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# Novel SSF Process for Ethanol Production from Microcrystalline Cellulose Using the δ-Integrated Recombinant Yeast, *Saccharomyces cerevisiae L2612δGC*

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**Abstract** A novel simultaneous saccharification and fermentation (SSF) process from the microcrystalline cellulose to ethanol was developed by using δ-integrated recombinant cellulolytic *Saccharomyces cerevisiae L2612δGC,* which can utilize cellulose as carbon and energy sources. The optimum amount of enzymes needed for the efficient conversion of cellulose to ethanol at 30°C was determined with commercial cellulolytic enzymes. By fed-batch cultivation, the heterologous cellulolytic enzymes were accumulated up to 42.67% of the total cellulase and 29% of the β-glucosidase needed for the efficient SSF process. When this δ-integrated recombinant yeast was applied to the successive SSF step for ethanol production, 20.35 g/l of ethanol was produced after 12 h from 50 g/l of microcrystalline cellulose. By using this novel SSF process, a considerable amount of commercial enzymes was reduced.

**Key words:** *Saccharomyces cerevisiae*, δ-integration, SSF process, microcrystalline cellulose, ethanol, fed-batch culture

Ethanol production from cellulosic materials has been widely studied. Among various approaches, the SSF process was found to be the most efficient way for producing ethanol from cellulosic materials [9, 24]. The SSF process is a combination of enzymatic saccharification and microbial fermentation. Therefore, in comparison with separate hydrolysis and fermentation processes, the SSF process makes it possible to cut the cost of ethanol production by the reduction in the number of reactors needed, as well as to enhance the hydrolysis rate of cellulose by removing product inhibition [10]. On the contrary, a major drawback of the SSF process is the discrepancy between optimal temperatures of commercial cellulases and ethanol-producing microorganisms. the However, the majority of the research on this subject focused mainly on the development of thermotolerant microorganisms [3, 4, 20]. If the SSF process with mild temperature can overcome this discrepancy without affecting the operational performance, this type of process can definitely be cost-effective in saving a large amount of ethanol production expenses by reducing the energy input. Another advantage of the SSF operation in mild temperature is the maintenance of the optimal viability of microorganisms for ethanol production.

Aside from the temperature discrepancy, the cost of commercial cellulase enzymes in the SSF process is still known to be a major barrier for the economically feasible production of ethanol from cellulose [23, 27]. In some bacterial and yeast strains, genes of cellulolytic enzymes were cloned and introduced to eliminate the need for commercial enzymes by using genetic engineering methods [1, 2, 13, 27]. Recently, cellobiose-utilizing yeast [17] and cellulolytic recombinant yeast strain, *Saccharomyces cerevisiae L2612δGC* [5], were developed by using a δ-integration method [26]. This recombinant yeast was found to utilize cellulose as a carbon and energy source necessary for growth, but the expression level did not reach to the sufficient level needed to convert the carbon flux from cellulose directly into ethanol. Therefore, for ethanol production from cellulose using this recombinant yeast, the supplementation of commercial enzymes is inevitable.

In the present study, a novel SSF process was suggested based on a two-step process for the minimal usage of commercial enzymes needed. First, the fed-batch cultivation of the recombinant yeast for accumulating cellulolytic recombinant enzymes was performed. Then, SSF operation was incorporated in order to convert cellulose directly to ethanol with the reduced amount of commercial enzymes. This new SSF process was developed under the temperature

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of 30°C. Fed-batch cultivation before the SSF operation may increase some production cost. However, the advantages of our new process, including low energy requirement and short operation time, can counterbalance the cost derived from the fed-batch operation.

## MATERIALS AND METHODS

### Recombinant Yeast

The cellulolytic recombinant yeast strain, *S. cerevisiae L2612δGC*, was constructed with the δ-integration method. As shown in Fig. 1, this vector (pδ-neoGC) carries bifunctional endo/exo-glucanase and β-glucosidase genes for the expression of cellulolytic enzymes. The developed recombinant yeast was found to have about 40 copies of these genes in its chromosomes [5].

### Media Composition and Experimental Conditions

The modified Semi Synthetic Minimal Medium (SSMM) was used for the ethanol production from cellulose. The composition of the SSMM was 3 g/l of yeast extract, 5 g/l of bacto-peptone, 2 g/l of $(NH_4)_2SO_4$, 1 g/l of $KH_2PO_4$, 1 g/l of $MgSO_4 \cdot 7H_2O$, 0.1 g/l of NaCl, 0.1 g/l of $CaCl_2 \cdot 2H_2O$, along with an appropriate amount of carbon source. Microcrystalline cellulose (Avicel PH101, Fluka Chem.) was used as a substrate for ethanol production.

Optimization of the amounts of enzymes needed for the SSF process was carried out in 300-ml shake flasks at 150 rpm with 50 g/l of Avicel in the SSMM media (100 ml). All the flasks were inoculated with the same amount of recombinant yeast which was grown overnight. For the ethanol production by this novel SSF process with the recombinant yeast, all the experiments were made in a 3-l fermentor (Bioflo IIc, New Brunswick Scientific) equipped with an on-line vent gas analyzer (FOCA-1, TOA Electronics Ltd.). As a result, on-line monitoring of vent gas was made possible by interfacing with the computer. Seed culture was made from the frozen stock in 100 ml SSMM containing 20 g/l of glucose, maintained for 24 h at 30°C, 300 rpm. The seed was then transferred to a fermentor containing 1.4 l of SSMM with 20 g/l of glucose. As soon as glucose was exhausted, the fed-batch cultivation immediately began. The concentrated SSMM (two-folds) containing 200 g/l of glucose was used as a feed medium for the fed-batch cultivation. The pH of all the culture processes was controlled at 5.5.

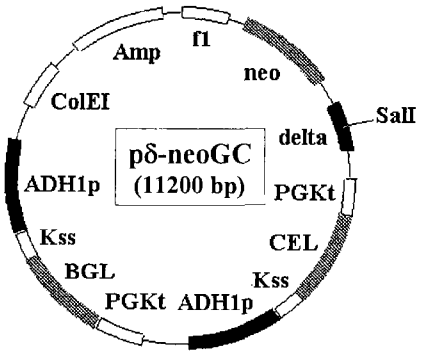


**Fig. 1.** Plasmid map of the δ-integrative vector, pδ-neoGC.
$ADH1_p$, Promoter of alcohol dehydrogenase 1; $K_{ss}$, Signal sequence of killer toxin; BGL, β-glucosidase gene; $PGK_t$, Termination sequence of phosphoglycerate kinase; CEL, Endo/exo-glucanase gene; delta, δ-sequence of Ty1; neo, G418 selection marker.

### Enzymes

The commercial cellulase (Celluclast 1.5 l, Novo Nordisk Ltd.) from *Trichoderma reesei* and β-glucosidase (Novozyme-188, Novo Nordisk Ltd.) from *Aspergillus niger* were utilized for the compensation of the deficits in enzyme activities during the SSF process. Their volumetric activities were 60 filter paper units (FPU)/ml and 45 IU/ml, respectively.

### Measurements of Cell Mass, Glucose, and Ethanol

The cell mass was measured with a spectrophotometer (Ultraspec III, Pharmacia LKB) at 600 nm. The measured OD value was converted to dry cell mass using a pre-determined calibration curve. Yeast cells can be separated from the solid fraction of cellulose by centrifugating at 5000 rpm for 1 min. The glucose concentrations in the culture broth were measured with a glucose analyzer (YSI2700, YSI Inc.). The ethanol concentration was determined with GC-FID (Shimadzu GC-14B). The temperatures of the column, injector, and detector were 180°C, 220°C, 220°C, respectively. The gas pressures of air, hydrogen, and helium were controlled at 50 kPa, 50 kPa, 150 kPa, respectively. In addition, an internal standard using isopropanol (10 g/l) was used.

### Determination of Cellulolytic Enzyme Activities

Total cellulase activity was determined by measuring the amount of total reducing sugars derived from the hydrolysis of Whatman filter paper [28]. The reaction mixtures consisted of 1 cm×6 cm of filter paper, 700 μl of 10 mM potassium phosphate buffer (pH 5.8), and 300 μl of cultured broth after centrifugation. This mixture was then incubated at 30°C for 30 min with shaking. After that, the total amount of reducing sugar derived from the hydrolysis of filter paper was determined by the DNS method [15]. One filter paper unit (FPU) was defined as the amount of enzyme required for releasing reducing sugars equivalent to 1 μmol of glucose per min.

The β-glucosidase (cellobiase) activity was determined by measuring the amount of glucose derived from cellobiose. The reaction mixtures consisted of 100 μl of 50 g/l of cellobiose, 600 μl of 10 mM potassium phosphate buffer (pH 5.8), and 300 μl of cultured broth after centrifugation.

The mixtures were incubated at 30°C for 30 min, and then the glucose derived from the hydrolysis of cellobiose was determined by the DNS method. One unit of the cellobiase activity was defined as the amount of enzyme required for releasing 1 μmol of glucose per min.

### Determination of the Optimum Amounts of Cellulolytic Enzymes

In the enzymatic hydrolysis of cellulose, the amount of fermentable sugars as degradation products increases with time, but on the other hand, it can reach its saturation points within 24 h [19]. For the purpose of determining the optimum amount of cellulolytic enzymes, SSF operations with various amounts of commercial enzymes were performed by inoculating 10 ml of the seed culture. The amount of one enzyme was varied in the condition that the amount of the other enzyme remained sufficient. This sufficient amount (total cellulase = 40 FPU/g-cellulose, β-glucosidase = 30 IU/g-cellulose) was determined from many previous reports [16, 19, 22, 29]. In this way, we can determine the optimum amount of each enzyme. The initial cell mass in these SSF operations was 0.54 g/l. After 24 h, the amount of ethanol produced from each SSF operation was analyzed and compared to determine the optimum values.

## RESULTS AND DISCUSSION

### Optimum Amounts of Enzymes Needed for the Efficient SSF Process

Table 1 showed the experimental result for the determination of the optimum cellulolytic enzymes. After considering the relative amount of the produced ethanol to the amount of enzymes used, the optimum values for total cellulase and β-glucosidase activities in the SSF process under the experimental conditions were identified to be 30 FPU/g-cellulose and 20 IU/g-cellulose, respectively. These optimum values were found to be somewhat higher than those of the processes previously done at higher temperatures, as can be seen in Table 2.

**Table 1.** Simultaneous saccharification and fermentation results for the determination of optimum amounts of enzymes.

| Total cellulase (FPU/g-cellulose) | β-glucosidase (IU/g-cellulose) | Ethanol[a] (g/l) | Yield[b] (%) | Relative amount of enzymes to the produced ethanol[c] |
|---|---|---|---|---|
| 10 | 30 | 12.54±1.10 | 44.15±3.87 | too low yield |
| 20 | 30 | 16.32±0.50 | 57.46±1.76 | too low yield |
| 30 | 30 | 19.94±0.75 | 70.21±2.64 | 0.6647 |
| 40 | 30 | 19.84±1.05 | 69.86±3.70 | 0.4960 |
| 50 | 30 | 20.12±0.45 | 70.85±1.58 | 0.4024 |
| 40 | 10 | 16.36±0.85 | 57.61±2.99 | too low yield |
| 40 | 20 | 19.64±0.90 | 69.15±3.17 | 0.9820 |
| 40 | 30 | 19.84±1.05 | 69.86±3.70 | 0.6613 |
| 40 | 40 | 19.88±1.10 | 70.00±3.87 | 0.4970 |
| 40 | 50 | 20.01±0.55 | 70.46±1.94 | 0.4002 |

[a]Ethanol analysis was done after 24 h. All the experiments were done in triplicate.
[b]Yield was the relative percentage for the theoretical ethanol yield on cellulose (0.568).
[c]Relative amount was the relative value of ethanol concentrations by dividing each amount of enzymes.

**Table 2.** Comparison of the amount of commercial enzymes used in previous reported SSF processes.

| Cellulosic substrate | (g/l) | Temp. (°C) | Operation time (h) | Microorganism | Total amount of commercial enzymes (FPU/g-cellulose) | Ethanol (g/l) yield[a] (%) | Reference |
|---|---|---|---|---|---|---|---|
| α-cellulose | 60 | 38 | 100 | *S. cerevisiae* | 25.0 | 10 (29.34) | [17] |
| Poplar wood | 60 | 38 | 80 | *S. cerevisiae* | 25.0 | 10 (47.62) | [16] |
| Pulp sludge | 50 | 30 | 30 | *S. cerevisiae* | 10.0 (100) | 10.6 (42) | [15] |
| Pretreated willow | 75 | 37 | 72 | *S. cerevisiae* | 18.0 (12.5) | 28.7 (84) | [8] |
| Sigmacell 50 | 100 | 37-43 | 72 | *S. cerevisiae* | 13.0 | 30 (60) | [19] |
| Woody crops | 75 | 37 | 72 | *S. cerevisiae* | 26.0 (208) | 21 (86) | [20] |
| Solka Floc | 100 | 37-42 | 100 | *Z. mobilis* | 24.5 | 32 (64) | [11] |
| Sugar cane bagasse | 160 | 35 | 168 | recom. *K.oxytoca* | 20.0 | 39.2 (73) | [7] |
| Dried grass | 10 | 30 | 72 | Fusant M14 | - | 0.17 (3.40) | [21] |
| Pretreated cellulose | 50 | 34 | 96 | *F. oxysporum* | - | 14.5 (53.9) | [6] |
| Avicel | 2.5 | 60 | 30 | *C. thermocellum* | - | 0.55 (44) | [13] |
| Avicel | 50 | 30 | 12(31[b]) | recom. *S. cerevisiae* | 17.2 (14.2[c]) | 20.35 (71.65) | This study |

[a]Yield was calculated based on the cellulose content in each cellulosic material.
[b]In case of including the fed-batch operation, the total SSF operation time would be 31 h.
[c]The figure in parenthesis is the amount of β-glucosidase.

## Design of a Novel SSF Process

For the efficient application of the δ-integrated recombinant yeast to ethanol production from cellulosic materials, an appropriate SSF process design must be preceded. This new SSF process must have a focus on the mimimal use of commercial enzymes by maximizing production of heterologous cellulolytic enzymes. Usually, fed-batch cultivation was used for the maximization of the recombinant proteins in yeast [12]. For this purpose, optimized fed-batch cultivation was chosen as the first step in the new SSF process. When the volume of the fermentor reached the limited process constraint (2 l), an accumulation of heterologous enzymes by fed-batch cultivation was discontinued. At this point, cellulose and the deficit amount of commercial enzymes were added in order to initiate the second step of the SSF operation. The schematic diagram of this new SSF process is depicted in Fig. 2. This novel SSF process was used for producing ethanol from microcrystalline cellulose.

## Fed-Batch Cultivation for Enzyme Accumulation

To reduce the amount of commercial enzymes used for the SSF process, an accumulation of cellulolytic recombinant enzymes was preceded by the fed-batch cultivation of the δ-integrated recombinant yeast, *S. cerevisiae L2612δGC*. The complete-step operation was started in the batch mode. After 7.67 h from the start of cultivation, the concentration of carbon dioxide from the on-line ventgas analysis system began to decrease drastically as shown in Fig. 3(a). This sharp drop indicated that glucose was in the starvation state. This point was selected as a turn-over time from the batch to the fed-batch mode. The concentrated feed medium was then fed to the fermentor with the determined feed rate as shown in Fig. 3(b). This exponential feeding strategy was derived from the calculation of glucose consumption rate during the exponential growth phase. This exponential feeding was replaced by subsequent linear feeding because there were some divergences between the predicted and the analyzed values of the glucose concentration that was observed after 12.67 h. During this fed-batch cultivation, glucose concentration was monitored every 15 min with the help of the glucose analyzer. Using this feeding strategy, glucose concentration in the culture broth was made possible to be maintained near 2.42 g/l as shown in Fig. 3(c), which was found to be the optimum

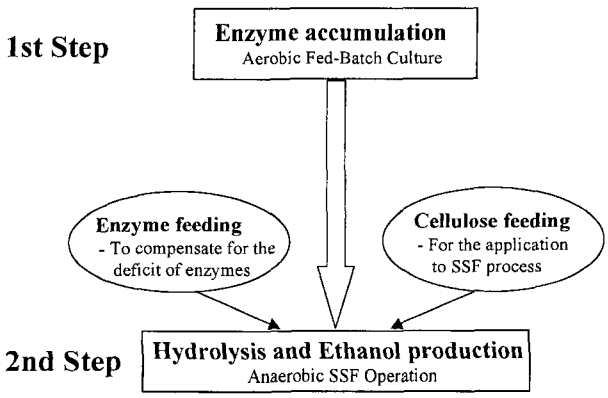


**Fig. 2.** Schematic diagram of the novel SSF process designed for this study.

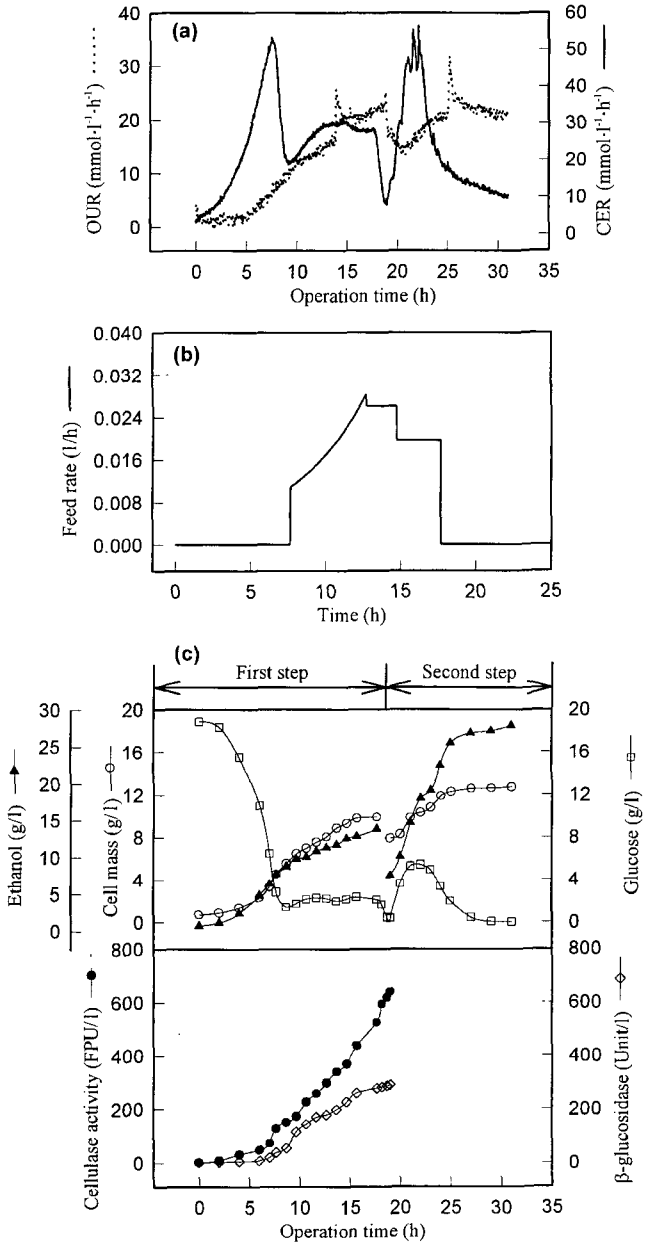


**Fig. 3.** Result of the novel SSF process for ethanol production from microcrystalline cellulose using the δ-integrated cellulolytic recombinant yeast, *Saccharomyces cerevisiae L2612δGC*.
(a) Time courses of the oxygen uptake rate (OUR) and $CO_2$ evolution rate (CER). (b) Feed rate profile during fed-batch cultivation in the process. (c) Time courses of cell mass, glucose, ethanol, and foreign cellulolytic enzymes.

concentration for the productivity of heterologous enzymes (unpublished data).

According to this controlled fed-batch cultivation of the δ-integrated recombinant yeast, *S. cerevisiae L2612δGC*, an accumulation of approximately 640 FPU/l of total cellulase and 290 IU/l of β-glucosidase activities was apparent, and this is shown in Fig. 3(c). What this means is that 42.67% of total cellulase (FPU) and 29% of β-glucosidase, which are needed for the efficient hydrolysis of cellulose, can be saved in the subsequent SSF process. The actual amount of commercial enzymes used in this study was 17.2 FPU/g-cellulose, 14.2 IU/g-cellulose as described in Table 2. When optimization of the first fed-batch cultivation step with an appropriate on-line control strategy is made, more recombinant cellulolytic enzymes may be accumulated. As a result, the cost of ethanol production from cellulose can be further reduced. In addition, this cost saving can be made possible by enhancing the genetic potential of the recombinant yeast.

### Cellulose Hydrolysis and Ethanol Production Using the δ-Integrated Recombinant Yeast

The fed-batch cultivation of the recombinant yeast for the accumulation of heterologous enzymes was discontinued when the working volume reached 2.0 l. After confirming the depletion of the residual glucose, 100 g of microcrystalline cellulose (Avicel PH101) dissolved in 200 ml of 50 mM citrate buffer (pH 4.8) and supplementary amounts of commercial enzymes (17.2 FPU/g-cellulose, 14.2 IU/g-cellulose of β-glucosidase) was added for the SSF process. These commercial enzymes were determined from the difference between the optimal amount of cellulolytic enzymes and the produced heterologous cellulolytic enzymes from the fed-batch cultivation. The result of the SSF process with 50 g/l of Avicel was also presented in Fig. 3(c). The decrease in the concentrations of cell mass and ethanol just before the SSF step was due to the dilution caused by adding cellulose as a substrate. As soon as the SSF operation got started, a significant increase in glucose concentration was detected. However, hydrolysis of cellulose ceased at around 27 h. From this time, no significant amount of glucose was observed. Along with the hydrolysis of cellulose to glucose, ethanol was produced rapidly. The ethanol production reached its saturation point at near 29 h, only 10 h after SSF began. The final ethanol yield on cellulose was about 0.41 (71.65% of the theoretical value).

In this study, the SSF process was conducted at 30°C for the recombinant yeast, in spite of some loss in the activities of commercial enzymes. However, in comparison with other SSF processes conducted at higher temperatures as shown in Table 2, the actual amount of commercial enzymes could be considerably reduced by using the recombinant cellulolytic yeast. Also, the ethanol production rate was higher than any other previous SSF process reported so far (Table 2). This higher rate of ethanol production may be due to the higher viability of the recombinant yeast at 30°C, in addition to the high concentration of the recombinant yeast derived from the fed-batch cultivation.

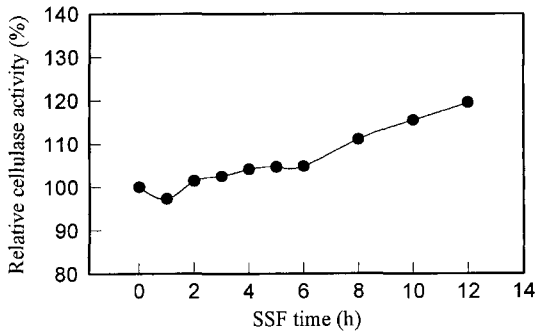


**Fig. 4.** Relative activity of the total cellulolytic enzymes during the SSF process.

The relative activities of the cellulolytic enzymes during the SSF operation were checked in order to identify the degradation of the cellulolytic enzymes. This relative activity was calculated from the relative ratio between the activity of each time and that of the initial time. From the analysis of relative enzyme activities as shown in Fig. 4, it was clear that this saturation kinetics in the ethanol production from cellulose was not caused by deactivation of enzymes but rather from the intrinsic digestibility of the cellulosic substrates. This digestibility of the cellulose depends on steps prepared for cellulosic materials. Therefore, for the enhancement of substrate utilization in addition to ethanol yield, development of the better pretreatment method must be made. The gradual increase in the activities of the cellulolytic enzymes during the SSF process was considered to be the successive expression of the cellulolytic genes by the recombinant yeast. This increase in cellulolytic enzymes with time presented the possibility of the SSF operation in fed-batch mode.

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