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Ethanol production by co-fermentation of hexose and pentose from food wastes using *Saccharomyces coreanus* and *Pichia stipitis*

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Abstract–To improve the conversion rate of a saccharification liquid from food wastes containing pentoses and hexoses into bioethanol, after selecting *Saccharomyces coreanus* and *Pichia stipitis*, the respective fermentation and co-fermentation properties were investigated. In the fermentation using *S. coreanus*, the result under anaerobic condition was better than under aerobic conditions. In the anaerobic fermentation, the concentration of the reducing sugar and glucose remaining after 24 hrs was 9.09 and 1.88 g/L, respectively, with 40.59 g/L of ethanol produced; the ethanol productivity was 1.69 g/L-h. Also, even with the fermentation using *P. stipitis*, the reducing sugars and glucose were rapidly reduced, with a marked production of ethanol, but the ethanol produced was lower than those under anaerobic and aerobic conditions with the use of *S. coreanus*. Therefore, for the production of a high concentration of bioethanol from food wastes, ethanol fermentation was induced using *S. coreanus* until the middle of the fermentation, with *P. stipitis* used during the latter stage of the fermentation, where the circumstance favored its use, and thus, the carbon source not converted by *S. coreanus* was later converted to ethanol. As a result, both ethanol production of 48.63 g/L and productivity of 2.03 g/L-h increased over those of the anaerobic fermentation using *S. coreanus*.

Key words: Bioethanol, Food Wastes, Hexose, Pentose, Co-fermentation

INTRODUCTION

In South Korea, interest has recently focused on food wastes as a concentrated source for energy regeneration, as it can be converted to bioethanol [1]. The production of bioethanol from food wastes may solve the problems of the lack, and high costs, of raw materials. The studies that have been conducted to date in relation to the production of bioethanol from food wastes have reported that food wastes when saccharified using an enzyme, contained 112-131 g/L of reducing sugar, of which glucose constituted 71-92 g/L. As much as 32-46 g/L of ethanol was produced with 24 hrs by fermenting the saccharification liquid under anaerobic conditions using *Saccharomyces coreanus* [2-4]. This concentration range would satisfy the minimum ethanol concentration of 4.5% (about 36 g/L) for the economical production of ethanol for use as a fuel using the adsorption-recovery process [5]. The saccharification liquid of the food wastes consisted of a mixture of sugars, including glucose. Although the distribution of sugars within the saccharification liquid differs depending on the composition of the food wastes, glucose normally constitutes 61-72% of the reducing sugar [2-4]. In view of the characteristics of the food wastes in South Korea, if cellulose-based substrates, such as fruits and vegetables, are effectively saccharified and fermented, a high concentration of ethanol can be obtained. However, pentose, such as xylose, in addition to glucose are contained in cellulose-based substrates, and cannot be converted to ethanol by *Saccharomyces*–based yeasts, which have traditionally been used in fermentation for the production of ethanol [6]. Although most bacteria, fungi and yeast can assimilate xylose, bacteria and fungi produce undesirable products or have a weak resistance to ethanol. Jefferies et al. [7] reported that yeast is most preferable as the microorganism for the conversions of pentose and hexose to ethanol. Among yeasts, *Pichia stipitis* [8,9], *Candida shehatae* [10] and *Pachysolen tannophilus* [11,12], etc. can directly ferment xylose to ethanol, but the reported rates of fermentation and yields of ethanol are generally very low. To solve these problems, recent studies are on-going using a new strain developed by a genetic engineering technique and conducting co-fermentation using two yeasts. Co-fermentation presents difficulties with respect to which microorganisms to use, as they will have different growth conditions. Among these difficulties, a diauxic phenomenon is also problematic, where the xylose-fermenting microorganism does not use xylose in the presence of glucose. Laplace et al. [13] co-cultured *S. cerevisiae* and *C. shehatae*, and obtained a 100% conversion rate of hydrolyzed glucose and a 27% conversion rate of xylose from lignocellulosic material using co-cultured yeasts. They also reported 100 and 69% conversion rates of glucose and xylose, respectively, via co-fermentation with a mutant of *S. cerevisiae* and *P. stipitis*. Grootjen et al. [14] conducted co-fermentation with a mixture of glucose and xylose using series reactors, and almost all of the substrates were converted, but with a low ethanol productivity of 0.51 g/L-h due to the long fermentation time. Lebeau et al. [15] simultaneously fermented a mixture of glucose and xylose through the co-immobilization of *S. cerevisiae* and *C. shehatae* using a two-chambered bioreactor, but the efficiency of the fermentation was no higher than that of the pure cultivation.

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Our aim was to improve the conversion rate of the saccharification liquid of food wastes, containing a mixture of pentose and hexose, to bioethanol using *S. coreanus*, which is generally used in the fermentation of glucose, and *P. stipitis*, which is used in the fermentation of pentose, such as xylose. Initially, the respective fermentation properties of *S. coreanus* and *P. stipitis*, which have different growth conditions, were investigated, with the co-fermentation property depending on the mixing ratio of the two yeasts also examined. Finally, the results from this study could be used to help formulate a process for high efficiency ethanol production.

EXPERIMENTAL SECTION

1. Microorganism and Culture Media

For the production of ethanol in this study, *S. coreanus* produced at S-Development Institute was used, which is known as the yeast of the turbid rice-wine (a kind of Korean traditional wine). Lodder [16] classified *S. coreanus* as an independent species, but Barnett et al. [17] described it as a *S. cerevisiae var*, *coreanus* and thus classified it as a mutant of *S. cerevisiae*. The optimum temperature of *S. coreanus*, heat-resistant yeast broadly growing up to 37 °C, is 30 °C. The optimal growing pH is 5.5, but it can grow over the broad pH range of pH 3.0-7.0 [18].

P. stipitis NRRL Y-7124 (CBS 5773) was obtained from the Korean Culture Center of Microorganisms. Stock cultures were streaked onto YM solid medium, cultured for 48 hrs at 30 °C, and then stored at 4 °C. For pre-cultivation, 50 mL of YM liquid medium was placed into a trypsinizing flask, infused with a loop full of cells and used in the experiment. *P. stipitis* provides the optimal biomass and ethanol productivity within the range 26-35 °C, and with glucose as the carbon source, the optimal temperature is 34 °C, but when xylose is used, the optimal temperature is 25-26 °C and the optimal pH range is 4.0-7.0 [19].

2. Production Media, Saccharification and Fermentation

The food wastes were used by taking grain, vegetables, fruits and meats, including fish, which were milled with a home mixer, and mixed in the ratio of 7 : 9 : 2 : 2, respectively [4]. These food wastes were taken from a cafeteria of M-Maritime University in Korea within the day discarded. The food wastes provide good conditions for diverse and various microorganisms to thrive. Therefore, if suitable sterilization is not performed as a pre-treatment, a high rate of the saccharification will not be obtained. Pre-treatment was performed as a matter of course to allow easy hydrolysis and sterilization, which was conducted for 15 min at 121 °C. From the results of a previous study, the saccharification was undertaken for 8 hrs at 55 °C and 100 rpm, with a 0.01 mL/g-FW (wet basis) of Spirizyme Plus FG and Viscozyme L, respectively [2]. For enzymes to produce hydrolyzate from food waste, Spirizyme Plus FG and Viscozyme L are currently sold in the market. Spirizyme Plus FG is 1, 4-α-D-glucan-glucohydrolase derived from *Aspergillus niger*, a kind of enzyme that breaks down and saccharizes 1, 4- and 1, 6-α bonds in the liquefied substrates containing starch. Its optimal activity is in the pH range of 4.2 and 4.5, and its optimal temperature is between 60 and 63 °C. Viscozyme L is a multi-complex enzyme derived from *Aspergillus aculeatus*, a kind of carbohydrase in broader range including arabanase, cellulase, β-glucanase, hemicellulase, and xylanase, and its optimal activity is in pH range of 3.3 and 5.5, and its optimal temperature is between 25 and 55 °C. This is an enzyme mainly used to break down plant cell walls in order to extract useful substances from plants.

All fermentation was performed by using a serum bottle. The fermentation using *S. coreanus* was conducted for 24 hrs at 30 °C and 100 rpm by infusing 0.2% (w/w) based on the reducing sugar in the saccharification liquid [2]. The anaerobic fermentation was performed by sucking air with a pump prior to the fermentation using a vial stopper, with the carbon dioxide produced during the fermentation process removed by attaching a needle to a stopper 8 hrs after the fermentation [2]. In the aerobic fermentation, a sili stopper was used, but the carbon dioxide was not separately removed.

Fermentation experiments using *P. stipitis* were performed at 30 °C and 100 rpm. The fermentation experiments using the two yeasts were performed for 24 hrs at 30 °C and 100 rpm by infusing 0.2% (w/w) of *S. coreanus* based on the reducing sugar in the saccharification liquid, with *P. stipitis* in the range 0.5-10% (v/v) based on the saccharification liquid. The aerobic state was maintained by using a sili stopper. To investigate the fermentation when *P. stipitis* was infused in an extraordinary amount, the experiment was performed, even under the anaerobic condition, after infusing 20% (v/v) of *P. stipitis* based on the saccharification liquid.

3. Analytical Methods

The amount of reducing sugar was determined by the DNS method [20] and the glucose analyzed via the reagent prepared for this measurement by A Pharmaceuticals, Inc. Qualitative and quantitative analyses of the sugar were performed with HPLC (Agilent 1200 series). A portion of the sugar was taken, filtered through a 0.45 μm membrane filter, and the acetic acid and ethanol analyzed by gas chromatography (YL 6100) with an FID. The items included in the analyses were as hexose, glucose, fructose, mannose and galactose; as pentose, ribose, xylose and arabinose; as disaccharides, maltose, lactose and sucrose. The conditions for the HPLC analyses were as follows: a Zorbax carbohydrate column (150 mm×4.6 mm×5 μm), with water/acetonitrile at a 20/80 ratio at a flow rate of 1 mL/min, used as the mobile phase, and ELSD as the detector, operated at a temperature of 40 °C and a pressure of 3.6 bar. The conditions for the GC-FID analyses were: a Nukol (30 m×0.25 mm×0.25 μm) column, with the oven temperature ramped from 110 to 185 °C at 8 °C/min. The temperatures of the injection port and detector were 185 and 220 °C, respectively. Argon gas was used as the carrier at a flow rate of 1 mL/min. The yeast biomass was determined by its OD value (OD 600) at 600 nm by using a UV-spectrophotometer.

RESULTS AND DISCUSSION

1. Fermentation Property of Ethanol by *S. coreanus*

The anaerobic and aerobic fermentation properties of ethanol produced via *S. coreanus* were investigated by using the saccharification liquid of the food wastes. The initial reducing sugar and glucose in the saccharification liquid were 107.53 and 79.12 g/L, respectively. The amounts of sugar remaining and ethanol produced after the fermentation are shown in Fig. 1. All the anaerobic and aerobic fermentations caused a rapid decrease of the reducing sugar and increase of the ethanol production, and thereafter showed a moderate decrease of the reducing sugar. After 24 hrs of the anaerobic fermentation, the reducing sugar and glucose remaining were 9.09 and

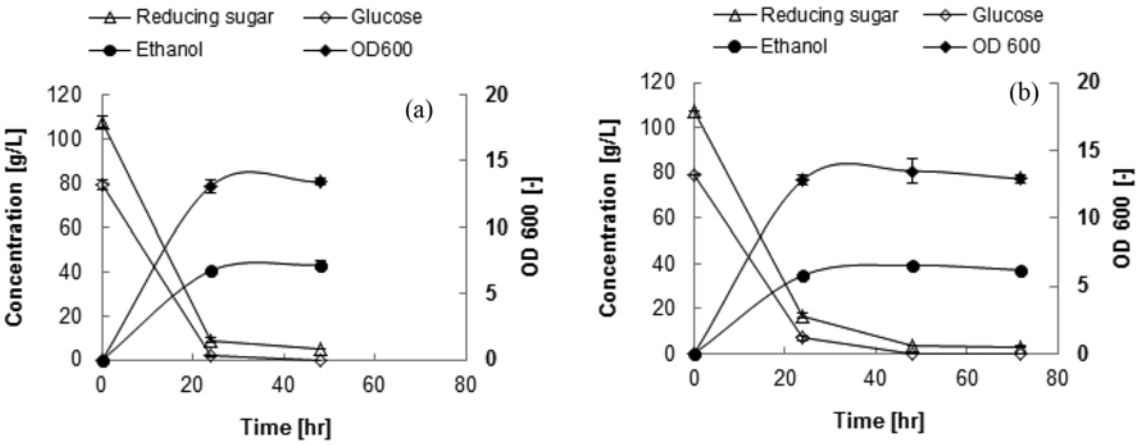

Fig. 1. Ethanol fermentations using saccharified liquid of food wastes by *S. coreanus*. Fermentation was carried out at 30 °C and 100 rpm. *S. coreanus* was injected with 0.2% (w/w) of initial reducing sugar in saccharified liquid. Data points and error bars represent the average and the standard deviation of at least three separate experiments: (a) anaerobic condition, (b) aerobic condition.

1.88 g/L, respectively. The ethanol produced at this time was 40.59 g/L, with an ethanol productivity of 1.69 g/L-h, within the range of 1.33-1.92 g/L-h found in the previous study. Conversely, in the aerobic fermentation, 16.7 g/L of the reducing sugar and 6.94 g/L of the glucose remained after 24 hrs, with 34.88 g/L of ethanol produced. In all the fermentations, under both anaerobic and aerobic conditions, similar amounts of reducing sugar and glucose remained, but 43.18 and 39.24 g/L of ethanol were produced from the anaerobic and aerobic fermentations, respectively. The biomass, as indicated by the OD 600 value, after 24 hrs of the anaerobic fermentation was higher than that of the aerobic fermentation, but after 48 hrs similar values were observed. In the anaerobic fermentation, the biomass remained unchanged with time. As a result, in the ethanol fermentation of *S. coreanus*, the growth of the yeast was not affected by oxygen, but the ethanol production was affected. Also, considering the sugars remaining and ethanol produced after 24 and 48 hrs of fermentation showed a minor difference, the sugar remaining after 24 hrs was considered not easily used by *S. coreanus*, or as being sugar that cannot be used.

2. Fermentation Property of Ethanol by *P. stipitis*

To improve the fermentation efficiency of *P. stipitis*, for which the fermentation rate was slow, oxygen is necessary [21]. The injection of air increased the biomass growth, ethanol productivity and maximum ethanol concentration, but shortened the fermentation time [22-24]. However, excessive air injection decreased the ethanol production [24]. Also, the maximum amount of air that could increase the yeast biomass and improve the ethanol production was 0.5 vvm [22-24]. Although the ethanol production by *P. stipitis* was improved by the injection of air, it was still lower than the 1-3 g/L-h shown by *Saccharomyces* [25].

Based on the results in the reference review, the properties of the aerobic fermentation of ethanol by *P. stipitis* were investigated, as shown in Fig. 2. The initial amounts of reducing sugar and glucose in the saccharification liquid were 95.19 and 77.62 g/L, respectively. In the fermentation using *P. stipitis*, the reducing sugar and glucose rapidly decreased until 72 hrs, with well defined ethanol production. After 72 hrs, however, the carbon source was moderately decreased, with only a small amount of ethanol produced. Even after 168 hrs,

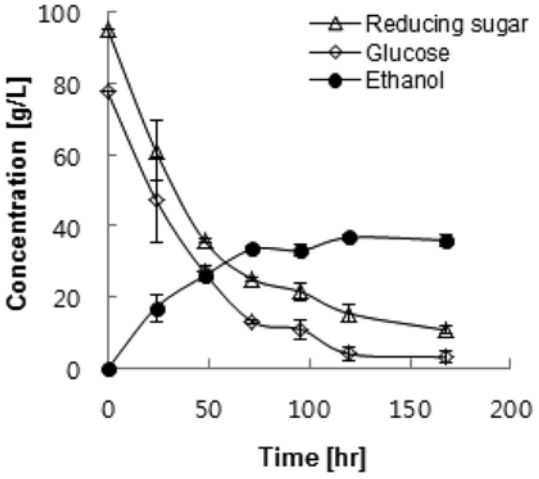

Fig. 2. Aerobic ethanol fermentations using saccharified liquid of food wastes by *P. stipitis*. Fermentation was carried out at 30 °C, 100 rpm for 168 hrs. Yeast was injected with 0.5% (v/v). Data points and error bars represent the average and the standard deviation of at least three separate experiments.

glucose still remained in the fermentation liquid, and the amount of ethanol produced was much lower than in the anaerobic and aerobic fermentations using *S. coreanus*. The ethanol yield and ethanol productivity after 24 hrs, based on the initial reducing sugar, were 0.18 and 0.71 g/L-h, and those after 168 hrs were 0.38 and 0.20 g/L-h, respectively. Kilian and van Uden [26] reported that glucose acted as competition and inhibition factors in the xylose fermentation process using *P. stipitis*. Also, under the conditions that pentose and hexose were mixed, hexose was firstly used, with pentose sequentially used after all the hexose had been consumed [27]. The fermentation rate using *P. stipitis* was noticeably slower than that using *S. coreanus*, and even after seven days from the start of the fermentation, glucose still remained. This resulted from the high concentration of initial carbon source. Vallet et al. [28] reported that *P. stipitis* did not consume all of the glucose when initial glucose concentration was high. Besides, maximum ethanol yield rate was

changed according to concentration of initial carbon source on same fermentation condition using *Saccharomyces* strain. Therefore, an investigation on the conversion of the remaining glucose to ethanol for the highly efficient production of ethanol will be required.

3. Fermentation Property of Ethanol by the Mixed Yeast of *S. coreanus* and *P. stipitis*

From the results mentioned above, the process of mixing two yeasts, *S. coreanus* and *P. stipitis*, with their use in fermentation was considered rather than using single yeast for fermentation to improve the ethanol productivity from the saccharification liquid of food wastes. Also, the forced injection of air was determined to increase the fermentation rate by *P. stipitis*. To minimize the effect of air on the fermentation by *S. coreanus*, 0.52 vvm of air was also injected at 17 hrs after the start of the fermentation to the end of fermentation. The vvm of aeration rate could be defined by the gas volume flow per unit of liquid volume per minute (consequently, volume per volume per minute). The point at which to inject the air was determined as follows. First, from the results of the previous study, maximum ethanol was produced after 21 hrs of fermentation using *S. coreanus* [29]. Second, Najafpour et al. [30] reported that the rapid consumption of glucose and production of ethanol occurred between 8 and 16 hrs in a batch fermenter using *S. cerevisiae*. Third, when glucose and xylose were mixed, the conversion of xylose to ethanol by *P. stipitis* started with 2.3 g/L or less of glucose [31]. Based on these findings, after 16 hrs, the available carbon source when using *S. coreanus* was considered to have been almost consumed; therefore, 17 hrs from the start of the fermentation was determined as a satisfactory point at which to inject air. In the fermentation with the mixture of *S. coreanus* and *P. stipitis*, the ethanol production and biomass, according to the amount of *P. stipitis* infused and the injection of air are shown in Fig. 3. The initial reducing sugar and glucose in the saccharification liquid of the food wastes were 107.53 and 79.12 g/L, respectively. In the mixed fermentation, wherein air was injected, more ethanol was produced than with *P. stipitis* infused at 20%, wherein air is not injected, despite the amount of the infused yeasts in the mixed fermentation with air injection being small. In the experiments with air injected, the ethanol production was generally stable, depending on the amount of yeast infused, with the maximum ethanol concentration observed with a 3% infusion. The ethanol produced at this time was 48.63 g/L; thus, an 8.04 g/L increase compared to the anaerobic fermentation using only *S. coreanus*, with an ethanol productivity of 2.03 g/L-h, an increase of 0.34 g/L-h. The ethanol yield based on the initial reducing sugar was 0.45, an improvement over the 0.38 yield in the anaerobic fermentation using *S. coreanus*. On reviewing the biomass, the OD 600 value increased with increasing amount of the yeast infused in the mixed fermentation with the injection of air. Although the mixed fermenter (amount of *P. stipitis* infused=20%) without the injection of air initially had a large amount of the yeast infused, the results were lower than the biomass of the fermenter with the injection of air. In the mixed fermentations with the injection of air, the production of ethanol was decreased, despite the increased biomass, which was considered to have resulted from the consumption of the ethanol produced as a carbon source of the yeast. From these results, it can be seen that the production of ethanol as well as the growth of *P. stipitis* increased due to the injection of air into the ethanol co-fermentation process using *S. coreanus* and *P. stipitis*.

4. Conversion of Sugar to Ethanol

The changes in the sugars throughout the ethanol fermentation process using the saccharification liquid from the food wastes are shown in Fig. 4. The reducing sugars remaining in the fermentation liquid after the anaerobic fermentation using *S. coreanus* and those in the mixed fermentation on the addition of 3% *P. stipitis* to the *S. coreanus* with the injection of air were compared. In the saccharification liquid prior to the fermentation, hexose and pentose were

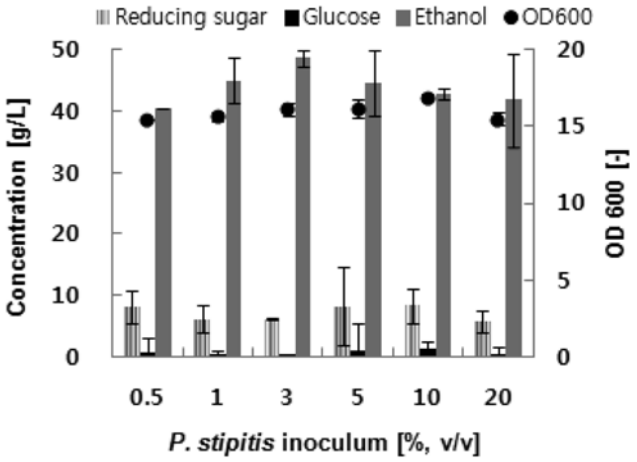

Fig. 3. Aerobic/forced aerobic consecutive ethanol fermentation using saccharified liquid of food wastes according to amount of *P. stipitis* inoculum into *S. coreanus* 0.2% (w/w). Fermentation was carried out at 30 °C, and aeration from 17-24 hrs. Fermentation with *P. stipitis* inoculum of only 20% was not aerated. Initial concentration of reducing sugar and glucose was 107.53 and 79.12 g/L, respectively. Data points and error bars represent the average and the standard deviation of at least three separate experiments.

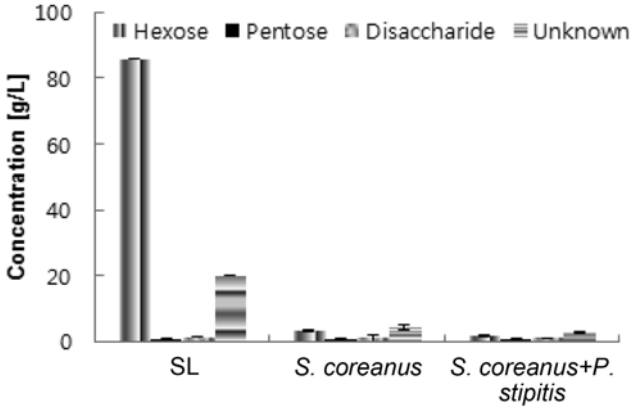

Fig. 4. Change of sugar concentration before and after fermentation according to yeast type added and oxygen condition. *S. coreanus* represents the results of anaerobic fermentation with S. coreanus of 0.2% (w/w). *S. coreanus+P. stipitis* represents the results of aerobic/forced aerobic (from 17-24 hrs) consecutive ethanol fermentation with *P. stipitis* of 0.3% (v/v) and *S. coreanus* of 0.2% (w/w). Fermentation was carried out at 30 °C, 100 rpm for 24 hrs. Data and error bars represent the average and the standard deviation of at least three separate experiments.

present in amounts of 85.67 and 0.73 g/L, respectively, and 1.18 g/L of disaccharide, with 19.93 g/L of unknown sugars. The hexose constituted 80% of the reducing sugars, with glucose constituting 92% of the hexose. It was thought that xylose derived from vegetables and fruits would be present in the saccharification liquid of the feed wastes, but no xylose was detected. However, trace amounts of ribose and arabinose were detected. In the anaerobic fermentation experiment using *S. coreanus* and the mixed fermentation experiment using both *S. coreanus* and *P. stipitis*, the consumptions of the hexose and unknown sugars were distinct. Mostly the hexose was consumed, but 4.23 and 2.63 g/L of the unknown reducing sugars remained in the single fermentation using *S. coreanus* and in the co-fermentation, respectively. Although the amount of disaccharides was very low, the amount remaining in the mixed fermentation was less than in the single fermentation. However, no quantitative change in the amount of pentose occurred. For the pentose to be consumed, and considering the fermentation rate by *P. stipitis* is very slow [22-24], a sufficient fermentation time would be required. In addition, considering the viability of *P. stipitis*, even under anaerobic conditions [21,31], if the mixed fermentation using *S. coreanus* was put into practice via anaerobic - aerobic steps, the fermentation efficiencies of hexose and pentose would be considerably increased.

CONCLUSION

This study aimed to improve the rate of the conversion of the saccharification liquid obtained from food wastes, containing a mixture of pentose and hexose, to the bioethanol, by selecting *S. coreanus*, which is generally used in the fermentation of glucose, and *P. stipitis*, which is used in the fermentation of pentose, such as xylose. Initially, the respective fermentation properties of *S. coreanus* and *P. stipitis*, which have different growth conditions, were investigated, with the co-fermentation properties also examined with respect to the mixing ratio of the two yeasts.

In the fermentation using *S. coreanus*, the result of the fermentation under anaerobic conditions was better than under aerobic conditions. In the anaerobic fermentation, the concentration of the reducing sugar and glucose remaining after 24 hrs was 9.09 and 1.88 g/L, respectively, with 40.59 g/L of ethanol produced; the ethanol productivity was 1.69 g/L-h. In the fermentation using *P. stipitis*, the reducing sugar and glucose rapidly decreased until 72 hrs, with marked ethanol production. However, after 72 hrs, a moderate decrease was observed in the amount of the carbon source, but almost no ethanol was produced. Even after 168 hrs, glucose still remained in the fermentation liquid, with less ethanol produced than in both the anaerobic and aerobic fermentations using *S. coreanus*.

Therefore, to produce a high concentration of bioethanol from food wastes, the ethanol fermentation should be induced by *S. coreanus* until the middle of the fermentation, with *P. stipitis* used during the later part of the fermentation; thus, the carbon source not converted by *S. coreanus* would be converted to ethanol by *P. stipitis* under the conditions that favor its use. As a result, 48.63 g/L of ethanol was produced, with an ethanol productivity of 2.03 g/L-h, thus resulting in increases of 8.04 g/L and 0.34 g/L-h of ethanol produced and productivity, respectively, over those of the anaerobic fermentation using *S. coreanus*.

This study could be used to help produce a high concentration of ethanol from biomass, wherein monosaccharide and disaccharide, as well as glucose are contained in the saccharification liquid from food wastes.

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