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Bioethanol Production from the Hydrolysate of Rape Stem in a Surface-Aerated Fermentor

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In this study, we investigated the feasibility of producing bioethanol from the hydrolysate of rape stem. Specifically, the most ideal yeast strain was screened, and the microaeration was performed by surface aeration on a liquid medium surface. Among the yeast strains examined, *Pichia stipitis* CBS 7126 displayed the best performance in bioethanol production during the surface-aerated fermentor culture. *Pichia stipitis* CBS 7126 produced maximally 9.56 g/l of bioethanol from the initial total reducing sugars (about 28 g/l). The bioethanol yield was 0.397 (by the DNS method). Furthermore, this controlled surface aeration method holds promise for use in the bioethanol production from the xylose-containing lignocellulosic hydrolysate of biomass.

Keywords: Surface aeration, hydrolysate of rape stem, bioethanol, *Pichia stipitis*

Production of bioethanol and biodiesel has received a great deal of attention by the energy industry owing to their potential use as alternatives to fossil fuels [7, 35]. In particular, there has been enormous interest in using lignocellulosic material from agricultural by-products as a C-source in the production of bioethanol [6, 16, 34]. Among these by-products, the rape stem is a waste from the production of rapeseed oil, which is a resource for biodiesel production [11, 12, 33]. Therefore, we hypothesized that if this rape stem is used as biomass for bioethanol production, the energy conversion efficiency of rape stem to bioenergy (*i.e.*, biodiesel and bioethanol) will be remarkably increased. There are currently three major bottlenecks in the production of bioethanol from agricultural by-products, which include preparation of the hydrolysis of lignocellulosic material, a microbial system that can utilize both glucose and xylose, and a bioreactor system with finely controllable aeration equipment.

Because xylose utilization in yeast is carried out through the pentose phosphate pathway, which enters this pathway *via* xylitol and xylulose, the reducing power is supplied from the tricarboxylic acid cycle. Therefore, fine-tuning and controlling aeration during fermentation are essential for maximal bioethanol production using xylose-containing lignocelluloses [3, 10, 21]. In this study, to achieve a small amount of air supply, surface aeration was carried out to control the aeration rate at the microaeration level. Surface aeration has been extensively studied theoretically in reactor systems [18, 19, 26, 32]. In particular, studies using bioreactor systems have been widely reported [9, 15, 17, 22, 36]. In addition, the hydrolysate of the agricultural by-product (*i.e.*, the hydrolysate of rape stem) was produced by a two-step, high temperature, pretreatment process [8, 24]. The best yeast strain was screened from yeast stocks available in domestic yeast stock organizations. Finally, this study was performed to investigate the feasibility of producing bioethanol from rape stem, in which the fermentor was equipped with a surface aeration system.

The yeast strains used in this study are as follows; *Pachysolen tannophilus* ATCC 32691, *Candida shehatae* ATCC 58779, *Pichia stipitis* ATCC 58376, *Pichia stipitis* ATCC 58784, *Pichia stipitis* ATCC 58784, and *Pichia stipitis* CBS 7126, respectively. These strains are abbreviated as 32691, 58779, 58376, 58784, 58784, and 7126, respectively.

Yeast extract and peptone were purchased from Becton Dickinson and Co. (Sparks, MD, USA). Glucose, xylose, *n*-butanol, acetonitrile, and sulfuric acid were from Daejung

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Chemical Co. (Shiheung, Gyeonggi-Do, Republic of Korea). Phosphoric acid and ammonia water were from Wako Chemical Co. (Osaka, Japan) and Junsei Chemical Co. (Tokyo, Japan), respectively. Dinitrosalicylic acid and helium gas were from Samchun Chemical Co. (Seoul, Republic of Korea) and Daesung Gas Co. (Seoul, Republic of Korea), respectively. α-Naphtol was from Sigma-Aldrich (St. Louis, MO, USA).

The aforementioned two-step pretreatment process using a high temperature liquefying system (Ilshin, Republic of Korea) was carried out to efficiently hydrolyze the rape stem [8, 24].

The seed culture for the shaking bottle culture was performed in a 250-ml Erlenmeyer flask with 50 ml of YPD medium (yeast extract, 10 g/l; peptone, 20 g/l; glucose; 20 g/l) in a shaking incubator at 30°C and 150 rpm. The shaking bottle culture was started in a 250 ml media bottle (Schott Duran) containing 50 ml of YDX medium (yeast extract, 10 g/l; glucose, 5 g/l; xylose, 20 g/l) in a shaking incubator at 30°C and 150 rpm. The inoculation volume was 2% (v/v) of the volume of the shaking bottle culture and, instead of using a screw cap, the media bottle mouth was stopped by a silicone plug. After glucose was completely consumed, the silicone plug was exchanged with a screw cap and then the media bottle mouth was tightly capped by the screw cap. At this time, the agitation speed of the shaking incubator was reduced to 80 rpm.

The medium for bioethanol production, yeast extract (10 g/l) and peptone (20 g/l), were dissolved in a 3-fold concentrated hydrolysate of rape stem. The fermentor culture was conducted in a 2.5-l jar fermentor (KoBiotech, Republic of Korea) with a working volume of 800 ml. During fermentation, the temperature, pH, and agitation speed were maintained at 30°C, 5.0, and 200 rpm, respectively. The pH was maintained by adding a phosphoric acid solution [10% (v/v)] or ammonia water, as necessary. For finely controlled aeration, the surface aeration was carried out using silicon tubing (inside diameter=3.1 mm). Tubing (L=6.7 cm) was inserted from the head plate into the inner headspace of the fermentor vessel (inside diameter=14.2 cm). When agitation was not carried out, the distance from the surface of the medium to the end of the tubing was approximately 7 cm. The air supply rate was finely controlled through an air-flow meter (Cole-Parmer, USA).

Cell growth was monitored by measuring the optical density at 600 nm using a spectrophotometer (Spectronic, Thermo Scientific, USA). The residual reducing sugar and ethanol concentration in the culture broth were measured using the dinitrosalicylic acid (DNS) method [2] and gas chromatography (8610C; SRI, USA), respectively [30]. The monosaccharide was analyzed *via* thin-layer chromatography (TLC) using a 20×10 cm Partisil K5F (Whatman) as a TLC plate and acetonitrile solution [acetonitrile:water=85:15 (v/v)] as the mobile phase with a sample loading volume of 1.0 μl. Specifically, the amounts of glucose and xylose in the yeast culture broth were quantitatively determined *via* TLC, based on the rationale for quantitative determinations of monosaccharides [27]. The oxygen transfer rate coefficient (k_La) was measured by the unsteady-state method [31], in which 800 ml of distilled water was filled in the fermentor, which was equipped with a dissolved oxygen probe (Mettler-Toledo).

As presented in Fig. 1, five yeast strains, which possess the ability to utilize glucose and xylose, were evaluated in a shaking bottle culture. Cell growth of strains 58376,

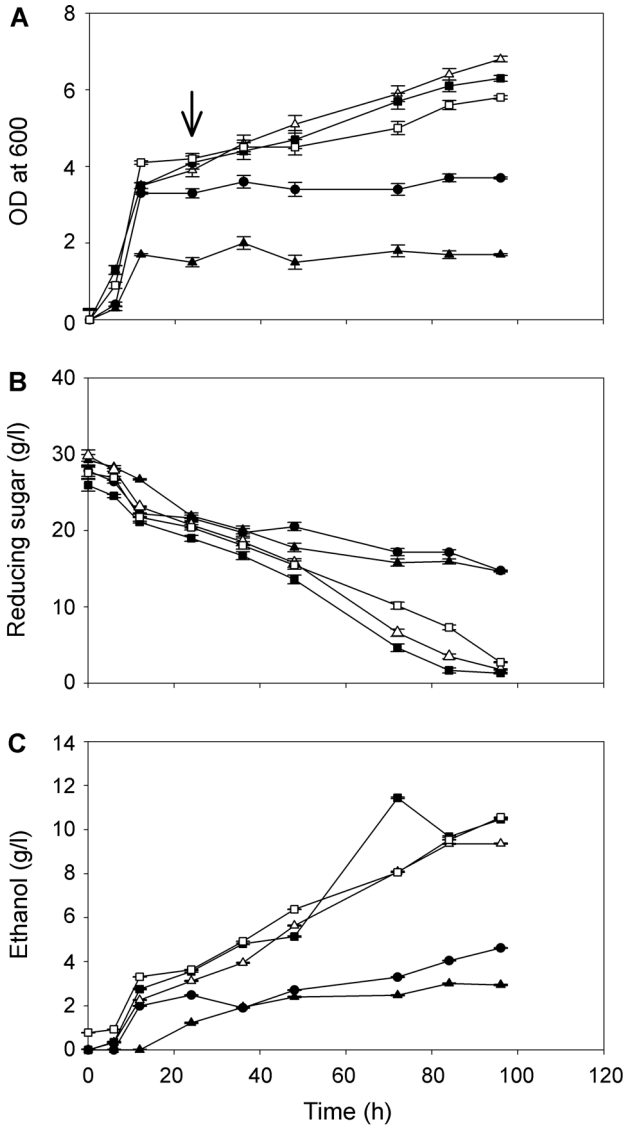

Fig. 1. Bottle cultures for the selection of yeast strain, in which xylose and glucose were used as carbon sources.
(**A**) Cell growth, (**B**) residual reducing sugar consumption, and (**C**) ethanol production. The yeast strains used in this study are described in panel **B**. The arrow indicates the time at which the screw cap was completely closed. All measurements were performed three times (n=3) using the same sample, and the average and standard deviation were calculated.

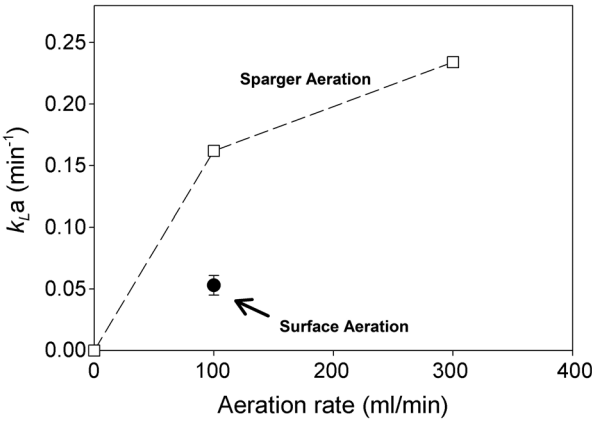

Fig. 2. The oxygen transfer rate coefficient (k_La) when surface aeration (closed circle) and sparger aeration (open square) were carried out.

Data of sparger aeration were taken from our previous work [30]. The measurement of k_La when surface aeration was carried out was performed three times (n=3), and the average and standard deviation were calculated.

58784, and 7125 were superior to those of strains 32691 and 58779. Ethanol production by these three strains ranged from 9–11 g/l, in which the total reducing sugar (glucose and xylose) was completely consumed. Therefore, strains 58376, 58784, and 7126 were selected as candidates for bioethanol production from the hydrolysate of rape stem in a fermentor culture.

Microaeration is essential for ethanol production from xylose [3, 10, 21]; that is, a small amount of air should be supplied in a finely controlled manner, regardless of the culture system. We determined that if air was not supplied, xylose was not consumed in the fermentor culture (data not shown). Next, we supplied air to only the medium surface of the fermentor culture, in which the silicone tubing was inserted through the head plate of the fermentor vessel. In these experiments, a surface air supply rate of 100 ml/min was selected because this value was the lowest among the controllable values. This air supply rate resulted in an oxygen transfer coefficient (k_La) of 0.053 min^{-1} (Fig. 2). This k_La corresponds to a fairly small value when compared with those found in flask culture. In the shaking flask culture, it is known that k_La ranges from 0.4 to 3.3 min^{-1} [1].

As shown in Fig. 3, the strains 58376, 7126, and 58784 were cultured for bioethanol production from the hydrolysate of rape stem, in which the surface aeration was carried out at 100 ml/min. Cell growth and bioethanol production of strain 7126 were superior to those of strains 58376 and 58784 (Fig. 3A, 3C, 3D, 3F, 3G, and 3I), in which the initial total reducing sugars were about 28 g/l in all three cultures (Fig. 3B, 3E, and 3H). In particular, strain 7126 produced a maximum of 9.56 g/l of bioethanol (Fig. 3F). In constrast, strains 58376 and 58784 only produced a maximum of 6.69 g/l and 4.89 g/l, respectively (Fig. 3C and 3I). Furthermore, strain 7126 produced the greatest amount of bioethanol during xylose utilization from 24 h of elapsed time to the last sampling time. In the hydrolysate of rape stem, it has been previously shown that glucose and xylose were the major monosaccharides [8]. In this study, glucose and xylose were also shown in the TLC analysis to be the major monosaccharides and these two monosaccharides were consumed completely (Fig. 4). However, as depicted in Fig. 3B, 3E, and 3H, the residual reducing sugar may not have been completely consumed. This may have occurred because unknown substances affected the DNS assay. At the elapsed time of 24 h, glucose was consumed completely, and at longer time periods, xylose was consumed (Fig. 3B, 3E, 3H, and 4). At the time of glucose consumption, xylose consumption was repressed by glucose. This phenomenon has been reported in previous studies [25].

Finally, similar to the bioethanol production profiles in Fig. 3, the bioethanol yield of strain 7126 was superior to those of strains 58376 and 58784. The bioethanol yields (*Ye*/s(d) and *Ye*/s(t)) of strain 7126 were calculated to be approximately 78% and 72%, respectively, of the theoretical value (*Ye*/s(d)=0.397±1.9E-4 and *Ye*/s(t)=0.368±1.7E-4). In addition, *Ye*/g(t) and *Ye*/x(t) were 0.45±1.1E-4 and 0.281±3.0E-4, respectively. These were approximately 88% and 55%, respectively, of the theoretical value. In the case of bioethanol production from xylose, the concurrent consumption of ethanol in the cultivation of yeast *Pachysolen tannophilus* has been reported [23]. Therefore, it was deduced that this phenomenon was the reason why bioethanol conversion was about half the theoretical value. In addition, microaeration was not optimized in this study; that is the surface aeration rate (100 ml/min) used in this study was not optimal. Previous studies reported that the *Ye*/x values were 0.28, 0.41, and 0.29–0.49, when using recombinant xylose-utilizing *Saccharomyces cerevisiae* [13, 14, 29], 0.28 when using *Candida shehatae* [5], and 0.42 when using *Pichia stipitis* [20]. The controlled microaeration rates were reported to vary from 0.01–0.1 vvm for *Pichia stipitis* [4], 0.075–0.3 vvm for *Candida shehatae* [28], 0.05 vvm for *Pichia stipitis* [20], and 0.2 vvm for recombinant xylose-utilizing *Saccharomyces cerevisiae* [14]. These reports suggest that microaeration is a tedious variable to control for bioethanol production, and thereby microaeration should be established according to the yeast strains and culture conditions.

Although this study was not carried out under optimal microaerations conditions, we demonstrated that the bioethanol production from the hydrolysate of rape stem is feasible and that microaeration can be successfully achieved by surface aeration. There have only been a limited number of studies that have examined this phenomenon, and based on the results of this study, we can conclude that both bioethanol production from the hyrolysate of rape

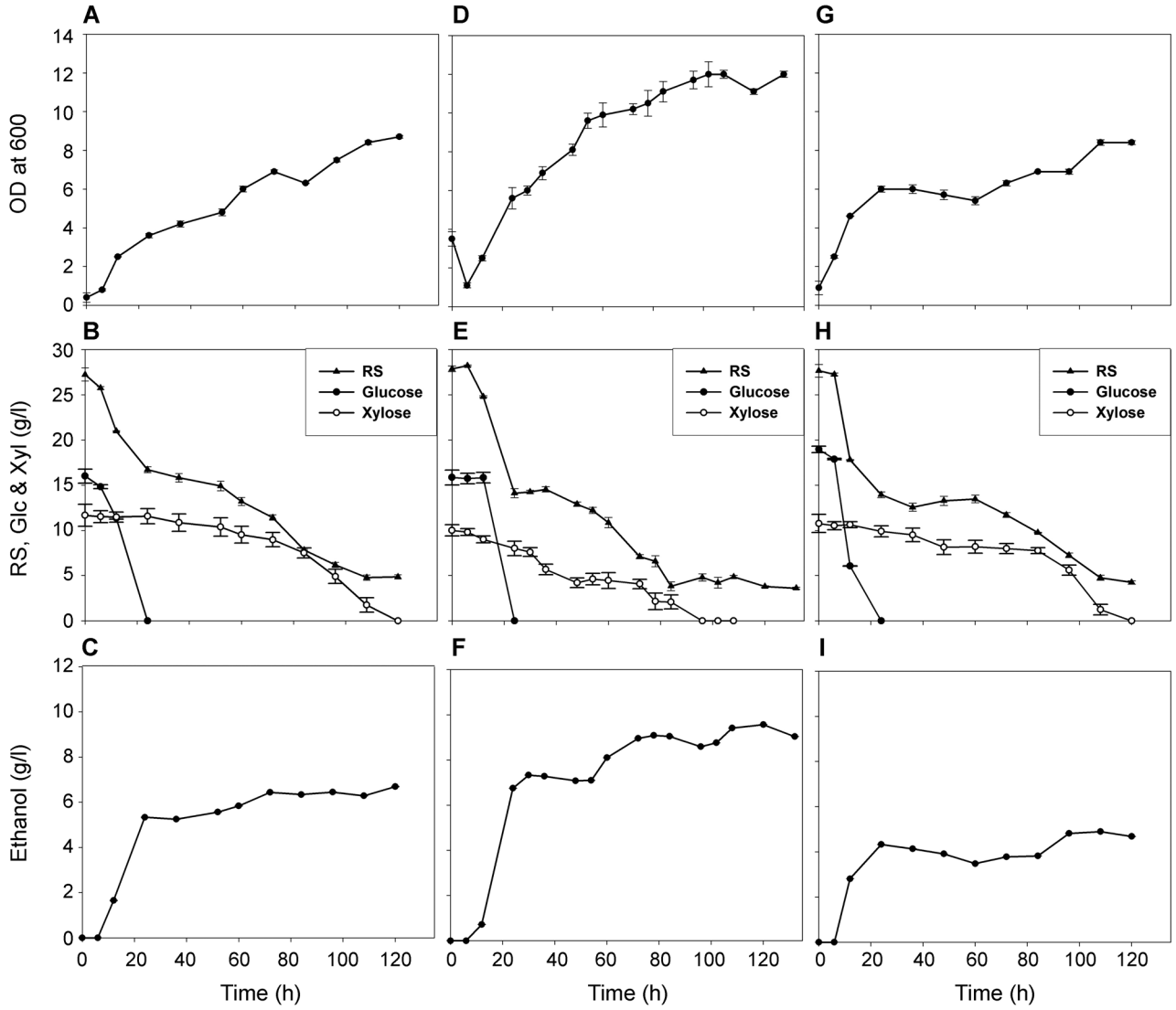

Fig. 3. Fermentor cultures of strains 58376 (**A**, **B**, **C**), 7126 (**D**, **E**, **F**), and 58784 (**G**, **H**, **I**), in which the hydrolysate of rape stem was used as a carbon source.
(**A**, **D**, **G**) Cell growth, (**B**, **E**, **H**) residual reducing sugar (RS), glucose (Glc), and xylose (Xyl) consumptions, and (**C**, **F**, **I**) bioethanol production. All measurements were performed three times (n=3) using the same sample, and the average and standard deviation were calculated.

stem and microareation are possible. In future studies, it will be necessary to optimize the surface aeration rate, which could be applied to larger scale fermentors. In addition, this controlled surface aeration method holds promise for use in other bioprocesses that require microaeration, as well as bioethanol production from the xylose-containing lignocellulosic hydrolysate of biomass.

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Nomenclature

k_La	Oxygen transfer rate coefficient (min^{-1})
Ye/s(d)	Bioethanol yield based on total reducing sugars measured by the DNS method (g/g)
Ye/s(t)	Bioethanol yield based on total monosaccharides measured by quantitative TLC (g/g)
Ye/g(t)	Bioethanol yield based on glucose measured by quantitative TLC (g/g)

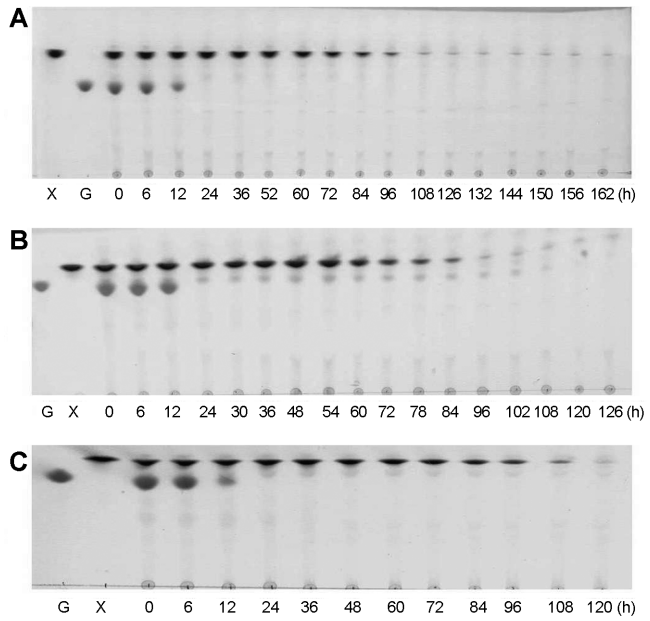

Fig. 4. TLC of the culture supernatant from Fig. 3.
(**A**) Strain 58376, (**B**) strain 7126, and (**C**) strain 58784. G and X indicate xylose and glucose, respectively, as the standards.

Ye/x(t)	Bioethanol yield based on xylose measured by quantitative TLC (g/g)
Ye/x	Ethanol yield based on xylose (g/g)
vvm	Air supply rate (l/l/min)

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