stipitis* was immobilized on 3.0 g of DEAE-corncob and 0.4 g of DEAE-cotton [22, 23], it was cultured in 30 ml of YPX medium (10 g/l yeast extract, 20 g/l peptone, 10 g/l xylose) in a 250 ml Erlenmeyer flask on a shaking incubator (30°C and 60 rpm).

**Continuous Column Reactor for the Immobilized *S. cerevisiae***

When 3 and 6 g of DEAE-corncobs were used as the immobilization carrier, a polypropylene column (1.5 cm ID x 14 cm H; Bio-Rad) was used as the column reactor and, if necessary, the height of the column was adjusted by cutting off the upper part. When 16 and 64 g of DEAE-corncob were used as the immobilization carrier, two kinds of glass columns (2.5 cm ID × 10 cm H, and 5.0 cm ID × 10 cm H; Bio-Rad) were used as the column reactors. Prior to continuously supplying medium into the column reactor, 30–120 ml of YPD medium was circulated at a flow rate of 1.0 ml/min in recycling mode, as shown in our previous study [22]. After recycling, the medium for ethanol production was supplied continuously. YPD medium (10 or 20 g/l glucose) was supplied or the following medium (YCD medium) was used: 10 g/l glucose, 20 g/l CSL, 5 g/l yeast extract, 1.2 g/l $(NH_4)_2SO_4$, 2.4 g/l $KH_2PO_4$, and 1.2 g/l $MgSO_4{\cdot}7H_2O$, for the optimization study on medium flow rate. After the medium flow rate was optimized, the *U. pertusa* Kjellman hydrolysate was used as the carbon source for ethanol production. We dissolved some medium ingredients in this hydrolysate for continuous ethanol production in the column reactor. The composition of the medium prepared using the *U. pertusa* Kjellman hydrolysate (YCH medium) was as follows: 20 g/l CSL, 5 g/l yeast extract, 1.2 g/l $(NH_4)_2SO_4$, 2.4 g/l $KH_2PO_4$, and 1.2 g/l $MgSO_4{\cdot}7H_2O$, dissolved in the *U. pertusa* Kjellman hydrolysate. The entire system was operated in a 30°C incubator. We used an industrial-grade yeast extract. In addition, the scale-up of the continuous column reactor was based on the design equation of a packed-bed immobilized cell bioreactor [31]. We first determined the medium flow rate in a polypropylene column on the condition that glucose was consumed completely. The medium flow rates in the larger column reactor were determined based on the following linear relationship between the medium flow rate in the column reactor (F) and the square of the column diameter ($D^2$). We fixed the height of the column reactor as a constant value.

$$F \propto D^2 \quad (H = \text{constant})$$

where F, D, and H are the medium flow rate, diameter, and height in the continuous column reactor, respectively.

**Repeated-Batch Reactor for Immobilized *P. stipitis***

Effluent from the continuous column reactor was used as medium for the repeated-batch reactor. The operation was started by adding 1 L of the first-step effluent and 6.0 g of DEAE-cotton, on which *P. stipitis* was immobilized, into a 3.0 L beaker. Then, 800 ml of the first-step effluent was added and withdrawn repeatedly for each batch. The operation was conducted in a shaking incubator at 30°C and 60 rpm. The beaker was covered tightly with sterile aluminum foil. We operated the repeated-batch reactor three times using YCH medium as a comparative study.

**Electron Microscopy**

Scanning electron microscopy (ESEM, FEI Quanta 400, Hillsboro, OR, USA) observations were made after the yeast cell-immobilized DEAE-corncobs and DEAE-cotton were washed with deionized water and dried for 24 h at 60°C [22].

**Analytical Methods**

Cell growth was assessed by measuring the OD at 600 nm using a spectrophotometer (Spectronic, Thermo Scientific, Rockford, IL, USA). Total reducing sugar was measured using the dinitrosalicylic acid method [4], and ethanol concentration was measured by gas chromatography, as described previously [38]. In addition, glucose and xylose were measured by quantitative thin-layer chromatography (TLC), which has been described previously in detail [24].

**Statistical Analysis**

All experimental data were measured three times using the same sample, and means and standard deviations were calculated, except for the cell growth monitoring OD at 600 nm.

## Results and Discussion

**Co-Culture of *S. cerevisiae* and *P. stipitis***

We investigated whether *S. cerevisiae* and *P. stipitis* co-cultured stepwise in YPDX medium. As shown in Fig. 1A, co-culture of the two yeasts was conducted, in which *S. cerevisiae* was initially cultured, and *P. stipitis* was subsequently cultured at 6, 12, and 24 h. After glucose was completely consumed within 6 h, xylose was gradually consumed in three co-cultures, in which the glucose and xylose were monitored using TLC (data not shown), and the reducing sugar assay was performed (Fig. 1B). All three co-cultures showed that ethanol production increased gradually by about 1 g/l during consumption of about 10 g/l xylose after complete consumption of glucose (Fig. 1C). This result demonstrated that glucose and xylose could be consumed stepwise and completely by *S. cerevisiae* and *P. stipitis*. In particular, *S. cerevisiae* and *P. stipitis* worked in a stepwise manner to produce ethanol. That is, after *S. cerevisiae* consumed the glucose, *P. stipitis* subsequently consumed xylose to produce ethanol. Therefore, we used this result as the experimental basis for the following reactor operation to produce ethanol.

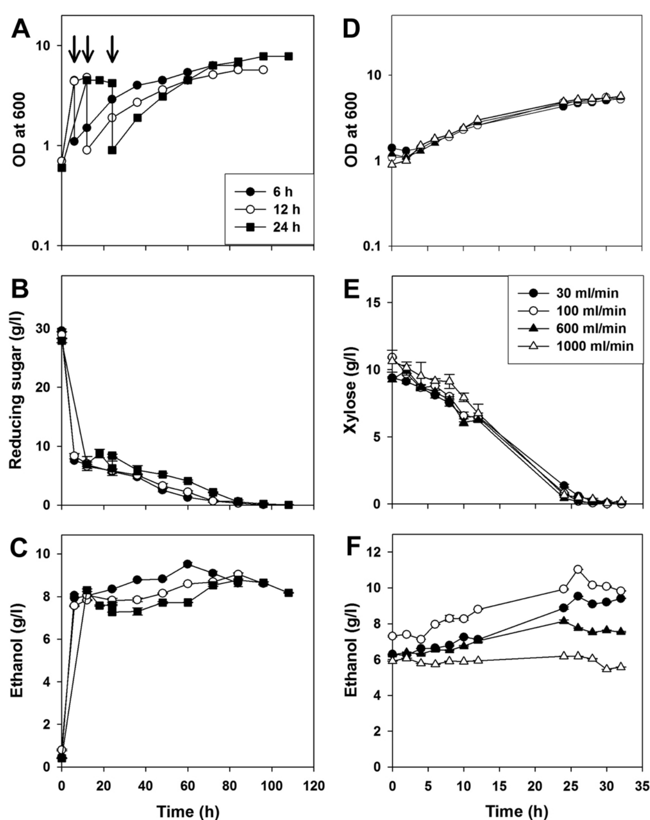


**Fig. 1.** Co-culture of *S. cerevisiae* and *P. stipitis* in YPDX medium in an Erlenmeyer flask (**A**, **B**, **C**). Fermentor culture of *P. stipitis* in YPEX medium (**D**, **E**, **F**).

(**A**) Cell growth of *S. cerevisiae* and *P. stipitis*; (**B**) residual reducing sugar concentration determined by the DNS method; (**C**) ethanol concentration. Arrows in panel A indicate the time (6, 12, and 14 h) when the *P. stipitis* culture was added. Box in panel A shows the symbols indicating the times. (**D**) Cell growth of *P. stipitis*; (**E**) residual xylose concentration determined by the DNS method; (**F**) ethanol concentration. Aeration was controlled using various surface aeration rates (30, 100, 600, and 1,000 ml/min). Box in panel E shows the symbols indicating surface aeration rates.

**Fermentor Culture of *P. stipitis* in Xylose- and Ethanol-Containing Medium**

We investigated ethanol production by *P. stipitis* using YPEX medium (Figs. 1D–1F). This strategy assumed that we might be able use culture broth containing xylose and ethanol, which was supplied from a culture of *S. cerevisiae* using YPDX medium. As shown in Fig. 1E, we found that although about 7 g/l ethanol was added initially to the culture medium, ethanol production increased gradually throughout consumption of about 10 g/l xylose in the surface-aerated fermentor culture. However, the increase in ethanol production depended remarkably on the surface aeration rate. Ethanol production was about 1–2 g/l when the surface aeration rates were 30, 100, and 600 ml/min; however, no increase in ethanol production was observed when the surface aeration rate was 1,000 ml/min (Fig. 1F).

This result demonstrated two important principles. First, if we used culture broth containing xylose and ethanol, prepared from the YPDX medium by *S. cerevisiae*, additional ethanol production was possible from xylose in the YPEX medium. In addition, aeration needed to be finely controlled to produce ethanol from xylose, particularly when xylose and ethanol were simultaneously present. Consequently, Fig. 1 demonstrated that a stepwise ethanol production process from YPDX medium using *S. cerevisiae* and *P. stipitis* was possible, and these results presented the rationale of a two-step process for ethanol production from *U. pertusa* Kjellman hydrolysate.

**Flask Culture of Immobilized *P. stipitis* in Xylose-Containing Medium**

DEAE-corncobs have been verified as a *S. cerevisiae* carrier for ethanol production, in which the yeast was

adsorbed both on the surface and into the pores of corncobs [22]. Ethanol production from glucose in a medium-recycled column reactor was observed using immobilized *S. cerevisiae* on DEAE-corncob. It was necessary to develop an appropriate carrier to immobilize *P. stipitis*. Because finely controlled aeration was needed for ethanol production using xylose by *P. stipitis*, we deduced that a porous carrier would not be appropriate for *P. stipitis*. In other words, we deduced that if *P. stipitis* was entrapped in holes of a porous carrier, oxygen would not be supplied. We decided that *P. stipitis* should be immobilized on the surface of a carrier. As shown in Fig. 2, we investigated whether DEAE-cotton was an appropriate carrier for ethanol production using *P. stipitis* in YPX medium, based on our previous study [23], and DEAE-cotton was compared with DEAE-corncob. In our previous study, we discovered the fibrous structure of cotton with no pores on the surface.

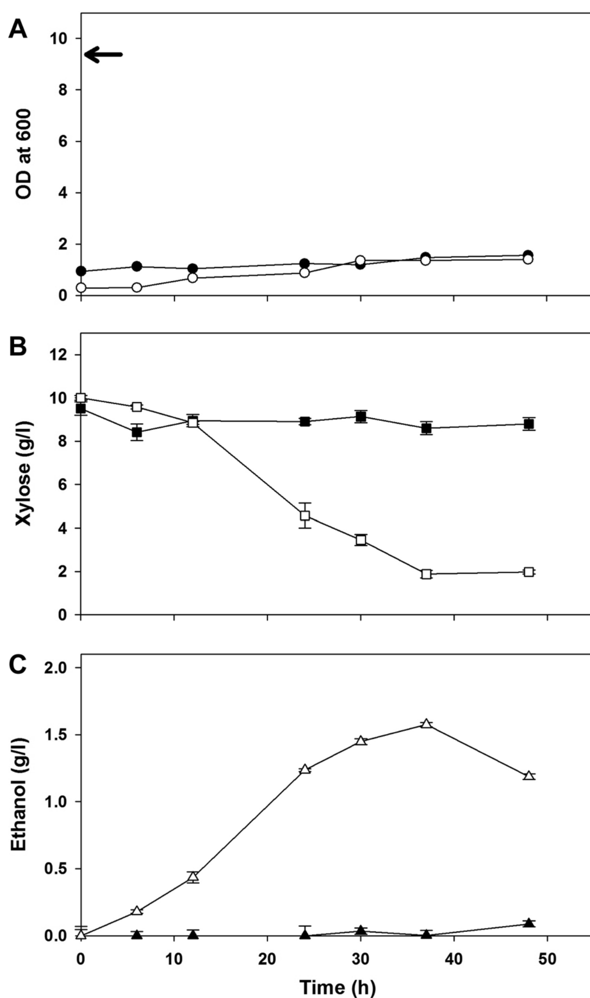


**Fig. 2.** Flask culture of immobilized *P. stipitis* in YPX medium, in which DEAE-corncob (●, ■, ▲) and DEAE-cotton (○, □, △) were used as a carrier for cell immobilization.

(**A**) Cell growth; (**B**) residual xylose concentration determined by the DNS method; (**C**) ethanol concentration. The arrow in panel A indicates the OD of seed culture at 600 nm.

As shown in Fig. 2A, cells were immobilized properly on the two carriers. However, when DEAE-corncobs were used a carrier for *P. stipitis*, only a very small amount of ethanol was produced using xylose (Figs. 2B, 2C). In contrast, when DEAE-cotton was used as the carrier, about 1.5 g/l ethanol was produced through the consumption of about 10 g/l xylose (Figs. 2B, 2C). These results indicate that DEAE-cotton was a more suitable carrier for ethanol production using xylose by *P. stipitis*. It was probable that *P. stipitis* immobilized on DEAE-corncob did not produce ethanol because of limited oxygen, whereas it was adsorbed on the DEAE-cotton fiber and adequate oxygen was available. Thus, we decided to used DEAE-cotton as a carrier to immobilize *P. stipitis*.

**Optimal Conditions for DEAE-Corncobs in a Continuous Column Reactor**

Because we used immobilized *S. cerevisiae* for ethanol production from glucose, optimal conditions were investigated when the continuous column reactor was operated and DEAE-corncob was used as the carrier for immobilization. *S. cerevisiae* was immobilized on 3.0 g of DEAE-corncob in the column reactor and we determined the optimal flow rate of YPD medium for the continuous column reactor (Fig. 3). Cells were successfully immobilized on the DEAE-corncob (Fig. 3A). In addition, when 20 g/l glucose-containing YPD medium was continuously supplied at medium flow rates of 0.22 and 0.4 ml/min, the glucose consumption and ethanol production in the effluent did not change during the latter period of the operation (Figs. 3B, 3C); that is, the operation entered into a steady state. However, the glucose consumption and ethanol production at medium flow rates of 0.8 and 0.95 ml/min were much less than those at 0.22 and 0.4 ml/min. In particular, we were unable to lower the speed of the medium pump because of a mechanical limitation on the pump operation. More than half the amount of glucose in the effluent was consumed at medium flow rates of 0.22 and 0.4 ml/min; therefore, we decided to decrease the glucose concentration in the medium reservoir to completely consume the glucose. As shown in Fig. 3B, when 10 g/l glucose-containing YPD medium was supplied to the continuous column reactor at medium flow rates of 0.25 and 0.475 ml/min, the residual glucose concentration in the effluent decreased remarkably during the latter period of the operation. However, complete consumption

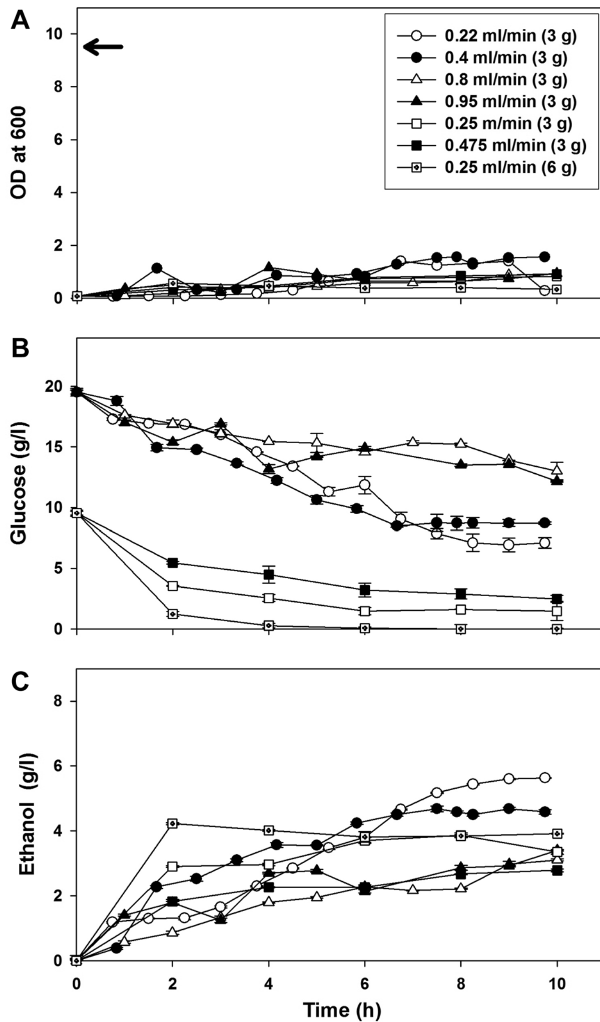


**Fig. 3.** Effects of medium flow rates during continuous column reactor operation, in which immobilized *S. cerevisiae* was packed.
(**A**) Cell concentration, (**B**) residual glucose concentration determined by the DNS method, and (**C**) ethanol concentration in the effluent are shown. DEAE-corncob was used as the carrier for immobilization, and corncobs of 3 and 6 g were used for preparing the column reactor. YPD medium was supplied continuously, in which the glucose concentration was 10 or 20 g/l. The arrow in panel A indicates the OD of the seed culture at 600 nm. The box in panel A shows the symbols indicating the medium flow rates, and the amounts of corncobs packed are in parenthesis.

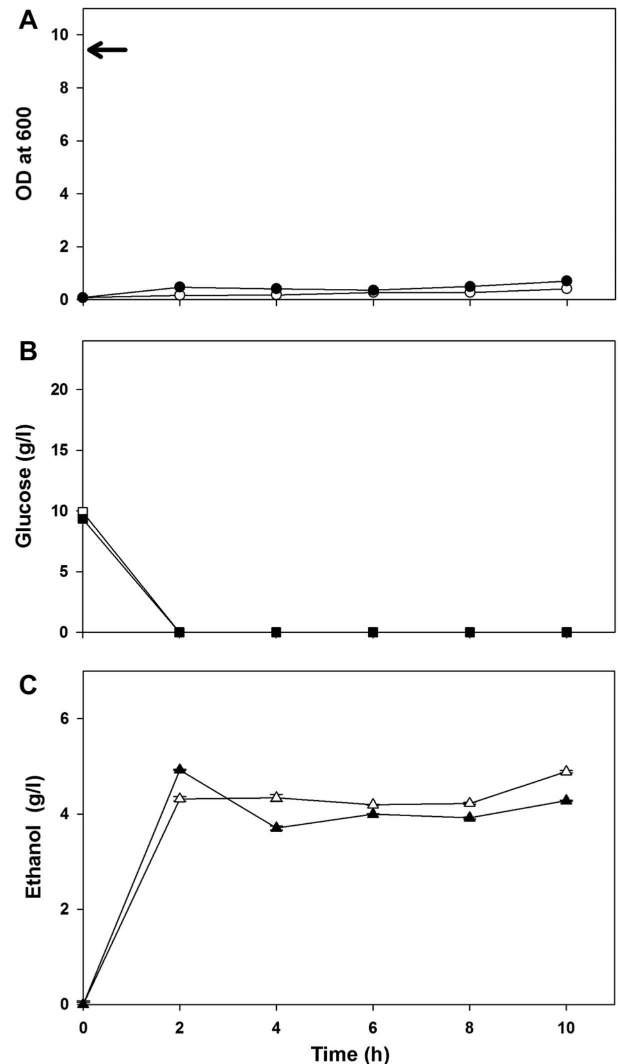


**Fig. 4.** Continuous column reactor of immobilized *S. cerevisiae* in which DEAE-corncobs were was used as the carrier.
(**A**) Cell concentration (○, ●), (**B**) residual glucose concentration determined by the DNS method (□, ■), and (**C**) ethanol concentration (△, ▲) in the effluent are shown. Corncobs of 16 and 64 g were used for preparing the column reactor. YPD medium was supplied continuously, in which the glucose concentration was 10 g/l. White symbols indicate that the medium flow rate and amount of corncobs packed were 0.69 ml/min and 16 g, respectively. Black symbols indicate that the medium flow rate and amount of corncobs packed were 2.55 ml/min and 64 g, respectively. The arrow in panel A indicates the OD of the seed culture at 600 nm.

of glucose was not observed, and more glucose was consumed at a medium flow rate of 0.25 ml/min than that at 0.475 ml/min. Therefore, we increased the amount of DEAE-corncob from 3 to 6 g, without changing the size of the column reactor, and the medium was supplied at 0.25 ml/min. As shown in Figs. 3B and 3C, when 6 g of DEAE-corncob was used for immobilizing *S. cerevisiae* to produce ethanol from 10 g/l glucose-containing YPD medium, the glucose was completely consumed, and about 3.9 g/l ethanol was produced in the effluent. Therefore, we finally decided to use these conditions as optimal conditions for the continuous column reactor using immobilized *S.*

*cerevisiae*. That is, to convert 10 g/l glucose completely to ethanol in the continuous column reactor, we used 6 g of DEAE-corncob to immobilize the *S. cerevisiae*, and the medium flow rate was 0.25 ml/min.

Then, we conducted a scale-up of the continuous column reactor, in which 16 and 64 g of DEAE-corncob were packed into column reactors. As shown in Fig. 4, the column reactors reached a steady state in 2 h. After 2 h, glucose was completely consumed, and about 3.7–4.9 g/l ethanol was produced in the effluent for 10 h (Figs. 4B, 4C). Medium was supplied at flow rates of 0.69 and 2.55 ml/min to the column reactors containing 16 and 64 g DEAE-corncob, respectively.

## Operation of the Continuous Column Reactor Using Immobilized *S. cerevisiae*: First Step

To establish the protocol for industrial application, we operated the continuous column reactor containing 64 g of DEAE-corncob in which *S. cerevisiae* was immobilized. We first verified the performance of the continuous column reactor using YCD medium containing glucose, CSL, and industrial yeast extract, *etc*., in which the medium was supplied at 2.5 ml/min (Figs. 5A–5C). The average ethanol production was about 4.6 g/l (standard deviation = 0.27, $n$ = 11) in the effluent during 240 h of operation. The 10.5 g/l glucose in the feeding medium was completely consumed.

Based on the performance data shown in Fig. 4, we conducted the first-step operation of the continuous column reactor in which *S. cerevisiae* was immobilized on DEAE-corncob and the YCH medium containing the *U. pertusa* Kjellman hydrolysate (glucose = 10.4 g/l, xylose = 3.1 g/l) for ethanol production. During 164 h of operation, the cells were successfully immobilized, and glucose was completely consumed (Figs. 5D, 5E). Ethanol was produced at a average concentration of 4.4 g/l (standard deviation = 0.25, $n$ = 17) in the effluent (Fig. 5F). Xylose was not consumed by *S. cerevisiae* during this operation (Fig. 5E), as the residual xylose concentration in the effluent was about 3.3 g/l. Throughout this operation, the hydrolysate of about 26.6 L flowed through the immobilized column reactor, and the ethanol yield from glucose was about 0.423. Finally, this effluent was transferred to the second step of the operation, in which we produced ethanol from glucose and xylose using immobilized *P. stipitis*.

## Operation of Repeated-Batch Reactor Using Immobilized *P. stipitis*: Second Step

In the previous experiment, we chose DEAE-cotton as the *P. stipitis* carrier. However, during the operation of continuous column reactor, we encountered clogging of the reactor, probably due to the fine fibrous structure of cotton (data not shown). Therefore, we chose a repeated-batch operation as the second step of the operation, because the long-term operation performance of the repeated-batch reactor is eventually similar to that of a continuous reactor; that is, the performance of a repeated-batch reactor reaches a quasi-steady state during long-term operation.

Therefore, we conducted the repeated-batch immobilized reactor operation, in which *P. stipitis* was immobilized on

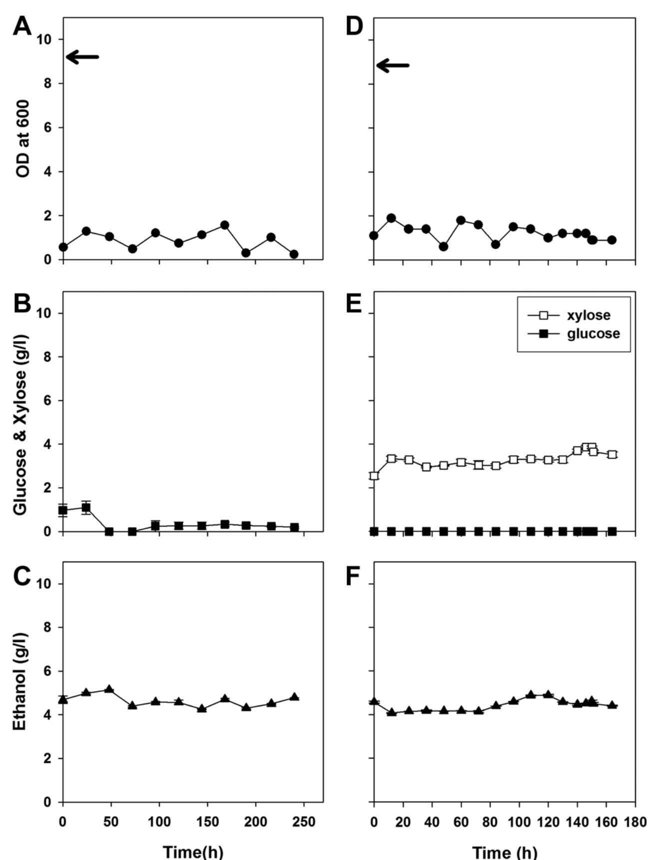


**Fig. 5.** Operation of continuous column reactor packed with immobilized *S. cerevisiae*.

(**A**, **D**) Cell concentration, (**B**, **E**) residual glucose and xylose concentrations determined by TLC, and (**C**, **F**) ethanol concentration in the effluent are shown. DEAE-corncob of 64 g was used as the immobilization carrier. Symbols in the box of panel E indicate glucose and xylose concentrations in panels B and E. In panels A, B, and C, the glucose concentration in the medium reservoir was 10.5 g/l (YCD medium), and the medium flow rate was 2.5 ml/min. In panels D, E, and F (first step), *U. pertusa* Kjellman hydrolysate was used for preparing the YCH medium, in which the glucose and xylose concentrations were 10.4 and 3.1 g/l, respectively. The medium flow rate was 2.7 ml/min. Arrows in panels A and D indicate the OD of the seed culture at 600 nm.

6 g of DEAE-cotton to convert this residual xylose to ethanol, because the effluent from the first step of the operation contained ethanol and xylose. As shown in Fig. 6, *P. stipitis* was successfully immobilized, and 25 repeated-batch runs were accomplished. Throughout this repeated-batch operation, the first-step effluent of 20 L was used to convert residual xylose to ethanol during the 544 h of operation. The average ethanol concentration in the harvested culture broth of each batch in the repeated-batch operation was 5.0 g/l ethanol (standard deviation = 0.24, $n$ = 25), and the ethanol yield from xylose was about 0.33 in the harvested broth. Finally, we established a two-step process for ethanol production from the medium containing the *U. pertusa* Kjellman hydrolysate (total reducing sugar = 13.5 g/l, based on medium from the first step of the operation). In particular, we were able to conduct the two-step process using at least 20 L of the hydrolysate-containing medium, and the ethanol concentration was about 5.0 g/l after the second step of the process. In addition, the overall ethanol yield from glucose and xylose in the hydrolysate-containing medium was 0.37. In other words, we were able to estimate the production of 100 g of ethanol (5.0 g/l × 20 L) from 270 g of reducing sugar (13.5 g/l × 20 L).

We conducted a control experiment for comparison in which the immobilized *P. stipitis* on DEAE-cotton was used for ethanol production from the YCH medium containing

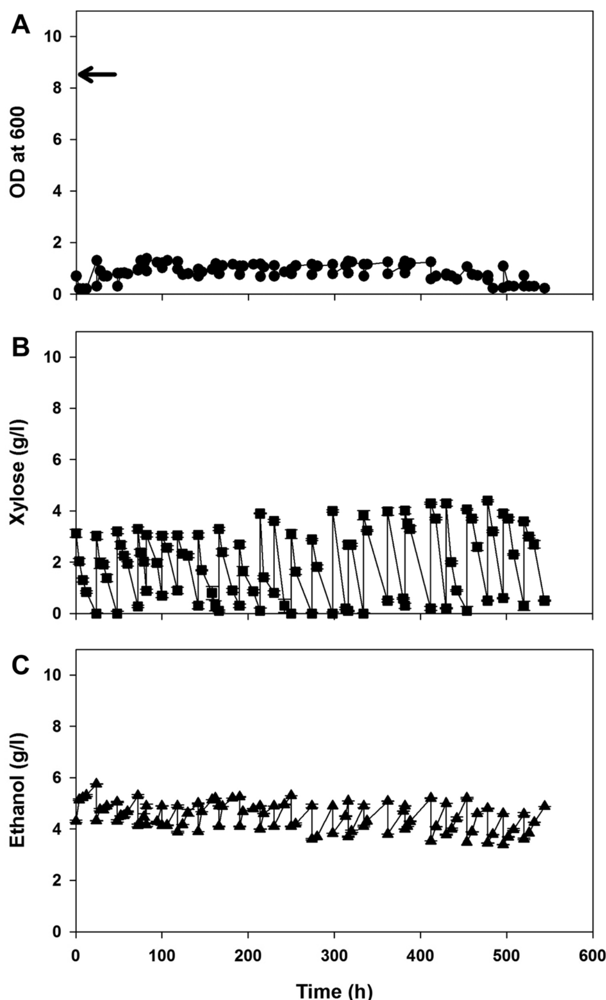


**Fig. 6.** Operation of repeated-batch reactor with immobilized *P. stipitis* (second step).

(**A**) Cell growth; (**B**) residual xylose concentration determined by TLC; (**C**) ethanol concentration. DEAE-cotton of 6 g was used as the immobilization carrier. Effluent from the first step was used for ethanol production. Arrow in panel A indicates OD of seed culture at 600 nm.

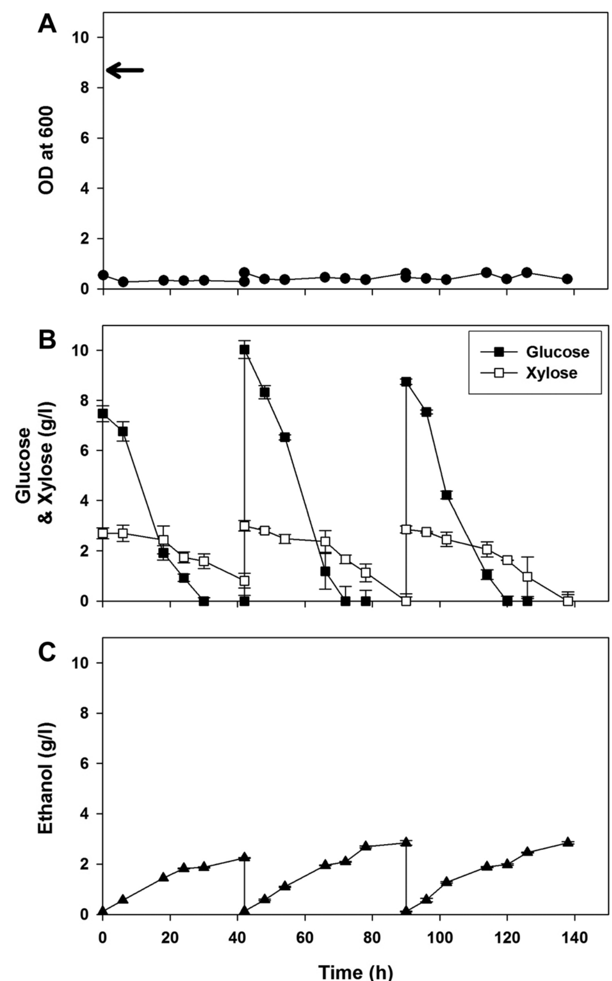


**Fig. 7.** Operation of repeated-batch reactor with immobilized *P. stipitis*.

DEAE-cotton of 6 g was used as the immobilization carrier. *U. pertusa* Kjellman hydrolysate was used for preparing the YCH medium. The arrow in panel A indicates the OD of the seed culture at 600 nm.

the *U. pertusa* Kjellman hydrolysate during the repeated-batch operation (Fig. 7). Because *P. stipitis* can utilize glucose and xylose stepwise for ethanol production, the results from Fig. 7 were compared with those of the above two-step process. Although there were several different operational strategies and conditions between the two processes, the volumetric ethanol productivity of the above two-step process was calculated to be 0.126 g/l/h when we assumed that 20 L of hydrolysate was used for the two-step process (Figs. 5D–5F, and Fig. 6). In contrast, the volumetric ethanol productivity of Fig. 7 was about 0.046 g/l/h during 138 h of operation. That is, the volumetric ethanol productivity of the two-step process was about 2.7 times greater than that shown in Fig. 7. The overall ethanol yield from glucose and xylose in Fig. 7 was about 0.233 during three batch runs, which was lower than that of the two-step process. Consequently, both an increase in volumetric ethanol productivity and ethanol yield was observed during the two-step process; that is, *S. cerevisiae* converted glucose to ethanol in the continuous column reactor as the first step, and *P. stipitis* converted xylose to ethanol in the repeated-batch reactor as the second step. *P. stipitis* converted both glucose and xylose alone in the repeated-batch reactor (Fig. 7). However, we were unable to simultaneously control the optimal conditions for the two carbon sources for ethanol production. The reason was because *P. stipitis* requires microaeration to convert xylose to ethanol, whereas *S. cerevisiae* did not require microaeration to convert glucose to ethanol.

The electron microscopy photographs of the immobilized yeast cells from the first and second steps of the process are shown in Fig. 8. We found that it was possible to increase the amount of *P. stipitis* cells immobilized on DEAE-cotton, whereas *S. cerevisiae* fully occupied the DEAE-corncob sites. In our previous study, we optimized the *P. stipitis* adsorption conditions on DEAE-cotton [23], and we used this immobilization protocol in the second step. Therefore, if we improved the DEAE-cotton immobilization protocol for *P. stipitis* or developed a more efficient immobilization method, we may be able to increase ethanol productivity.

Unfortunately, we were unable to accomplish an integrated one-step continuous process; however, we were able to alternatively and successfully operate the two-step process, using conditions for two yeasts with fairly different physiologies. In addition, the glucose and xylose in the seaweed *U. pertusa* Kjellman hydrolysate were consumed completely for ethanol production. It has been reported frequently that xylose is consumed incompletely in co-culture/co-fermentation systems of two yeasts such as *Saccharomyces*/*Pichia* or *Saccharomyces*/*Candida* [12, 18–21]. However, we were able to overcome this problem using the two-step operation. This two-step process was the first attempt to utilize DEAE-corncobs and DEAE-cotton as carriers for yeast cells to produce ethanol using *U. pertusa* Kjellman hydrolysate. In addition, increases in both volumetric ethanol productivity and ethanol yield were shown throughout the two-step process, compared with those of the process in which immobilized *P. stipitis* was used alone to produce ethanol. In addition, because the volumetric ethanol productivity depended on the sugar concentration in the medium, it is expected that volumetric ethanol productivity will increase more, if we develop

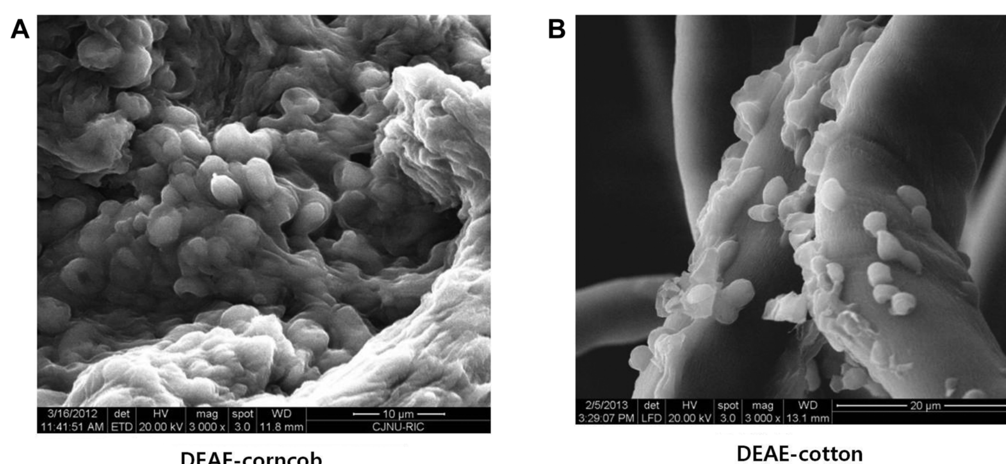


**Fig. 8.** Electron microscopy photographs.

(**A**) *S. cerevisiae*-immobilized DEAE-corncobs (from Figs. 5D, 5E, and 5F); (**B**) *P. stipitis*-immobilized DEAE-cotton (from Fig. 6). Magnification of the electron micrographs: 3,000×. Samples for observation were collected at the time when the first and second steps were finished.

effective hydrolysis methods or another biomass resource containing a high concentration of fermentable carbon source.

Chen [5] reviewed co-culture/co-fermentation using both glucose-utilizable and xylose-utilizable yeasts for ethanol production from glucose- and xylose-containing hydrolysates. Despite the different physiology between the two yeasts, many show batch operation of co-culture and co-fermentation. In contrast, there are few reports involving continuous operation using immobilized yeast. In addition, alginate [12] and agar [19–21] are rarely used for yeast immobilization; that is, various carriers for yeast immobilization have not been investigated and are not available for practical application. Nevertheless, it is expected that this two-step process will not only contribute to the development of an integrated process for ethanol production from glucose- and xylose-containing hydrolysates but could also be used as an alternative choice for ethanol production, such as the many processes introduced by Chen [5].

We developed a two-step process for ethanol production from the hydrolysate of *U. pertusa* Kjellman (containing glucose and xylose), in which immobilized *S. cerevisiae* on DEAE-corncob was used for ethanol production from glucose in the first step and immobilized *P. stipitis* on DEAE-cotton was used for ethanol production from xylose in the second step. The first step was operated in a continuous column reactor and the second step was conducted in a repeated-batch reactor, in which the operation completely consumed the glucose and xylose. It was verified that the glucose and xylose, in 20 L of medium that contained the *U. pertusa* Kjellman hydrolysate, was able to be converted to about 5.0 g/l ethanol throughout the two-step process, in which the overall ethanol yield was 0.37 and the volumetric ethanol productivity was 0.126 g/l/h. These values are remarkably better than those obtained when *P. stipitis* is used alone for ethanol production from a *U. pertusa* Kjellman hydrolysate.

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