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Invertase Production by Fed-batch Fermentations of Recombinant *Saccharomyces cerevisiae*

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Abstract Fed-batch fermentations with different feeding media were carried out in order to increase the productivity of invertase expression using a recombinant *Saccharomyces cerevisiae* containing plasmid pRB58. Two batch cultures showed the expression of the *SUC*2 gene at a low concentration of glucose, suggesting that glucose concentration could be used as a control variable in a fed-batch operation mode. In the fed-batch culture by feeding the basal medium, cell mass and specific invertase activity did not increase much as compared with the simple batch culture. A series of fed-batch cultures revealed that the sucrose-supplemented medium increased cell mass whereas the enriched medium did specific invertase activity. To capitalize on the synergism of the sucrose-supplemented medium and the enriched medium, the sucrose-supplemented enriched medium was used as a feeding medium. The fed-batch culture using this medium resulted in a 2.4-fold increase in cell mass and a 1.9-fold enhancement in specific invertase activity compared with those of the batch culture. The increase in cell mass and specific invertase activity led to a marked increase in total invertase activity, 250 U/ml, which was 6.3 times higher than that of the batch culture.

Key words: Recombinant *Saccharomyces cerevisiae, SUC* 2, invertase, fed-batch fermentation

Invertase (β-D-fructofuranoside fructohydrolase; EC.3.2.1.26), which catalyzes the hydrolysis of sucrose to glucose and fructose, is produced by *SUC*1~7 genes in yeast. The extracellular glycosylated invertase consists of 50% carbohydrate and 50% protein with a molecular weight of 270,000 daltons. The extracellular enzyme is stable in a range of pH 3 to 7.5 [4].

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Saccharomyces cerevisiae as a host in genetic technology has some advantages such as wealth information in molecular genetics and microbial physiology, easy gene manipulation, fast cell growth, and nonpathogenic characteristics. Hepatitis B surface antigen (HBsAg), vaccine, and other proteins have been industrially produced in *S. cerevisiae* [14]. *S. cerevisiae* provides additional benefits such as glycosylation, post-translational folding, processing, modification, and secretion of proteins [1, 9].

A wild-type of *S. cerevisiae* has at least one among *SUC*1~7 genes coding for invertase . The *SUC*2 gene could be transcribed to either 1.9 kb mRNA or 1.8 kb mRNA. The former is glycosylated and includes the secretion signal sequence whereas the latter is not glycosylated and does not have the secretion signal sequence. As a result, the invertase expressed by the former can be secreted out of cells whereas the invertase by the latter cannot. The expression of the former is inhibited by a high concentration of glucose and is stimulated below 2.0 g/l of glucose, whereas that of the latter is independent of glucose concentrations [13]. Invertase production from the *SUC*2 gene in batch and fed-batch cultures has been extensively studied [7, 8, 11].

In this study, batch and fed-batch cultures were carried out to produce invertase using recombinant *Sacchromyces cerevisiae* SEY2102 containing plasmid pRB58, the *SUC*2 gene expression vector. In order to increase a total invertase activity, an increase in specific invertase activity and cell mass was attempted by varying medium compositions in fed-batch cultures.

MATERIALS AND METHODS

Yeast Strain and Plasmid

The yeast strain used in this study was *Saccharomyces cerevisiae* SEY2102 (MATα, *ura*3-52, *leu*2-3, 112 *his*4-

159), α-haploid and auxotroph of uracil, leucine, and histidine. The strain could not produce invertase without plasmid since its chromosome did not have any *SUC* genes.

The recombinant plasmid pRB58 used as an expression vector of the *SUC2* gene was constructed by Carlson and Botstein [2]. The plasmid consists of the *SUC2* promoter, the signal sequence and the structural gene. It contains the 2 μm origin of replication, the URA3 marker gene to produce uracil, and the ampicillin marker used for selection pressure. The plasmid pRB58 [2] is a derivative of SEY2102 which is a shuttle vector for *Escherichia coli* and *S. cerevisiae* [3].

Media and Culture Conditions

The basal medium consisted of 10 or 20 g/l of glucose, 6.67 g/l of yeast nitrogen base without amino acids, and 5.0 g/l of casamino acid [12]. The composition of the enriched medium used for feeding medium in fed-batch fermentations is shown in Table 1 [10].

A single colony was inoculated into a test tube containing 5 ml of the basal medium and cultivated at 30°C for 24 h. The culture broth was transferred to a 500 ml-baffled flask containing 100 ml of the basal medium and cultivated for 15 h. Pre-culture broth was transferred to the main culture medium with a ratio of 2% (v/v). Batch and fed-batch cultures were carried out in a 5-l fermentor (Korea Fermentor Co., Korea). A working volume for batch cultures was 3 l and an initial volume for fed-batch cultures was 2.5 l. The pH of all cultures was controlled at 5.5 with 1.0 M phosphoric acid and 1.0 M ammonium hydroxide. Agitation speed was gradually increased to prevent the limitation of dissolved oxygen. A medium feeding rate in fed-batch cultures was regulated to maintain a glucose concentration below 1.0 g/l.

Table 1. Composition of the enriched medium [10].

Component	Concentration
KH_2PO_4	10.0 g/l
$CaCl_2 \cdot 7H_2O$	0.50 g/l
NaCl	0.50 g/l
$MgSO_4 \cdot 7H_2O$	3.00 g/l
$FeSO_4 \cdot 7H_2O$	8.34 mg/l
$ZnSO_4 \cdot 4H_2O$	8.64 mg/l
$CuSO_4 \cdot 4H_2O$	2.40 mg/l
$Na_2MoO_4 \cdot 2H_2O$	7.64 mg/l
$CoCl_2 \cdot 6H_2O$	7.14 mg/l
$MnSO_4 \cdot 4H_2O$	5.07 mg/l
Biotin	0.20 mg/l
Ca-pantothenate	3.60 mg/l
Inositol	12.0 mg/l
Pyridoxine-HCl	3.60 mg/l
Thiamine-HCl	3.60 mg/l

Invertase Activity Assay

Cells from the culture broth in the fermentor were harvested by centrifugation at 5,000×g for 10 min. After washing twice with 0.2 M sodium acetate buffer (pH 4.9), the cells were disrupted by grinding in a vortex for a total time of 3 min. Cell homogenates were centrifuged at 5,000×g for 10 min and the supernatant solution was used for the measurement of invertase activities. After 300 μl of 0.2 M sodium acetate buffer (pH 4.9) and 300 μl of 0.5 M sucrose solution were transferred to a test tube prewarmed at 30°C, the reaction was started by adding 100 μl of crude invertase to the reaction mixture at 30°C. After 10 min, the reaction was stopped by adding 2.0 ml of 0.5 M phosphate buffer and boiling for 3 min. Invertase activity was determined by measuring glucose concentration generated by the hydrolytic reaction of sucrose. One unit of invertase activity was defined as one μmole of glucose produced per minute under the reaction conditions [5].

Analytical Methods

A dry cell concentration was estimated by using a calibration curve obtained from the relationship between optical density at 600 nm and dry cell mass. A glucose concentration was determined by a glucose kit (Sigma Chemical Co., U.S.A.) and glucose analyzer (Yellow Spring Instruments 1500 Sidekick, U.S.A.).

Results and Discussion

Invertase Production in Batch Cultures

Two batch fermentations were performed by using different initial glucose concentrations, which results were shown in Figs. 1 and 2. After a short lag period, the cells grew rapidly in the first exponential phase by using glucose as a carbon source. The maximum specific growth rate of 0.35 h^{-1}, dry cell mass of 6.58 g/l, and total invertase activity of 40.0 U/ml were attained. Since the glucose concentration in the medium was high, the cells utilized the fermentative pathway of glucose metabolism, resulting in ethanol production (data not shown). Invertase activities were determined from the cell samples taken during the fermentation. For acceptable accuracy in the invertase assay, a minimum optical density of about 0.5 is required. If the cell concentation is below this level, the pellet obtained after centrifugation is very small and difficult to handle. Thus, for the first few samples, invertase assay was not performed. When the initial glucose concentration was increased from 10 to 20 g/l, the dry cell weight increased by 20% but the specific invertase activity (SIA) decreased by 55%, and consequently the total invertase activity decreased. As noted before, the expression of the yeast chromosomal *SUC2* gene is

repressed at high glucose concentrations. The batch fermentations showed that the same is true for the cloned *SUC2* gene in the plasmid pRB58. At the beginning of the fermentation, where the glucose concentation was high, the SIA was very low. Near the end of the first exponential phase, where a glucose concentration dropped to a very low value of 2 g/l, the SIA increased sharply, indicating the derepression of the *SUC2* promoter. Thus, a glucose concentration must be controlled below 2.0 g/l for efficient invertase expression.

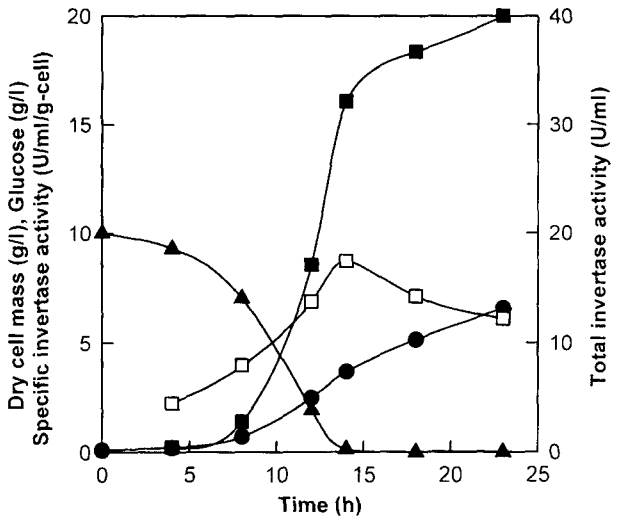

Fig. 1. Invertase production by batch fermentation of recombinant *Saccharomyces cerevisiae* in the basal medium containing 10 g/l glucose.
●, dry cell mass; ▲, glucose; ■, total invertase activity; □, specific invertase activity.

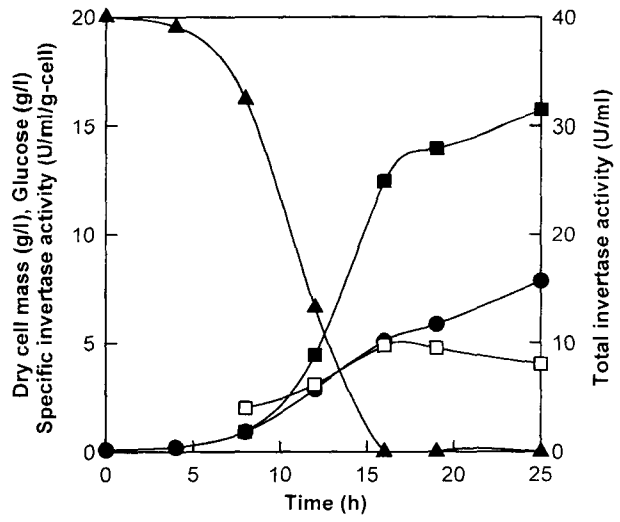

Fig. 2. Invertase production by batch fermentation of recombinant *Saccharomyces cerevisiae* in the basal medium containing 20 g/l glucose.
●, dry cell mass; ▲, glucose; ■, total invertase activity; □, specific invertase activity.

Invertase Production in Fed-batch Cultures by Feeding Different Media

Fed-batch cultures are usually used for controlling a glucose concentration during fermentations. A fed-batch culture using the basal medium containing 10 g/l of glucose was carried out to improve the productivity of invertase expressed in recombinant *S. cerevisiae* containing plasmid pRB58 (Fig. 3). After 14 h of the batch mode, the fed-batch operation was started by feeding the basal medium with 100 g/l of glucose. Glucose concentrations were maintained around 2 g/l during the fed-batch period of 8 h. Unlike batch fermentations, the specific invertase activity was kept constant. As a result, 46.9 U/ml of the total invertase activity was obtained which was a 1.2-fold increase compared with the first batch fermentation.

The fed-batch culture with the basal medium did not increase specific invertase activity. Another fed-batch culture was attempted to increase specific invertase activity by changing a feeding medium to the enriched medium (Fig. 4). The feeding rate was increased from 17.5 to 60.8 ml/h during 13 h of the fed-batch mode with the enriched medium containing 60 g/l of glucose and the glucose concentration was controlled around 0.5 g/l. The feeding of the enriched medium stimulated an increase in specific invertase activity (13.2 U/ml/g-cell) rather than cell growth (8.28 g/l). The total invertase activity was enhanced 2.6 times higher than that of the first batch culture due to the increased cell mass and specific activity.

It was reported previously that specific invertase activity could be increased by feeding a sucrose solution [6]. The sucrose supplemented fermentation was undertaken

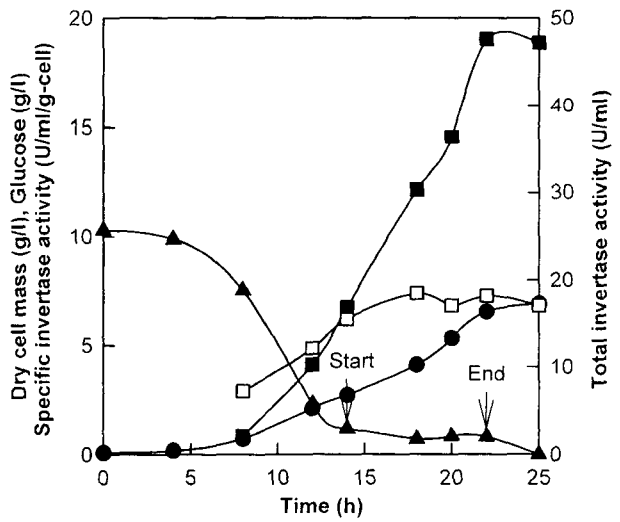

Fig. 3. Invertase production by fed-batch fermentation of recombinant *Saccharomyces cerevisiae* with the basal medium.
Arrows indicate the points of medium feeding at start and end. ●, dry cell mass; ▲, glucose; ■, total invertase activity; □, specific invertase activity.

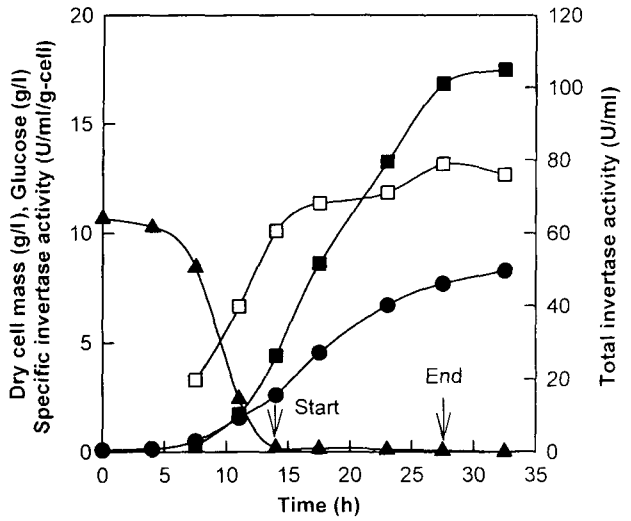

Fig. 4. Invertase production by fed-batch fermentation of recombinant *Saccharomyces cerevisiae* with the enriched medium.

Arrows indicate the medium feeding points at start and end. ●, dry cell mass; ▲, glucose; ■, total invertase activity; □, specific invertase activity.

by feeding the basal medium containing 60 g/l of glucose and 60 g/l of sucrose (Fig. 5). When glucose was depleted at 14 h of fermentation, this medium was fed to control a glucose concentration below 0.5 g/l during 25 h of the feeding period. As a result, 19.8 g/l of cell mass, 11.0 U/ml/g-cell of specific invertase activity, and 218 U/ml of total invertase activity were obtained which were 2.9-, 1.5-, and 4.6-fold increases, respectively, compared with those of the first fed-batch fermentation. In particular, the feeding of the sucrose supplemented medium caused a marked increase of cell mass.

Invertase Production in Fed-batch Culture by Feeding the Sucrose-Supplemented Enriched Medium

The feeding of the enriched medium increased the specific invertase activity whereas the feeding of sucrose-supplemented medium increased cell mass dramatically. To increase both cell mass and specific invertase activity, the sucrose-supplemented enriched medium was used as a feeding medium (Fig. 6). The enriched medium containing 60 g/l of glucose and 60 g/l of sucrose was fed to control a glucose concentration below 0.5 g/l. The feeding of the sucrose-supplemented enriched medium increased cell mass and specific invertase activity 2.4 and 1.9 times, respectively, as compared with those of the first batch culture. The marked increase of the specific invertase activity resulted in 250 U/ml of total invertase activity which was 6.3 times higher than that of the first batch culture.

Kinetic parameters of fermentations to produce invertase in recombinant *S. cerevisiae* are summarized in Table 2. It was found that total invertase activity and productivity of the first fed-batch culture was higher than those of two batch cultures. However, cell mass and specific invertase activity were not increased much. In order to increase both cell mass and specific invertase activity, the feeding media in fed-batch cultures were changed. Cell mass showed a high concentration in the

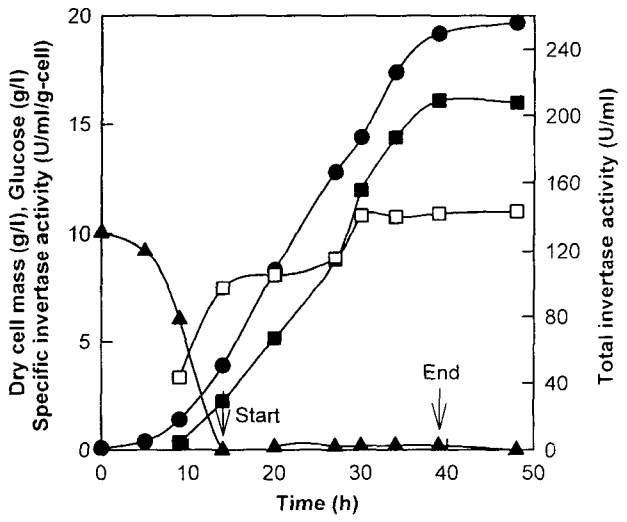

Fig. 5. Invertase production by fed-batch fermentation of recombinant *Saccharomyces cerevisiae* with the sucrose-supplemented medium.

Arrows indicate the medium feeding points at start and end. ●, dry cell mass; ▲, glucose; ■, total invertase activity; □, specific invertase activity.

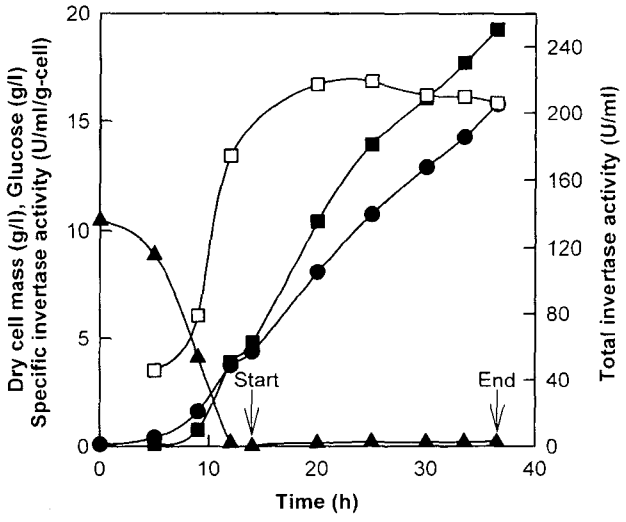

Fig. 6. Invertase production by fed-batch fermentation of recombinant *Saccharomyces cerevisiae* with the sucrose-supplemented enriched medium.

Arrows indicate the medium feeding points at start and end. ●, dry cell mass; ▲, glucose; ■, total invertase activity; □, specific invertase activity.

Table 2. Kinetic parameters of invertase fermentation by recombinant *Saccharomyces cerevisiae* in batch and fed-batch cultures.

Run Medium	Cell mass (g/l)[a]	Specific invertase activity (U/ml/g-cell)[a]	Total invertase activity (U/ml)[a]	Productivity (U/ml/h)
Batch				
Glucose 10 g/l	6.58	8.73	40.0	1.74
Glucose 20 g/l	7.89	4.86	31.6	1.26
Fed-batch (feeding medium)				
Basal	6.89	7.38	46.9	2.16
Enriched	8.28	13.2	105	3.68
Sucrose-supplemented	19.8	11.0	218	5.28
Sucrose-supplemented and enriched	15.8	16.7	250	6.86

[a]Maximum value.

sucrose-supplemented medium whereas the specific invertase activity exhibited a high level in enriched medium. For the synergism of the sucrose supplemented medium in combination with the enriched medium, the sucrose supplemented enriched medium was used as a feeding medium. Feeding this medium significantly improved the total invertase activity of 250 U/ml and the invertase productivity of 6.86 U/ml/h. These numbers correspond to a 6.3-fold increase in total invertase activity and a 3.9-fold enhancement in invertase productivity compared with those of the first batch culture, respectively.

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