

## Enhancement of secreted production of glucoamylase through fed-batch bioreactor culture of recombinant yeast harboring glucose-controllable *SUC2* promoter

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**Abstract**—Glucoamylase that hydrolyses starch to glucose is one of the important industrial enzymes for ethanol production industry. Therefore, genetic production of recombinant glucoamylase has been widely studied. Previously, we reported secreted production of *Saccharomyces diastaticus*-originated glucoamylase in *Saccharomyces cerevisiae* expression system using its own signal sequence and the *SUC2* promoter that is regulated by glucose level in culture medium. In the present work, we performed a comparative study between batch and fed-batch bioreactor cultures for secreted production of recombinant glucoamylase. Through maintaining low glucose levels in the culture broth, we obtained about 7-fold higher secreted production levels of glucoamylase in fed-batch culture. Fed-batch culture strategy also enhanced (~3.1-fold) secretion efficiency of recombinant glucoamylase in *S. cerevisiae*.

Key words: Recombinant Yeast, Glucoamylase, *SUC2* Promoter, Secreted Production, Fed-batch Culture

### INTRODUCTION

The yeast *Saccharomyces cerevisiae* is widely used as a recombinant host for the production and secretion of foreign genes owing to its several advantages [1]. The particular importance of *S. cerevisiae* lies in its wide-spread use in ethanol fermentation. Ethanol production can be divided into three steps: liquefaction that hydrolyzes starch to oligosaccharides using  $\alpha$ -amylase, saccharification that produces glucose using glucoamylase [2] and debranching enzymes, and fermentation that produces ethanol from glucose using yeasts. Glucoamylase (EC 3.2.1.3) is used to saccharify starchy feed stocks in commercial processes for glucose and ethanol production, and is not produced naturally by *S. cerevisiae*. Therefore, many attempts have been made to produce recombinant glucoamylase in *S. cerevisiae*.

Previously, we reported secreted production of recombinant glucoamylase in *S. cerevisiae* [3-6]. The *STA* gene (glucoamylase gene of *Saccharomyces diastaticus*) was chosen as a glucoamylase gene source for the research [7]. Also, the *SUC2* promoter, which is repressed at high glucose concentration and enhanced at low glucose concentration, was employed [8,9]. This *SUC2* promoter has the following advantages. First, since its action can be regulated, common problems such as reduced host cell growth rate and segregational plasmid instability can be reduced. Second, since the *SUC2* promoter is affected only by glucose, the costly addition of an inducer is unnecessary. Third, there is no medium substitution which is necessary for other repressible promoters. Finally, the product yield can be improved by decoupling cell growth from the gene expression phase during the course of fermentation [10]. In addition, the original *STA* signal sequence from yeast *S. diastaticus* glucoamylase was used for glucoamylase secretion in the work because

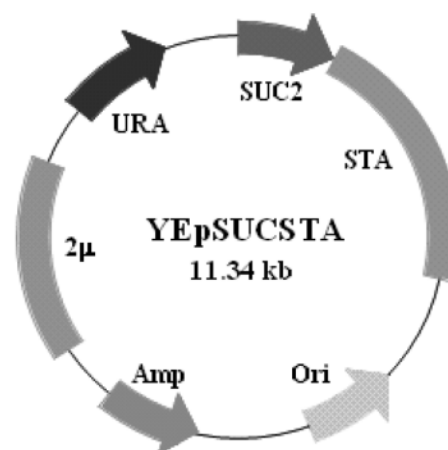


Fig. 1. Gene maps of recombinant plasmid YEpsUCSTA. Abbreviations: *SUC2*, *SUC2* promoter; *STA*, *STA* glucoamylase gene; *URA*, *ura3* gene; *2μ*, yeast replication origin; *Ori*, *E. coli* replication origin; *Amp*, ampicillin resistance gene.

recovery of desired genetic product is difficult due to thick cell wall when working with a eukaryotic organism like yeast.

In the present work, we performed comparative fed-batch bioreactor culture of recombinant *S. cerevisiae* to enhance secreted production of glucoamylase, which takes advantage of the peculiar control mechanism of the *SUC2* promoter by glucose levels.

### MATERIALS AND METHODS

#### 1. Strains and Culture Medium

*S. cerevisiae* MMY2 (*a*, *ura3-52*, *sta0*, *sta10*) strain was used as a host. The 2m yeast-based episomal recombinant plasmid YEps-SUCSTA [11] containing glucoamylase coding the *STA* gene fused with the *SUC2* promoter and the original *STA* signal sequence, were

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transformed into the host. In batch culture, the yeast cells were grown in a complex rich medium (YPD) containing 1% (w/v) yeast extract (Difco, USA), 2% (w/v) bacto-peptone (Difco), and 1% (w/v) glucose. For fed-batch culture, 10% (w/v) glucose was supplemented in the medium when glucose was almost consumed. Cultures were carried out in a 5-L fermentor (KoBiotech, Korea) with a working volume of 3 L. The bioreactor was equipped with all the control devices and pumps required to maintain environmental parameters at their set points. Agitation rate was 350 rpm and temperature was 30 °C. Aeration rate was set at 0.5 vvm (volume of air/volume of liquid/min).

## 2. Analytical Assays for Cell Density and Glucoamylase Activity

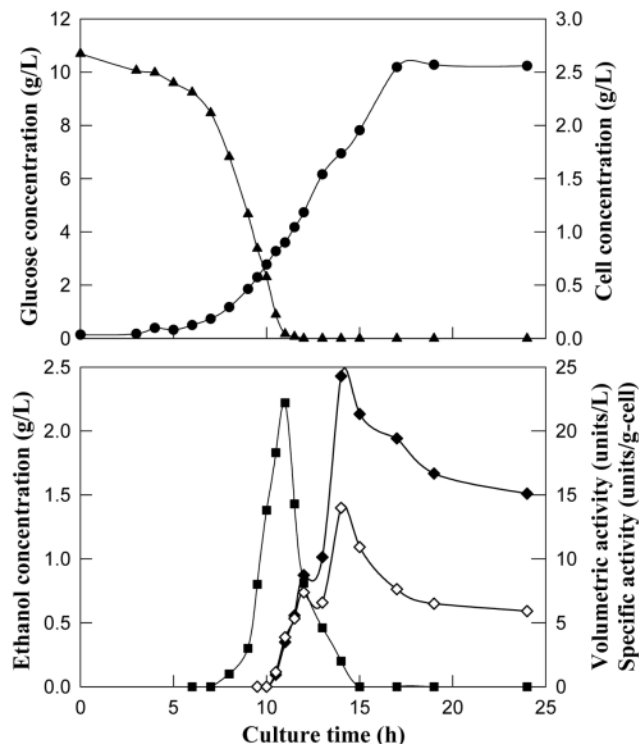
Optical density (OD) of culture at 600 nm was measured with a spectrophotometer (Kontron, Swiss) to determine cell density. Dry cell weights were measured after drying cells in the oven and calibrated with ODs. The conversion factor between OD and dry cell weight was found to be 0.43 g/L·OD. To measure glucoamylase activity, 0.7 mL sample was incubated in 0.1 mL of 1 M sodium acetate buffer (pH 5.0) and 0.2 mL of 8% soluble starch (Junsei Chemical, Japan) at 50 °C for 30 min and boiled at 100 °C for 5 min to inactivate glucoamylase. Glucose produced by the action of glucoamylase on soluble starch was assayed with a glucose-diagnostic kit (Sigma, USA). One unit of glucoamylase activity was defined as a corresponding amount to catalyze the release of 1  $\mu$ mol of glucose per 1 min. Ethanol concentration was measured by gas chromatography (Young Lin Instrument, Korea) equipped with flame ionization detector.

## 3. Cell Fractionation

Cells were harvested and the cleared medium was defined as an extracellular fraction. The harvested cells were washed with 10 mM sodium azide, suspended in a lysis buffer (0.1 M sodium acetate (pH 5.0), 10 mM sodium azide, 1 mM EDTA, and 0.1% v/v Triton X-100) and mechanically lysed by vortexing with glass beads (425–600 microns; Sigma). After intermittent vortexing and cooling on ice, the suspension was harvested by centrifugation with 12,000 rpm at 4 °C for 5 min, and the supernatant was operationally defined as an intracellular fraction. Secretion efficiency was defined as a ratio of extracellular activity per total (extracellular and intracellular) activity.

## RESULTS AND DISCUSSION

Batch and fed-batch cultures of recombinant *S. cerevisiae* were comparatively performed in the complex YPD medium containing 10 g/L glucose as carbon source for cell growth and glucoamylase production. Because we employed the *SUC2* promoter that is down-regulated by glucose concentration in the culture broth [8,9], maintenance of low glucose levels is important for high transcriptional action for this peculiar promoter [4]. Thus, fed-batch culture might be suitable for this system. In the case of batch bioreactor culture, cells showed lag time (~5 h) to initiate their growths and entered exponential phase until glucose concentration was depleted at around 11 h (Fig. 2A). However, the cells showed diauxic-type growth by utilizing 'by-product' ethanol (Figs. 2A vs 2B). After depletion of ethanol as carbon source at ~16 h, the cells finally entered stationary phase. Because we used the glucose-controllable *SUC2* promoter,



**Fig. 2. Time profiles for batch reactor culture of recombinant yeast.** Complex rich medium containing 1% (w/v) glucose was used. Symbols; ●: cell concentration, ▲: glucose concentration, ■: ethanol concentration, ◆: volumetric glucoamylase activity, ◇: specific glucoamylase activity. Each value represents the mean of two independent experiments.

glucoamylase expression was repressed in early culture stage, cells grew through consuming glucose, and secreted expression of glucoamylase was begun at around 11 h, which is coincident with glucose depletion (Fig. 2B). After depletion of all energy sources (glucose and ethanol), glucoamylase activity in the extracellular medium was decreased. This reduction might be due to stop of glucoamylase synthesis and proteolysis. Through batch culture of recombinant yeast in 10 g/L glucose medium, we obtained maximum volumetric glucoamylase activity of 24 units/L and maximum specific glucoamylase activity of 14 units/g-cell. For fed-batch bioreactor culture of recombinant yeast, feeding with 100 g/L glucose was started at the point of glucose depletion (~12 h) to maintain a low level (0.1–0.4 g/L) in the medium and finished at 21 h (Fig. 3A). During feeding periods, cell growth was slightly slower than that in batch stage and ethanol was continuously produced during all culture periods. Glucoamylase production was also begun late when glucose level was lowered (~12 h) and its level also showed a maximum point at around 19 h (Fig. 3B). Importantly, through fed-batch culture, we obtained significantly higher maximum volumetric glucoamylase activity of 180 units/L (~7.4-folds higher; Fig. 4A) and maximum specific glucoamylase activity of 95 units/g-cell (~6.8-folds higher; Fig. 4B) than that from batch culture.

Several systems of recombinant protein secretion using the *STA* signal sequence have been reported [12,13]. Most of these reports state that the use of *STA* signal sequence was effective in the secretion of recombinant yeast protein. Secretion efficiency is known to

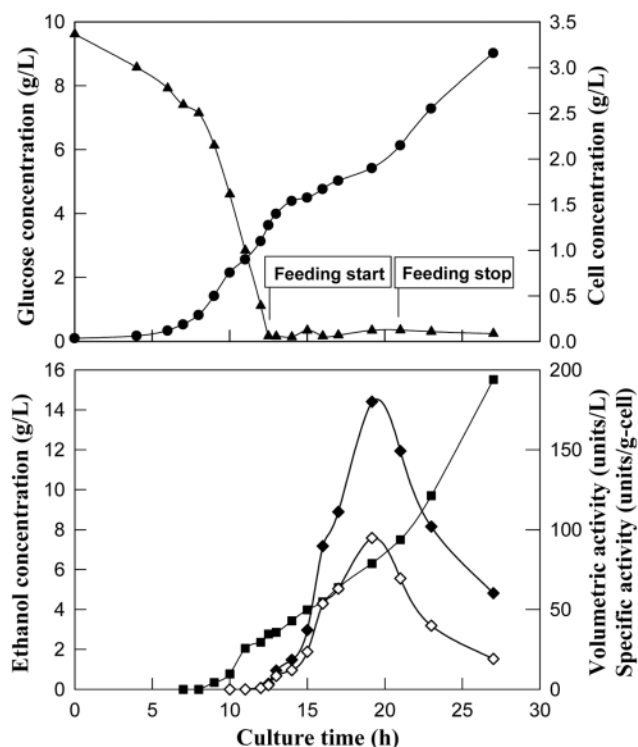


Fig. 3. Time profiles for fed-batch reactor culture of recombinant yeast. Complex rich medium containing 1% (w/v) glucose was used. 10% (w/v) glucose was supplemented in the medium when glucose was almost consumed. Symbols; ●: cell concentration, ▲: glucose concentration, ■: ethanol concentration, ◆: volumetric glucoamylase activity, ◇: specific glucoamylase activity. Each value represents the mean of two independent experiments.

be affected by the type of promoter and secretion signal sequence, signal sequence size, net charge and degree of glycosylation of secreted protein, and the type of used host strains [14]. We compared secretion efficiency of recombinant glucoamylase from batch and fed-batch cultures (Fig. 4C). In this work, we defined secretion efficiency as a ratio of glucoamylase activity in the culture broth per total glucoamylase activity. Both time profiles of secretion efficiencies showed maximum points that were coincident with the points of maximum glucoamylase production. While batch culture showed ~26% of maximum secretion efficiency, fed-batch culture also highly increased secretion efficiency to ~82% (3.1-fold). We surmise that this high increase of secretion efficiency in fed-batch culture might be from continuous synthesis of secretion machinery by supplying energy source and/or change of glycosylation pattern that can facilitate secretion due to the different mode of culture. This enhanced secretion efficiency might also confer higher secreted production of recombinant glucoamylase in fed-batch culture.

In summary, we demonstrated that high enhancement of expression and secretion of glucoamylase from recombinant yeast harboring the glucose-controllable peculiar *SUC2* promoter was possible through maintaining of low glucose levels in the culture broth by fed-batch bioreactor culture strategy. This result can be applied to the fields of useful recombinant protein production and bioethanol fermentation.

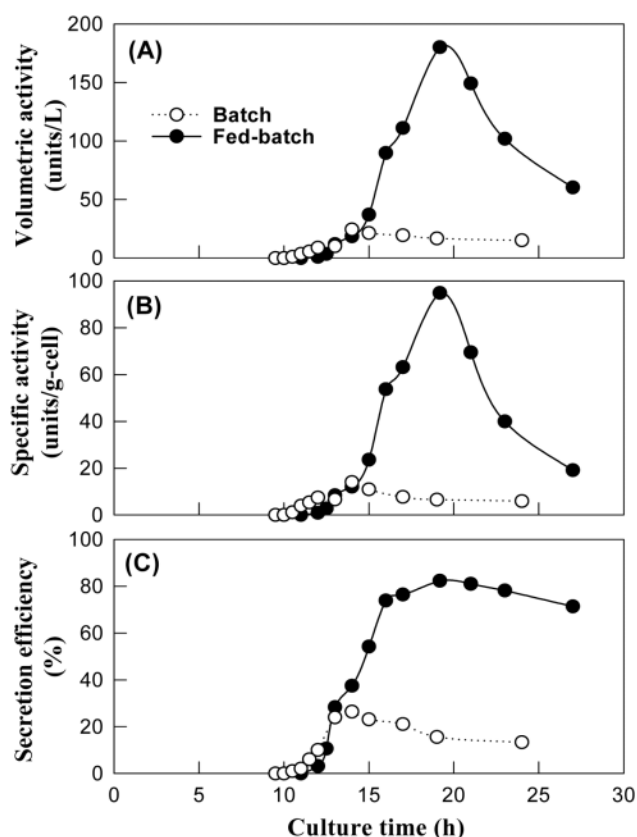


Fig. 4. Comparison of (A) volumetric glucoamylase activity, (B) specific glucoamylase activity, and (C) secretion efficiency in batch and fed-batch cultures. Symbols; ○: batch culture, ●: fed-batch culture. Each value represents the mean of two independent experiments.

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