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Ethanol Production from Lignocellulosic Biomass by Simultaneous Saccharification and Fermentation Employing the Reuse of Yeast and Enzyme

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Abstract Simultaneous saccharification and fermentation (SSF) experiments were carried out with a lignocellulosic biomass. The effects of temperature on enzymatic saccharification and the ethanol fermentation were also investigated. The batch SSF process gave a final ethanol concentration of 10.44 g/l and equivalent glucose yield of 0.55 g/g, which was increased by 67% or higher over the saccharification at 42°C. The optimal operating condition was found to vary in several parameters, such as the transmembrane pressure, permeation rate, and separation coefficient, related to the SSF combined with membrane system (semi-batch system). When the fermentation was operated in a semi-batch mode, the efficiency of the enzymes and yeast lasted three times longer than in a batch mode.

Key words: Semi-batch simultaneous saccharification and fermentation, lignocellulosic biomass, fuel ethanol, ultrafiltration

The production of ethanol from lignocellulosic biomass can play an important role in compensating for diminishing fuel reserves, and in reducing carbon-dioxide emission to the atmosphere. Because biomass is a cheaper and more abundant resource than corn and sugar cane, it is thought to be a promising source for producing ethanol by fermentation [9]. The first step in the fermentation is an acid or enzymatic hydrolysis by which the cellulosic biomass is degraded into fermentable carbohydrate [23]. However, this enzymatic hydrolysis is disadvantageous being slow due to product inhibition. One way of reducing the product inhibition is to saccharify and ferment the substrate simultaneously. Since the fint repart by Takagi *et al.* [24], several researchers have studied such simultaneous saccharification and fermentation (SSF) which aimed to decrease enzyme loadings and to increase hydrolysis reaction rates along with production yields, by relieving the product inhibition and contamination problems [7, 10, 21]. However, there is not enough information available on the industrial production of fuel-grade ethanol from lignocellulosic biomass, because many different enzymes are required in the hydrolysis of cellulose, which increase the production cost of ethanol. This problem has been tackled by lowering the enzyme cost, increasing the reactivity of the substrate after pretreatment, and improving enzyme production systems and its activity [17, 20]. The ultimate goal of this study was to increase and maximize the ethanol productivity by operating the SSF process in a semi-batch mode. In the SSF process combined with the membrane system, the reusable enzyme and yeast are separated from the enriched ethanol and higher cell, and enzyme concentrations can be achieved by recycling them to the fermenter. Compared with the prevailing cell recycle system such as the Biostill process [6], the membrane offers an advantage for economic ethanol production [2-5, 14, 15].

In this work, a hollow fiber membrane module was adopted to a semi-batch fermenter to separate cells and enzyme efficiently. This method was performed intermittently because of the difficulty of the feeding methodology of exploded cellulosic biomass as substrate. Therefore, the yeast and enzymes were separated at the end of each run and recycled to the next step. The objective of this study was to find optimum conditions through a series of experiments under various conditions including transmembrane pressure, permeation rate, and separation coefficient, using a batch and semi-batch SSF processes.

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MATERIALS AND METHODS

Enzymes and Microbial Strain

Cellulase from *Trichoderma reesei* (Celluclast 1.5 l) and β-glucosidase (Novozym-188) were purchased from Novo. Co., Denmark, and they were used without further purification. Their activities were 200 FPU (filter paper unit)/ml and 350 CBU (cellobiase unit)/ml, respectively [8]. *Brettanomyces custersii* H1-55, derived through genetic improvements of *Bretanomyces custersii* CBS 5512, was used for fermentation.

Substrate

The oak wood [16] used as a substrate was obtained from the Korea Institute of Energy Research (KIER, Teajon, Korea). It was heated directly by steam up to the desired explosion temperature (215°C), and maintained there for 3 min. Then, the material was fragmented by applying a sudden release of pressure. The cellulose content of these treated oak wood chips was about 54.5% on a dry weight basis.

Analytical Methods

Filter paper and β-glucosidase activities were assayed using 50 mg (1×6 cm) filter paper (Whatman No. 1) and 15 mM cellobiose, respectively, in 50 mM citrate buffer of pH 4.8, as described by Mandels and Weber [18]. Total reducing sugars were measured by the DNS method in which glucose was used as the standard [19]. Glucose was measured by the glucose oxidase/peroxidase method (Glucose-E kit, Yeongdong Pharm. Co., Korea). Ethanol was analyzed by gas chromatography (Packard, Hewlett Packard Co., Palo Alto, U.S.A.) equipped with a flame ionized detector. Nitrogen was used as a carrier gas with a flow rate of 30 ml/min. In addition, the combustion gas was a mixture of hydrogen and air. Yeast cell viability was measured by methylene blue staining [22].

Hydrolysis of Cellulose

The cellulose suspension was prepared by mixing steam-exploded wood chips with 0.05 M citrate buffer at pH 4.8. Saccharification was carried out by adding the mixed enzyme solution to the flask. Enzyme activities of Celluclast 1.5 l and Novozym-188 for the enzymatic hydrolysis of cellulose were diluted to 30 FPU/ml and 18 CBU/m, respectively.

Test Fermentations, SSFs, and Semi-Batch SSFs

Test fermentations were carried out during 80 h in 250-ml Erlenmeyer flasks at four different temperatures; 30°C, 37°C, 40°C, and 42°C. The basal medium consisted of 1.5% yeast extract and 2.0% peptone with an initial sugar concentration of 20 g/l which was a mixture of cellobiose and glucose of the same quantity. The flasks were inoculated with 10% (v/v) seed culture grown aerobically for 24 h at 30°C. The medium used was the same except with 20 g/l glucose. Batch and Semi-batch SSFs were implemented at 42°C for the further experiment. One reactor was used for batch SSF and two reactors for semi-batch SSF. As soon as the reaction in the first reactor was finished, the cell and enzyme were transferred through hollow fiber module to the second reactor containing 80 g/l of substrate. The operating volume of bioreactor (Korea Fermenter Ltd.) was 2.0 l. The substrate for SSF was the steam-exploded wood chips and its concentrations were 7, 8, and 9% (w/v). Cellulase and β-glucosidase loadings were 30 FPU/g of substrate and 18 CBU/g of substrate.

Experimental Apparatus

Figure 1 shows a schematic diagram of the semi-batch SSF system with the hollow fiber membrane module. A variable-stroke plunger pump (Weco Co. Korea) was used for separation and a cylindrical damper was used to smooth out the flow rate and pressure. The two hollow fiber modules of a different pore size were tested for the semi-batch SSF: 1. SKC-103 (Sunkyong Industries, Korea); total active filtration area of 1000 cm^2; material: polysulfone; MWCO: 30,000. 2. Microsampler (Microgon Co. Laguna Hills, U.S.A.); total active filtration area of 680 cm^2; material: polysulfone; MWCO: 50,000. To reduce the membrane fouling phenomena by the cellulosic biomass, the pre-filter system was used. The most suitable and effective operating condition was selected in order to operate the hollow fiber modules properly. The operating pressures through ultrafiltration were adjusted to 1.0, 1.5, and 2.0 kg_f/cm^2 by a pressure valve. The operating temperatures were adjusted to 30, 35, and 45°C by a thermoregulator. The activities of enzymes and the viability of yeast were measured at various operating transmembrane pressures and temperatures. The enzymes and yeast were recycled to the next reactor at the end of each round for increasing the

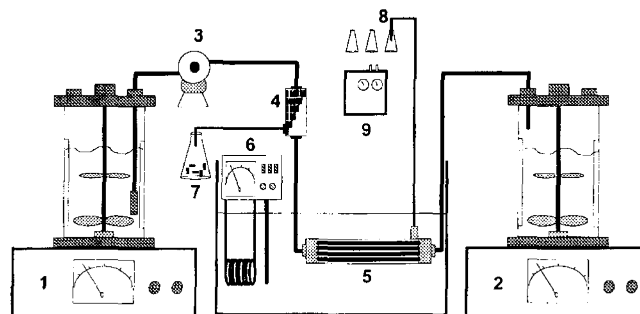

Fig. 1. Schematic diagram of the semi-batch SSF system with a cross flow membrane unit.
1. 1st SSF vessel; 2. 2nd SSF vessel; 3. Pump; 4. Pre-filter; 5. Hollow fiber module (UF); 6. Thermoregulator; 7. Rejected material; 8. Separated ethanol; 9. Gas-chromatography.

ethanol productivity. The permeation flux of enzymes and the yeast were measured for both membranes.

RESULTS AND DISCUSSION

Optimal Conditions of SSF Process

One major problem associated with the SSF process is the difference in the optimum temperature of the hydrolysis and the fermentation. In general, while the optimum temperature for the ethanol fermentation is around 30°C, that for the hydrolysis of cellulose is nearly 50°C [1, 11]. Thus, these two temperatures must be compromised in the SSF process. Figure 2 shows the effects of temperature on the rate of hydrolysis. At 30°C, the initial hydrolysis rate was as low as 0.06 g/l/h, while at 50°C, it was 0.17 g/l/h. Thus, the rate of dependence of hydrolysis increased as the temperature rose from 30°C to 50°C.

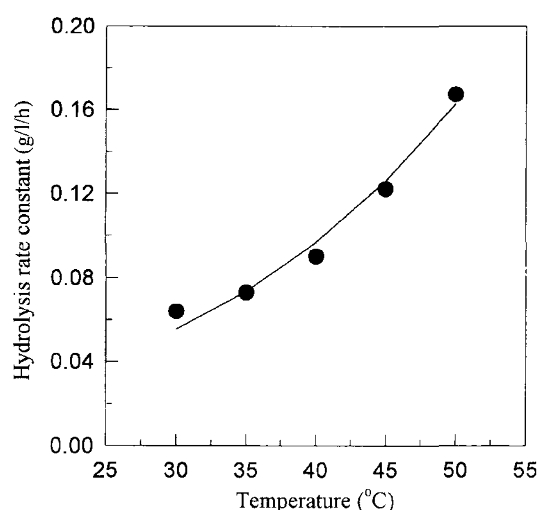

Fig. 2. Effect of temperature on initial hydrolysis rate of cellulase (30 FPU/g cellulose) with α-cellulose (8%).

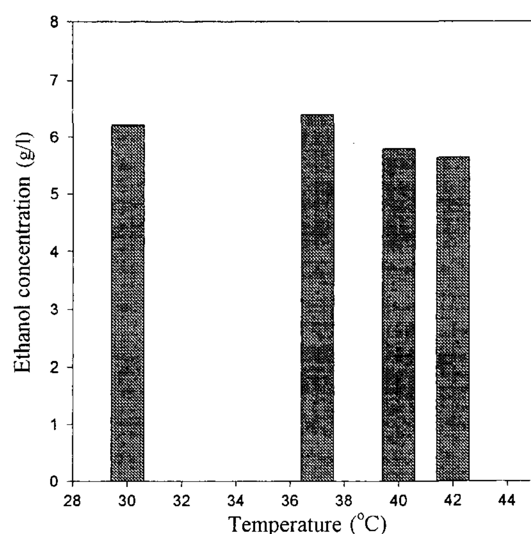

Fig. 3. Effect of temperature on the ethanol fermentation of 20 g/l mixed sugars by *B. custersii* H1-55.

Test fermentations were carried out at various temperatures, between 30°C and 42°C, for 80 h. The fermentative performance decreased remarkably at a temperature above 42°C. As shown in Fig. 3, the highest fermentative performance was at 37°C. As the temperature increased, the fermentative performance decreased slightly. These results showed that the most effective SSF could be carried out at the temperatures between 37°C and 42°C. Table 1 summarizes the results of SSFs at 48 h and 80 h. Ethanol concentration produced was 1.34 and 1.47% at the substrate concentrations of 80 and 90 g/l, respectively. Table 1 also shows that the ethanol concentration increased as the substrate concentrations increased. However, when the substrate concentration was extremely high, the SSF process did not work properly because of the difficulty in mixing the wood chips. Through this investigation, the optimum substrate concentration was found to be 80 g/l.

Yeast and Enzyme Separation by Ultrafiltration

The two different membranes used rejected 100% of the yeast cell and more than 95% of enzymes (Celluclast 1.5 l, β-glucosidase). As shown in Table 2, both membranes were suitable for the recycling system. The 50,000 MWCO membrane had a higher permeate flux than that of the 30,000 MWCO membrane. Considering the lignocellulosic biomass as a substrate, the 50,000 MWCO membrane module was suitable for the semi-batch SSF. Therefore, yeast and enzymes were recycled with the 50,000 MWCO ultrafiltration membrane at 42°C, which was the optimum temperature of the SSF process.

As shown in Fig. 4, the relative cell viability was maintained at greater than 95% during a 720 min operation. This means that there is no effect of shear on cell viability in the recycle system. Thus, the semi-batch SSF with ultrafiltration system could be used to increase cell concentration to the next SSF process.

In the case of Celluclast 1.5 l, the enzyme activity did not change at the chosen operating temperatures and transmembrane pressures (Fig. 5). Figure 6 shows β-glucosidase activity at transmembrane pressures of 1.0, 1.5, and 2.0 kg_f/cm^2, respectively. This suggests that β-

Table 1. Ethanol concentration (%, w/v) produced by *B. custersii* H1-55, with different concentration of steam-exploded wood chips at 42°C. Enzyme activity: 30 FPU/g wood chips.

Time (h)	Substrate concentration (g/l)		
	70	80	90
48	1.03	1.23	1.27
80	1.08	1.34	1.47

Table 2. Data on enzyme relative activity (%) and protein concentration of permeate and retentate solutions for two different membranes.

Membrane		30,000 MWCO[a]		50,000 MWCO[a]	
		0 min	720 min	0 min	720 min
Cellulase	Relative activity (%)	100.0	97.5	100.0	98.0
	Average permeate flux (ml/cm^2/min)	0.385	0.378	0.483	0.475
	Separation coefficient[b]	0.961	0.968	0.940	0.944
β-Glucosidase	Relative activity (%)	100.0	62.5	100.0	67.7
	Average permeate flux (ml/cm^2/min)	0.340	0.331	0.449	0.440
	Separation coefficient	0.975	0.977	0.950	0.951

[a]MWCO, Molecular Weight Cut-Off.

[b] $\frac{C_f - C_p}{C_f}$

C_f; initial enzyme concentration, C_p; enzyme concentration of permeate.

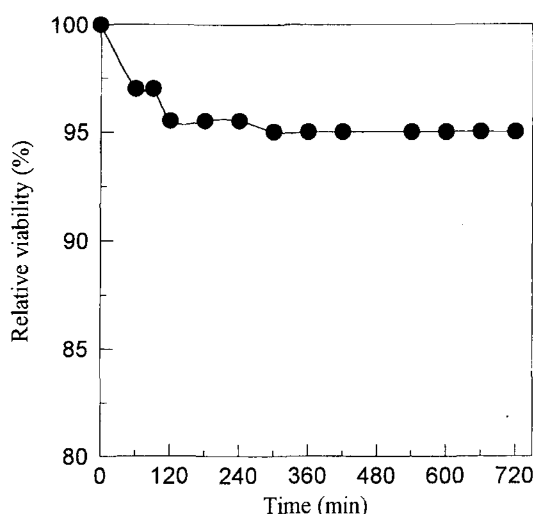

Fig. 4. Effect of shear on cell viability in the system with 50,000 MWCO membrane module at 1.0 kg$_f$/cm^2, 42°C.

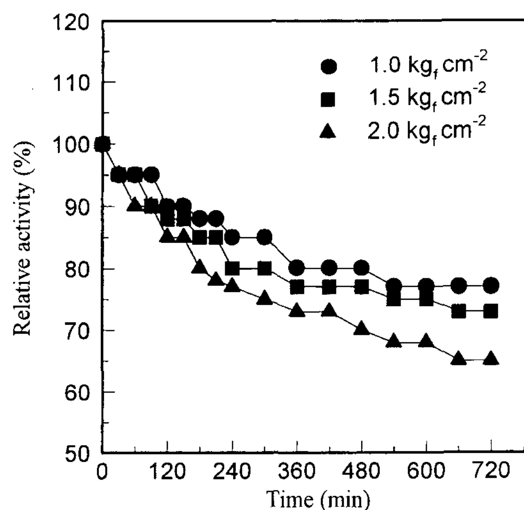

Fig. 6. Relative retention activity of the enzyme (β-glucosidase) solution through the 50,000 MWCO membrane at 42°C.

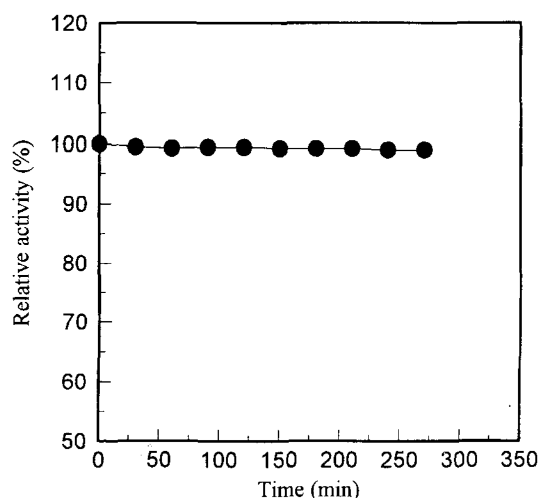

Fig. 5. Relative retention activity of the enzyme (cellulase) solution through the 50,000 MWCO membrane (42°C and 2.0 kg$_f$/cm^2).

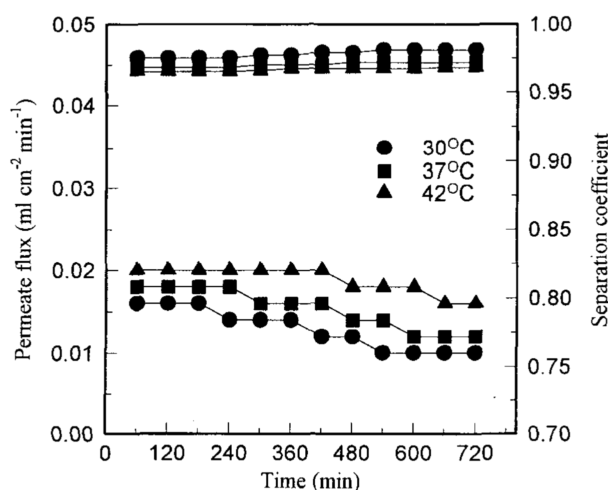

Fig. 7. Permeate flux and separation coefficient on the enzyme (β-glucosidase) solution through the 50,000 MWCO membrane at 1.0 kg$_f$ cm^{-2}.

glucosidase activity decreased as transmembrane pressures and time increased [12]. This means that enzyme activity was affected by shear through the membrane module. It could be caused by protein aggregation at the inner surface of the membrane fiber [13]. Therefore, the optimum transmembrane pressure was 1.0 kg_f/cm^2 for recycling enzyme with the pressure minimizing its deactivation. Figure 7 shows the permeate flux and the separation coefficient in the β-glucosidase solution at 1.0 kg_f/cm^2. It is noted that the permeate flux and separation coefficient had reasonable values for enzyme recycle. As a result of recycling yeast and enzymes for SSF, the optimum membrane operating condition was found to be the 50,000 MWCO ultrafiltration membrane, 1.0 kg_f/cm^2 transmembrane pressure, and 42°C.

Semi-Batch Ethanol Production

Figure 8 shows the change of ethanol and glucose concentrations in the semi-batch SSF where the 50,000 MWCO membrane was used with the operating pressure and temperature of 1.0 kg_f/cm^2 and 42°C, respectively. Three rounds of semi-batch SSFs including ultrafiltration process were performed. The initial substrate concentration was 80 g/l. After the first SSF was completed, residual materials were rejected by the pre-filter system. The final concentration of ethanol was 10.2 g/l and the overall productivity was 0.14 g/l/h at the first SSF batch. Yeast and enzyme were recycled to the new SSF batch process by membrane filtration. In the second SSF batch, fresh wood chips were used as substrate at a concentration of 80 g/l. The second SSF took 50 h to react completely, which was shorter than that of the first SSF. At the second SSF, ethanol concentration was 7.0 g/l and the productivity was also calculated to be 0.14 g/l/h. Ethanol was measured in the permeate stream at the first and the second batch SSFs. The third SSF batch was operated as the previous method and productivity was found to be similar to the previous

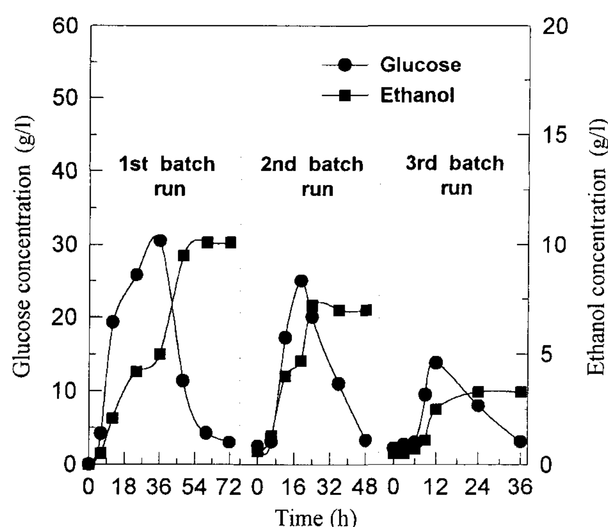

Fig. 8. The concentration change of ethanol and glucose using semi-batch SSF system.
Yeast and enzyme recycling system with the 50,000 MWCO membrane at 42°C.

experiments. The reduction of ethanol in the second and the third reactors was thought to be due to the deactivation of enzymes. However, the advantages of this process was that the enzyme and yeast lasted three times longer than in a batch mode. The saccharification, SSF, and the semi-batch SSF with ultrafiltration systems are compared in Table 3. Since it is difficult to compare the separate hydrolysis and fermentation (SHF) with SSF directly, the following equivalent values were used. The equivalent glucose concentration for SSF experiments was determined by dividing the ethanol concentration by the ethanol yield, and the equivalent glucose yield per amount of enzyme was determined by dividing the equivalent glucose concentration by the amount of cellulose and the amount of enzyme [19]. These results were used to decide the

Table 3. Comparison amongst saccharification, SSF, and semi-batch SSF with ultrafiltration system.

Processes	Reducing sugar concentration[a]	Ethanol concentration[a]	Equivalent glucose concentration[b]	Equivalent glucose yield[c]	Cellulose used per amount of enzyme[d]
Saccharification (30 FPU)	14.27	-	-	0.33	15.07
Saccharification (150 FPU)	31.94	-	-	0.15	6.75
SSF (30 FPU)	-	10.44	24.06	0.55	25.41
SSF (90 FPU)	-	11.46	26.41	0.20	9.30
Semi-batch SSF (30 FPU)					
1st	-	10.2	23.5	0.54	24.81
2nd	-	7.0	16.13	0.37	17.03
3rd	-	4.9	11.29	0.26	11.29
Total					53.13

[a]Concentration (g/l).
[b]For SSF process, equivalent glucose concentration was calculated as ethanol concentration divided by the ethanol yield of *B. custersii* H1-55 $Y_{p/s}$=0.434 at 42°C.
[c]Equivalent glucose yield was defined as the amount of equivalent glucose produced per gram of cellulose and enzyme amount.
[d]Amount of cellulose used was defined as the equivalent glucose multiplied by 1.056 per enzyme amount (the coefficient, 1.12 account for the incorporation of water molecules).

feasiblilty of cellulose utilization for ethanol production, and the SSF processes could be compared to the straight saccharification of cellulose. The highest equivalent glucose yield based on the enzyme activity was obtained in the SSF process with a cellulase activity of 30 FPU/g wood chips. This showed an increase of about 67% over the straight saccharification with the same enzyme activity. Therefore, the SSF process could be an alternative for producing ethanol from lignocellulosic biomass because of a higher rate and yield. The ultrafiltration system adapted to the SSF and semi-batch SSF was more economical in the view of enzyme utilization as shown in Table 3. Cellulose used per amount of enzyme was defined as the equivalent glucose concentration multiplied by 1.12 which accounts for the incorporation of water molecules. The total amount of cellulose used was 53.13 g. This value was higher by factors of 3.53 and 2.09 than those of a straight saccharification and SSF. Because the enzyme could be reused three times in this system, the cost of enzyme loading for ethanol production could be reduced. Overall, these results indicate that the semi-batch SSF with ultrafiltration system may be suitable for the conversion of lignocellulosic biomass to ethanol.

Acknowledgments

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