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Continuous Ethanol Production from Cassava Through Simultaneous Saccharification and Fermentation by Self-Flocculating Yeast *Saccharomyces Cerevisiae* CHFY0321

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Abstract In this study, a fermentor consisting of four linked stirred towers that can be used for simultaneous saccharification and fermentation (SSF) and for the accumulation of cell mass was applied to the continuous production of ethanol using cassava as the starchy material. For the continuous process with SSF, the pretreated cassava liquor and saccharification enzyme at total sugar concentrations of 175 g/L and 195 g/L were continuously fed to the fermentor with dilution rates of 0.014, 0.021, 0.031, 0.042, and 0.05 h^{-1}. Considering the maximum saccharification time, the highest volumetric productivity and ethanol yield were observed at a dilution rate of 0.042 h^{-1}. At dilution rates in the range of 0.014 h^{-1} to 0.042 h^{-1}, high production rates were observed, and the yeast in the first to fourth fermentor showed long-term stability for 2 months with good performance. Under the optimal culture conditions with a feed sugar concentration of 195 g/L and dilution rate of 0.042 h^{-1}, the ethanol volumetric productivity and ethanol yield were 3.58 g/L·h and 86.2%, respectively. The cell concentrations in the first to fourth stirred tower fermentors were 74.3, 71.5, 71.2, and 70.1 g dry cell/L, respectively. The self-flocculating yeast, *Saccharomyces cerevisiae* CHFY0321, developed by our group showed excellent fermentation results under continuous ethanol production.

Keywords Bioethanol · Continuous production · Simultaneous saccharification and fermentation · Cassava · Self-flocculating yeast

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Introduction

Bioethanol is used for medicines, cosmetics, and industrial materials as well as fuel and beverages, so the production amount thereof is increasing every year [1, 2]. Recently, bioethanol production has been the focus of attention, because of the increasing price of oil and global environmental problems [2, 3]. Therefore, techniques for producing ethanol more cheaply and with higher productivity are required. Although various studies have been conducted on bioethanol production, recent studies have focused on the production of bioethanol from lignocellulosic biomass and seaweeds [4–10]. Undoubtedly, it is necessary to conduct research on bioethanol production using alternative feedstocks. However, it is also important to study economic bioethanol production from sugary and starchy substrates, which have become the main feedstocks for the production of bioethanol at present [2, 3]. So far, the cost of ethanol obtained from lignocellulosic substrates is approximately two times higher than that obtained from starchy substrates [11].

Cassava is considered a particularly attractive raw material for bioethanol production, as it is inexpensive and is not affected by food and feed shortage concerns [12]. In general, the cost of bioethanol production using cassava for a ton of ethanol is approximately $40–$60 less than that using corn or wheat, which has been the common starch used for bioethanol production [13]. However, since cassava is a starchy substrate, an additional pretreatment process is required for its liquefaction and saccharification [14]. Moreover, the pretreated starch liquor should be filtered for the continuous or semi-continuous production of bioethanol, because the particle residues of the pretreated starch liquor may reduce the efficiency of the continuous or semi-continuous process [15]. Most ethanol production using starchy materials is conventionally performed by a batch process. Although the continuous processes used for enhancing the volumetric productivity need a pretreatment process such as liquefaction, saccharification, and filtration, they have several advantages compared to conventional batch processes, due to their reduced construction costs, lower maintenance, and operation requirements, and higher productivities. The bioethanol productivity can be enhanced by maintaining a high cell density and ensuring the continuous removal of ethanol from the fermentation broth [2, 16]. The volumetric productivity should be high for the economical production of fuel ethanol. In general, the volumetric ethanol productivity afforded by yeast fermentation is 1–2.5 g/L·h in batch fermentation and about 7 g/L·h in continuous fermentation [17]. In order to increase the volumetric ethanol productivity in continuous fermentation, a high cell concentration is necessary, since it is dominated by the specific productivity and the cell concentration. In continuous fermentation, it is possible to continuously feed the substrate and eliminate the product and this results in higher productivity compared to batch fermentation; however, it has the disadvantage of not being able to operate above a critical dilution rate, beyond which washout occurs. In order to solve this problem, various continuous processes have recently been suggested, including immobilized yeast cells using a carrier or packed bed fermentor and recirculating yeast cells using a separating device [18]. Continuous processes using a membrane, sedimentation tank, centrifuge, and high flocculating yeast were reported [19–23]. Although some literatures regarding the continuous production of bioethanol from filtered saccharification liquor have been previously reported [24–26], the continuous production of bioethanol with simultaneous saccharification and fermentation from starchy materials has rarely been reported so far.

This study was mainly focused on the continuous production of bioethanol through simultaneous saccharification and fermentation with cassava. We evaluated the continuous process by using a flocculating yeast, *Saccharomyces cerevisiae* CHFY0321, and optimized

dilution rate and substrate feeding concentration of the continuous process, in order to achieve a high volumetric ethanol productivity and stable continuous operation. Additionally, the continuous process studied in this work was compared with the batch process and repeated batch process in terms of the bioethanol productivity.

Materials and Methods

Microorganism and Pre-culture

S. cerevisiae CHFY0321, a fusion hybrid yeast, was developed in our laboratory by protoplast fusion between non-flocculent high ethanol fermentative *S. cerevisiae* and flocculent low ethanol fermentative *S. bayanus*. The protoplast fusion was carried out by culturing using a suitable buffer solution to remove the cell wall and mixing two protoplast-state cells with a buffer solution to induce fusion. This strain is stocked in the Korean Collection for Type Cultures (KCTC, Daejeon, Korea) under the collection number, KCTC 11249BP. The stock cultures were maintained at −20 °C in 5 ml vials containing 50% (*v*/*v*) glycerol.

The medium composition for cell growth is as follows (g/L): glucose 20, yeast extract 10, and peptone 20. The pre-culture medium for inoculation into the cassava-based medium was composed of liquefied cassava with a total sugar concentration of 100 g/L and 0.1 g/L of urea. The growth culture and pre-culture were performed in a 250-mL Erlenmeyer flask containing 100 mL of the medium and were cultivated at 33 °C and 200 rpm for 24 h on a shaking incubator (FMC-1000, Eyela, Tokyo, Japan).

Preparation of Liquefied Cassava for Continuous Feeding and Fermentation Medium

Liquefied cassava was prepared using cassava chips (starch content, approximately 72%) imported from Vietnam. The cassava chips were ground using a hammer mill and passed through a 1 mm screen. This ground cassava powder was provided by Changhae Ethanol Co., Ltd. (Jeonju, Korea). The cassava powder was mixed with tap water at a ratio of 1:3. For liquefaction, a commercial α-amylase (Termamyl SC, 120 KNU (kilo novo α-amylase unit)/g, Novozymes, Bagsvaerd, Denmark) was added at a dosage of 0.7 g/kg dry matter and then the mash was heated up to 100 °C and liquefied for 90 min. After the liquefying steps were completed, the resulting liquefied cassava mash was centrifuged at 10,000×*g* and the supernatant, which contained approximately 220 g/L total sugar, was diluted with distilled water to the desired total sugar concentration. Samples of liquefied cassava containing 175 g/L and 195 g/L total sugar were used as the fermentation medium for the continuous ethanol production.

Repeated Batch Fermentation for Initial Stability of Continuous Process

The repeated batch fermentation for initial stability of the continuous process was carried out using simultaneous saccharification and fermentation and performed individually at the same time in the four fermentors. For the saccharification, Spirizyme Fuel (750 AGU (amyloglucosidase unit)/g, Novozymes, Bagsvaerd, Denmark) was added at a dosage of 0.5 mg/g total sugar. The fermentations were performed in four stirrer tower fermentors manufactured by our laboratory containing 1 L of the fermentation medium. These stirred tower fermentors were composed of two sections: the upper section was 7 cm in height and

11 cm in diameter, and the lower section with a water jacket was 20 cm in height and 6 cm in diameter. The upper section acted as a settling zone to decrease the turbulence by separating the CO_2 and liquid, and the lower section served as a reaction zone to convert the sugar to ethanol. The culture temperature was maintained at 33 by a thermowater circulator (NCB-2300, Eyela, Tokyo, Japan) and the agitation speed was adjusted to 50 rpm. The pre-cultured broth was inoculated into the fermentation medium at a level of 5% (*v*/*v*). The repeated batch operations were carried out as follows. After the first batch fermentation was terminated, the agitation was stopped and the culture broth was allowed to stand for 30 min in order to sediment the cells. Then, the top phase of the broth was withdrawn (80% of total broth) and an equal volume of fresh medium (liquefied cassava) was charged into the fermentor. The agitation was restarted and then the next fermentation was initiated. These procedures were repeated until the concentration of cells was constant.

Continuous Process System with Four-Stage Cascade Fermentor

A schematic diagram of the continuous process system used in these experiments is shown in Fig. 1. The continuous fermentation was also carried out under similar fermentation conditions to those described above (repeated batch fermentation). Four tower fermentors containing a working volume of 1 L were linked in series and the total working volume was 4 L. For continuous fermentation with simultaneous saccharification and fermentation, the saccharification enzyme was continuously supplied along with the feeding medium at a constant ratio of feeding medium (0.5 mg/g total sugar) by a peristaltic pump (MP-1000, Eyela, Tokyo, Japan). When the repeated batch processes were terminated, continuous operation was initiated by feeding the fermentation medium and enzyme at the desired dilution rates (D=0.014, 0.021, 0.031, 0.042, and 0.05 h^{-1}). The fermented broth overflowed from the top of the first fermentor and continued into the bottom of the next fermentor. This continuous stream was kept in motion by the difference in height between the different fermentors without the need for any transportation device. Air was supplied into the four fermentors at 0.05 vvm for 10 min every other day for the activation of the flocculating yeast.

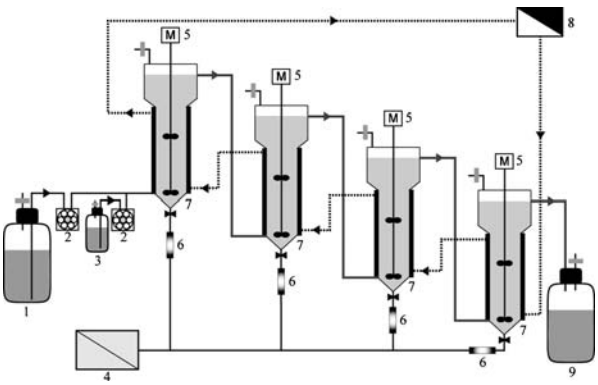

Fig. 1 Schematic diagram of the four-stage cascade system for continuous ethanol production with simultaneous saccharification and fermentation by self-flocculating yeast *Saccharomyces cerevisiae* CHFY0321. *1* substrate feeding tank, *2* peristaltic pumps, *3* enzyme feeding tank, *4* air compressor, *5* agitator, *6* air flowmeters, *7* stirrer tower fermentors, *8* thermostatted water bath, *9* broth storage tank

Analytical Methods

Samples were withdrawn aseptically at regular intervals for the reducing sugar, total sugar, biomass, and ethanol measurements. The sugar and ethanol concentrations were quantified using a high performance liquid chromatography (Waters, Milford, MA, USA) equipped with an Rspack KC-811 (8×300 mm) column (Showa Denko, Tokyo, Japan) and a refractive-index detector (Waters 2414, Milford, MA, USA). The mobile phase was 4 mM H_2SO_4 at a flow rate of 1 mL/min. The temperature of the column was maintained at 60 °C. The samples were subjected to membrane filtration. The samples used for total sugar analysis were hydrolyzed using 2 M HCl for 2 h. The biomass concentration was determined by the dry cell weight (DCW) method. For the measurement of the DCW, the cells from the sample were filtered through a filter paper with a pore size of 45 μm, washed with a sufficient amount of distilled water, then dried at 50 for a sufficient time until a constant dry weight was obtained.

Results and Discussion

Cell Accumulation Through Repeated Batch Process and Semi-Continuous Ethanol Production

For the continuous production of bioethanol, an initial stabilization step is needed. In order to secure a high cell density, several weeks were required in the tower reactor using flocculating yeast as well as a packed bed reactor and ethanol was not produced normally in this process [27]. However, in this study, repeated batch fermentation using *S. cerevisiae* CHFY0321 was carried out with a recycling volume of 20% (*v*/*v*), in order to produce ethanol stably and secure a high cell concentration. The production medium containing 175 g/L total sugar in the liquefied cassava under the same fermentation conditions and 0.1 g/L of urea was used for the semi-continuous production of bioethanol, and the pH of the culture broth was not controlled. After finishing the batch fermentation, the agitation was stopped for 30 min in order to sediment the cells. Then, the 80% top phase of the total broth was withdrawn and an equal volume of fresh medium was charged into the fermentor. Each of these procedures was repeated eight times and a maximum of 38.9 g/L of DCW could be accumulated through this repeated batch process. In addition, ethanol was steadily produced at a concentration of 82.6 g/L (Fig. 2). A higher cell mass can be accumulated by increasing the recycling volume up to 20% in the repeated batch process. Ethanol was produced steadily from the four linked fermentors through each of the repeated batch fermentation steps designed for use with a continuous process and a maximum cell concentration of 38.9 g/L was secured with 20% recycling volume. The volumetric ethanol productivity of this process was 2.79 g/L·h, which is 1.7 times higher than that obtained with batch fermentation. The fermentation time was 24 h. Moreover, the repeated batch process of *S. cerevisiae* CHFY0321 was found to be not only superior in terms of the initial stabilization for continuous ethanol production, but also in terms of the semi-continuous ethanol production, and made it possible to eliminate the propagation stage in the total process of conventional batch fermentation through a simple modification.

Continuous Production of Ethanol by Four Stirred Tower Fermentors In Series

The use of self-flocculating yeast with a high cell concentration of *S. cerevisiae* CHFY0321 made it possible to accumulate yeast cells with high flocculation in the

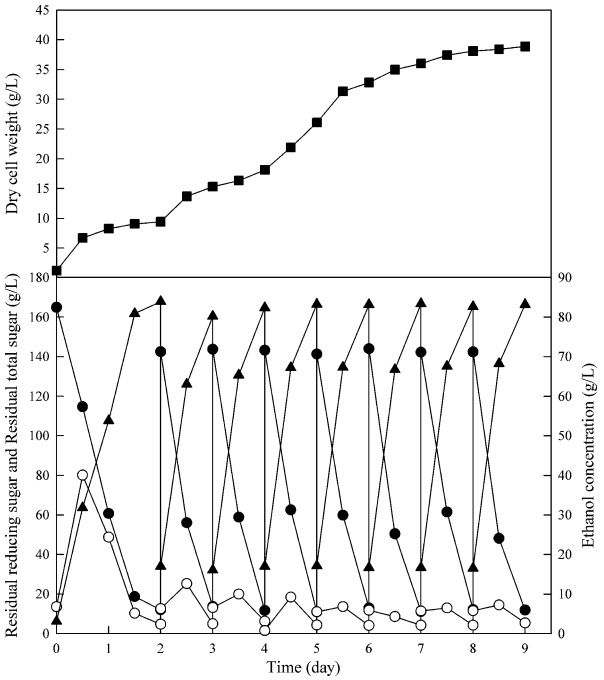

Fig. 2 Profiles of ethanol production and cell growth during repeated batch fermentation by self-flocculating yeast, *Saccharomyces cerevisiae* CHFY0321. *Filled square* biomass, *filled triangle* ethanol, *filled circle* total sugar, *open circle* reducing sugar

fermentor. Total sugar concentrations of 175 and 195 g/L were used for feeding the fermentation medium with dilution rates of 0.014, 0.021, 0.031, 0.042, and 0.05 h^{-1}. Figures 3 and 4 show the results obtained from the prolonged operation of the continuous ethanol fermentation process with various substrate feeding concentrations and dilution rates. The continuous process was preceded by an initial stabilization process, thereby drastically shortening the time taken to reach the pseudo steady state. The dilution rate was gradually raised through continuous operation after the completion of the repeated batch process, through which the ethanol production rate could be observed in relation to the dilution rate and the substrate feeding concentration. An ethanol concentration of 77–78 g/L was stably produced in the fourth fermentor at a total sugar concentration of 175 g/L, even though the dilution rate was varied from 0.014 h^{-1} to 0.042 h^{-1}. Although an ethanol yield of 86–88% was obtained at all dilution rates, at a total sugar concentration of 175 g/L, the maximum productivity was D=0.042 h^{-1}. On the other hand, a higher maximum productivity of D=0.05 h^{-1} was obtained at a total sugar concentration of 195 g/L, but the ethanol yield was only 74.5%, which is much lower than that observed at D=0.042 h^{-1} (Table 1). The balanced saccharification yield and ethanol yield that were obtained through the fermentors in the continuous process shows that it would be possible to establish a continuous process through SSF (Figs. 3 and 4). It is necessary to keep the dilution rate constant for 24 h in the continuous process through SSF to achieve an adequate ethanol yield. The volumetric ethanol productivity may increase at a higher dilution rate, but this will not allow enough time for saccharification (Table 1). As shown in Fig. 4, stable continuous fermentation could be obtained at a dilution rate of 0.042 h^{-1} even with a total feeding sugar concentration of 195 g/L. These results show that it is possible to effectively operate a continuous production process with only liquefaction as the preceding process for SSF by using

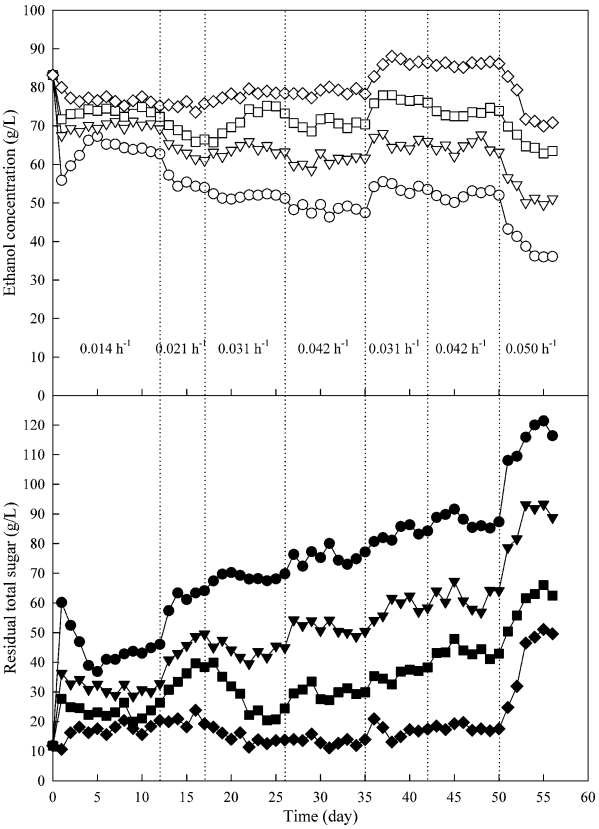

Fig. 3 Continuous fermentation profiles with simultaneous saccharification and fermentation of *Saccharomyces cerevisiae* CHFY0321 at different feeding concentrations and dilution rates; 0–12 days, D=0.014 h^{-1}; 13–17 days, D=0.021 h^{-1}; 18–26 days, D=0.031 h^{-1}; 27–35 days, D=0.042 h^{-1}; 36–42 days, D=0.031 h^{-1}; 43–50 days, D=0.042 h^{-1}; 51–56 days, D=0.050 h^{-1}. *Open symbols* are produced ethanol and *filled symbols* are total sugar. (*open circle*, *filled circle*: first fermentor), (*open triangle*, *filled triangle*: second fermentor), (*open square*, *filled square*: third fermentor), (*open diamond*, *filled diamond*: fourth fermentor)

high self-flocculating yeast. It is also important to retain the activity of the yeast for long-term operation when using self-flocculating yeast with a high cell concentration [15, 16, 24]. In this study, the yeast activity was maintained by stopping the feed of the substrate for 10 min every other day in the first to fourth fermentor and supplying air at 0.05 vvm without any extra activation devices or processes, because adequate aerobic culture provides advantages for cell activation and growth [26–29]. The 10 min of aeration used in this continuous process ensures that cells remain activated for 56 days in the continuous process without affecting the ethanol yield.

Effects of Dilution Rate on Cell Concentration for Continuous Ethanol Production

Considering the yield of saccharification, a dilution rate of 0.042 h^{-1} was found to be optimal at feeding concentrations of 175 and 195 g/L. Also, the concentration of the self-flocculating yeast, *S. cerevisiae* CHFY0321, used for cell recycling in this study was affected by the dilution rate. As shown in Table 2, the cell accumulation increased as the dilution rate increased to 0.042 h^{-1}, but decreased when the dilution rate was further increased (D=0.05 h^{-1}). In other words, the continuous fermentation using the self-flocculating yeast in SSF was affected not only by the saccharification rate, but also by the accumulation of the self-flocculating yeast. The optimal dilution for the self-flocculating yeast in the tower-type fermentor with agitator was found to be 0.042 h^{-1}. According to Xu

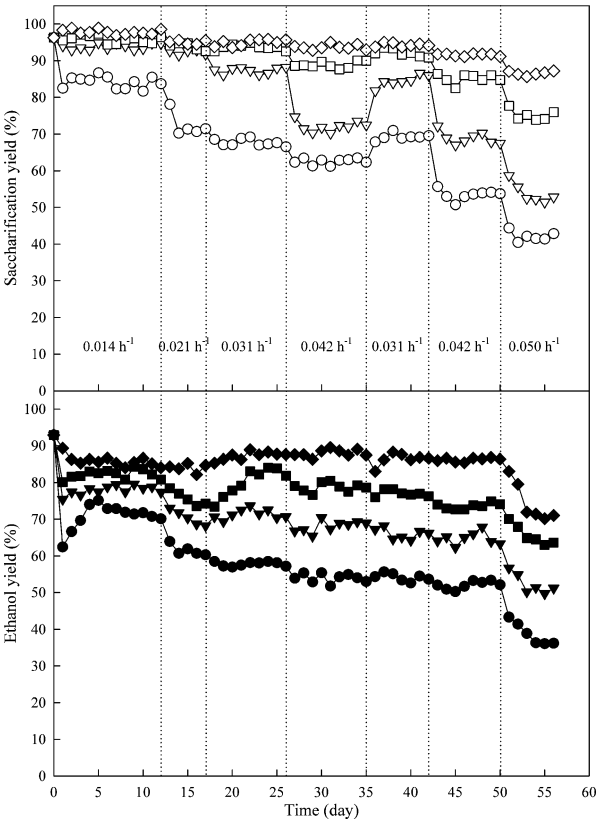

Fig. 4 Profiles of saccharification yield (*open symbols*) and ethanol yield (*filled symbols*) by continuous fermentation with simultaneous saccharification and fermentation at different feeding concentrations and dilution rates; 0–12 days, D=0.014 h^{-1}; 13–17 days, D=0.021 h^{-1}; 18–26 days, D=0.031 h^{-1}; 27–35 days, D=0.042 h^{-1}; 36–42 days, D=0.031 h^{-1}; 43–50 days, D=0.042 h^{-1}; 51–56 days, D=0.050 h^{-1}. (*open circle*, *filled circle*: first fermentor), (*open triangle*, *filled triangle*: second fermentor), (*open square*, *filled square*: third fermentor), (*open diamond*, *filled diamond*: fourth fermentor)

et al. [15], the optimal dilution rate for continuous fermentation in cascade fermentors using self-flocculating yeast was 0.033 h^{-1}. However, the stirred tower fermentor used in this study was able to maintain a high cell concentration of more than 70 g/L in the first to fourth fermentor at a higher dilution rate of 0.042 h^{-1}.

Table 1 Comparison of saccharification yield, theoretical ethanol yield, and volumetric ethanol productivity in each fermentor according to concentration of liquefied cassava and dilution rate.

S_f (g/L)	D (h^{-1})	First fermentor		Second fermentor		Third fermentor		Fourth fermentor		q (g/L·h)
		SY (%)	*EY* (%)	*SY* (%)	*EY* (%)	*SY* (%)	*EY* (%)	*SY* (%)	*EY* (%)	
175	0.014	85.0	72.5	94.0	79.1	95.8	83.1	97.7	86.4	1.07
	0.021	72.3	61.5	92.3	70.4	93.9	75.7	95.0	84.0	1.57
	0.031	67.7	57.8	87.3	71.4	93.7	80.2	94.9	87.3	2.44
	0.042	62.5	53.9	72.1	68.1	88.9	78.7	93.7	88.0	3.28
195	0.031	69.2	54.2	84.5	66.0	92.1	77.0	94.3	86.4	2.69
	0.042	53.4	52.1	68.9	64.7	85.1	73.6	91.6	86.2	3.58
	0.050	42.1	38.7	53.9	52.3	75.2	65.7	86.6	74.5	3.71

S_f feeding total sugar concentration, D dilution rate, *SY* saccharification yield, *EY* theoretical ethanol yield, q volumetric ethanol productivity

Table 2 Comparison of ethanol production, residual total sugar, residual reducing sugar, and biomass in each fermentor according to concentration of liquefied cassava and dilution rate.

S_f (g/L)	D (h^{-1})	First fermentor				Second fermentor				Third fermentor				Fourth fermentor			
		P (g/L)	S_t (g/L)	S_r (g/L)	X_i (g/L)	P (g/L)	S_t (g/L)	S_r (g/L)	X_i (g/L)	P (g/L)	S_t (g/L)	S_r (g/L)	X_i (g/L)	P (g/L)	S_t (g/L)	S_r (g/L)	X_i (g/L)
175	0.014	64.9	42.3	16.1	71.3	70.8	30.1	19.7	66.3	74.3	22.8	15.6	63.5	77.3	16.7	12.7	60.9
	0.021	55.0	61.9	13.5	72.3	63.0	45.5	32.0	70.3	67.8	35.7	24.9	68.2	75.2	20.4	11.7	65.8
	0.031	51.7	68.7	12.1	78.9	63.9	43.7	21.5	74.3	71.7	27.5	16.5	71.6	78.1	14.4	5.6	67.9
	0.042	48.3	75.6	10.1	73.3	60.9	51.6	2.8	70.7	70.4	29.9	10.4	69.2	78.8	13.3	2.3	68.7
195	0.031	54.0	83.3	29.5	77.6	65.8	58.4	31.3	73.9	76.8	36.0	22.1	71.2	86.2	16.9	6.9	68.6
	0.042	51.9	87.8	6.3	74.3	64.5	61.9	7.5	71.5	73.4	43.7	17.6	71.2	86.0	18.0	3.3	70.1
	0.050	38.6	115.2	13.9	62.2	52.2	87.9	7.1	59.5	65.5	59.9	16.4	57.3	74.3	42.0	18.5	58.2

S_f feeding total sugar concentration, D dilution rate, P ethanol concentration, S_t residual total sugar concentration, S_r residual reducing sugar concentration, X_i biomass concentration g dry cell weight/L

Comparison of Ethanol Productivity Between Various Fermentation Modes

This study was carried out in various fermentation modes to compare the fermentation characteristics of *S. cerevisiae* CHFY0321. Table 3 shows the fermentation results for the batch, repeated batch, and continuous processes. In most studies on the production of ethanol from starch material [15], [24–27], [30], the initial pH of the fermentation medium was regulated to less than 5.5 to prevent contamination and saccharification. However, the ethanol production process with *S. cerevisiae* CHFY0321, which was not purposely regulated prior to fermentation, performed well. The actual ethanol production, ethanol yield, and volumetric productivity were measured for the three operation modes using a fermentation medium with a total sugar concentration of 175 g/L and identical amounts of saccharification enzymes for SSF. Batch fermentation showed the maximum ethanol yield of 95.8% and continuous fermentation showed the highest productivity of 3.28 g/L·h. Each of the three fermentation modes has its advantages and disadvantages [2, 3] and could be chosen for industrial scale ethanol production depending on the type and availability of the raw materials [3]. *S. cerevisiae* CHFY0321 showed excellent fermentation results in all three modes. The continuous process with self-flocculating yeast could be an alternative cost-effective method for the production of fuel ethanol.

Conclusions

The continuous production of ethanol from the starch material, cassava, was accomplished by SSF with *S. cerevisiae* CHFY0321 and a high volumetric productivity and high cell concentration of 3.58 g/L·h and 74.3 g/L were obtained, respectively. This process does not require any additional devices or processes to maintain a high cell concentration. The cell concentration and viability can be enhanced by increasing the air supply, whereas the ethanol production decreases under aerobic conditions. Thus, in this study, only minimal air was provided so that a high cell concentration could be maintained for a prolonged period of time with ensuing high ethanol production. The stirred tower fermentor with *S. cerevisiae* CHFY0321 used in this study allows a higher cell concentration, volumetric ethanol productivity, and long-term stability to be achieved.

Table 3 Comparison of ethanol production performance according to fermentation mode.

	Batch fermentation[a]	Repeated batch fermentation[b]	Continuous fermentation[c]
Ethanol concentration (g/L)	80.83	65.89	78.8
Fermentation time (h)	48	24	24
Theoretical ethanol yield (%)	95.8	90.25	86.2
Volumetric ethanol productivity (g/L·h)	1.64	2.75	3.28

The fermentation medium used in the three modes is identical, consisting of liquefied cassava mash with a total sugar concentration of 175 g/L

[a] Batch fermentation with inoculum level of 5%

[b] Average data of repeated batch fermentation with recycling volume of 20% ratio

[c] Dilution rate, 0.042 h^{-1}

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