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Production and Secretion Patterns of Cloned Glucoamylase in Plasmid-Harboring and Chromosome-Integrated Recombinant Yeasts Employing an *SUC2* Promoter

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Abstract

To understand the differences in production and secretion patterns between plasmid-harboring and chromosome-integrated recombinant yeasts, the two recombinant *Saccharomyces cerevisiae* yeasts, containing the structural glucoamylase *STA* gene and the *SUC2* promoter, were investigated. Both systems were regulated by glucose concentration in the culture broth. First, the glucoamylase activity per gene copy number of the chromosome-integrated recombinant yeast was 2.8- to 5.6-fold higher than that of the plasmid-harboring recombinant yeast. Overburdened owing to high copy number, the plasmid-harboring recombinant yeast gave lower glucoamylase activity per gene copy number. Second, the efficiency of signal sequence was compared; the secretion efficiency of glucoamylase in the plasmid-harboring recombinant yeast was higher than that in the chromosome-integrated recombinant yeast at 96 h of cultivation (74 vs 65%). We postulated that the higher level of secretion efficiency of the plasmid-harboring recombinant yeast resulted because the production level did not reach the capacity of the secretory apparatus of the host yeast. However, the specific

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secretion rate was much higher in the chromosome-integrated recombinant yeast even though the final secretion efficiency was lower. The lower secretion rate in the plasmid-harboring recombinant yeast could be explained by an adverse effect caused by higher production rate. Finally, the optimal glucose concentration for glucoamylase production in the chromosome-integrated recombinant yeast culture was lower than that in the plasmid-harboring recombinant yeast culture owing to gene dosage effect.

Index Entries: Plasmid-harboring recombinant yeast; chromosome-integrated recombinant yeast; *SUC2* promoter; glucoamylase; production; secretion.

Introduction

One of the severe problems encountered in the use of plasmid-harboring recombinant cells is their inability to retain cloned genetic information *(1,2)*, causing a decrease in overall productivity. The cell growth and biosynthesis of genetic product depend on plasmid copy number and plasmid stability. Therefore, a strategy to overcome genetic instability is needed and there have been several approaches for this purpose. One of the strategies commonly used is the insertion of a regulated promoter that controls gene expression by changing the composition of the medium, addition of chemicals, or change in cultural conditions *(2)*. By this strategy, a competitive interaction between plasmids and host cells can be minimized. However, one of the most efficient and ideal methods for cloned gene stabilization might be a chromosomal integration of desired genes *(3,4)*.

When working with a eukaryotic organism such as yeast, recovery of desired genetic product is difficult because of its thick cell wall. In recent years, research has therefore been conducted to find efficient methods to secrete a desired product to the culture broth. The secretion of a target product is highly desirable from a biotechnological point of view for several reasons, such as simplicity of the recovery and purification process, capability of posttranslational modifications, avoidance of cell growth inhibition by toxic product, and prerequisition for the development of more efficient immobilized cell bioreactors. Because the secretion mechanism of yeast is similar to that of mammalian cells, yeast is a useful microorganism for large-scale production of mammalian proteins, which requires posttranslational modifications such as glycosylation and phosphorylation. Most secreted proteins in yeast are glycosylated and have different secretion characteristics depending on a target protein. The factors that affect the localization of secreted proteins are still not well understood. However, it is certain that a secretion signal sequence plays a very important role in localization of secreted proteins. The signal sequences, which are widely used for recombinant yeast, include *SUC2 (5)*, *PH05 (6)*, Killer toxin *(7)*, and *MFαl (8)*. The *STA* signal sequence is originated from yeast *Saccharomyces diastaticus* that secretes the glucoamylase into the culture broth. Recently, several systems of recombinant protein secretion using this *STA* signal sequence have been reported *(9,10)*. Most of these reports state that the use

of the *STA* signal sequence was quite effective in the secretion of recombinant yeast protein. Secretion efficiency is known to 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 host strains used *(8)*.

The yeast *Saccharomyces cerevisiae* is widely used as a recombinant host for the production and secretion of foreign genes *(1)*. Glucoamylase (EC 3.2.1.3) is used to saccharify starchy feedstocks for glucose and ethanol production in commercial processes, and is not produced naturally by *S. cerevisiae*. The *STA* gene (glucoamylase gene of *S. diastaticus*) was chosen as a glucoamylase gene source for the present study *(11)*. In the present study, an *SUC2* promoter, which is repressed at high glucose concentration and derepressed at low glucose concentration, was used *(12,13)*. This *SUC2* promoter has the following advantages. First, because it is regulatory, common problems such as reduced host cell growth rate and segregational plasmid instability can be reduced. Second, because the *SUC2* promoter is affected by glucose alone, costly addition of an inducer (e.g., IPTG for *Escherichia coli* systems) is not necessary. Third, there is no medium substitution, which is necessary for other repressible promoters. Finally, the product yield can be improved by decoupling the gene expression stage from the cell growth stage during fermentation *(14)*. In addition, the *STA* signal sequence from yeast *S. diastaticus* glucoamylase was used for glucoamylase secretion in the present study.

It is very important to decide what types of recombinant yeast (plasmid-harboring or chromosome-integrated) will be used for further process development. Therefore, we studied the production and secretion patterns of two different types of recombinant yeasts containing the *SUC2* promoter for engineering purposes.

Materials and Methods

Strains and Culture Medium

The *S. cerevisiae* MMY2 (*a*, *ura3-52*, *sta0*, *sta10*) strain was used as a host. The 2μ yeast-based episomal recombinant plasmid YEpSUCSTA *(15)* and integrating recombinant plasmid YIpSUCSTA *(16)*, containing glucoamylase coding the *STA* gene fused with the *SUC2* promoter and the original *STA* signal sequence, were transformed into the host. The obtained transformants were named MMY2SUCSTA (plasmid-harboring recombinant yeast) and MMY2SUCSTA-I (chromosome-integrated recombinant yeast), respectively.

In flask culture, the recombinant yeast strains were grown at 30°C in a complex-rich medium (YPDS) containing 1% yeast extract (Difco), 2% bacto-peptone (Difco), 1% dextrose (glucose), and 1% potato starch (Sigma, St. Louis, MO). Starch was used as a carbon source for cell growth and glucoamylase biosynthesis *(16)*. In all batch fermentation, the yeast cells were grown in a semisynthetic minimal medium buffered with suc-

cinic acid (Sigma) at an initial pH of 6.0. The medium contained 0.2% $MgSO_4 \cdot 7H_2O$, 0.2% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.025% $CaCl_2 \cdot 2H_2O$, 0.2% yeast extract, 0.3% bacto-peptone, and 1% glucose. A single colony grown on a selective agar plate was transferred to a T-flask containing 250 mL of the selective medium (0.67% yeast nitrogen base without amino acid [Difco], 0.6% casamino acid [Difco], and 2% glucose) in order to ensure the maintenance of plasmids.

Fermentations were conducted in a 5-L fermentor (Korea Fermentor, Korea) with a working volume of 3 L. The fermentor 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 volume of air/(volume of liquid·min).

Measurement of Cell Density, Glucoamylase Activity, and Genetic Stability

Optical density (OD) of culture at 600 nm was measured using a spectrophotometer (WICON930, Kontron, Swiss) to determine cell density. Dry cell weights were measured after drying cells in an 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 of culture supernatant 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 using a glucose diagnostic kit (kit no. 510; Sigma). One unit of glucoamylase activity was defined as a corresponding amount to catalyze the release of 1 μmol of glucose/min.

Genetic stability of transformed cells was determined as a ratio of the number of target gene–harboring cells:total viable cells. The culture broth was appropriately diluted by distilled water, and the diluted samples were plated on nonselective agar plate media. One hundred colonies on the nonselective medium were picked on a selective agar plate. The number of target gene–harboring cells was measured by counting the number of colonies formed on selective medium.

Cell Fractionation

Cells were harvested, washed with 10 m*M* sodium azide, and suspended in spheroplast buffer containing 0.1 *M* sodium acetate (pH 5.0), 1 *M* mannitol, 10 m*M* sodium azide, 1 m*M* EDTA, and 0.1 *M* mercaptoethanol. After the addition of 15 mg/mL of Zymolase 20T (Seikagaku, Japan) in TEN buffer containing 10 m*M* Tris-Cl (pH 7.6), 1 m*M* EDTA, and 10 m*M* NaCl, the mixture was incubated at 45°C for 1 h. Spheroplasts were harvested by centrifugation at 12,000 rpm at 4°C for 1 min, and then the supernatant was operationally defined as a periplasmic glucoamylase source. The spheroplast was resuspended in a lysis buffer (0.1 *M* sodium acetate [pH 5.0], 10 m*M* sodium azide, 1 m*M* EDTA, and 0.1% [v/v] Triton

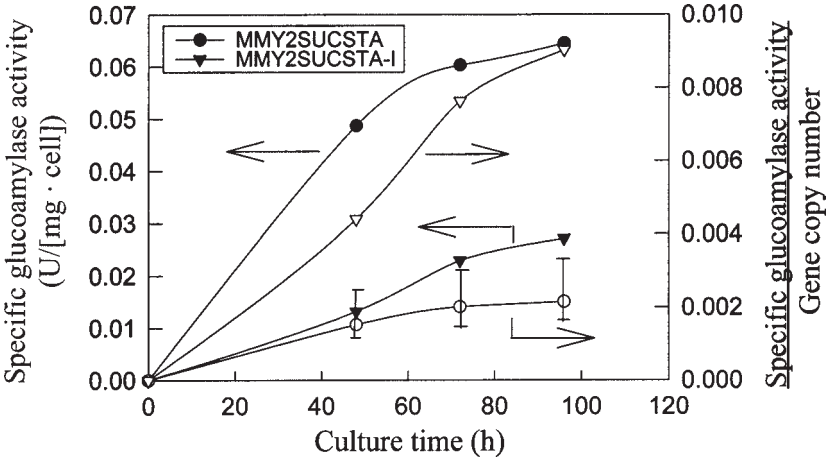

Fig. 1. Glucoamylase production in the flask cultures of the plasmid-harboring recombinant yeast MMY2SUCSTA (●, 20–40 gene copy number) and the chromosome-integrated recombinant yeast MMY2SUCSTA-I (▼, 3 gene copy number). Solid symbols: specific glucoamylase activity; open symbols: specific glucoamylase activity per gene copy number.

X-100) and mechanically lysed by vortexing with glass beads (425–600 μ) (Sigma). After intermittent vortexing and cooling on ice, the suspension was harvested by centrifugation at 12,000 rpm at 4°C for 5 min, and the supernatant was operationally defined as a cytoplasmic glucoamylase source.

Results and Discussion

Comparison of Production and Secretion Patterns in Flask Cultures

To investigate specific glucoamylase activities of the plasmid-harboring recombinant yeast MMY2SUCSTA and the chromosome-integrated recombinant yeast MMY2SUCSTA-I, flask cultures were performed in complex YPDS medium containing starch as a carbon source for cell growth and glucoamylase production after depletion of glucose. As shown in Fig. 1, MMY2SUCSTA-I that had three copies of integrated gene *(16)* had relatively lower glucoamylase activity (27.1 U/[g – cell] at 96 h) than MMY2SUCSTA (64.5 U/[g – cell] at 96 h) that had a higher number of plasmid copies (literally known as 20–40). This suggested that the multicopy (more than three copies) chromosomal integration techniques should be needed for high-level production of the *STA* gene.

However, the specific glucoamylase activity per copy number of the chromosome-integrated recombinant yeast (9.0 U/[g – cell]/copy number at 96 h) was 2.8–5.6 times higher than that of the plasmid-harboring recombinant yeast (1.6–3.2 U/[g – cell]/copy number at 96 h) (Fig. 1). This result indicated that the chromosome-integrated production system was more effective for the production of cloned glucoamylase than the plasmid-harboring system. The production of the plasmid-harboring recombinant yeast

was ineffective because the number of copies for the transformants was too high, resulting in the production system of the plasmid-harboring recombinant yeast being overburdened. The produced glucoamylase activity was thus not proportional to the number of copies. All the integrated transformants had about 99% genetic stability during a period of 50 generations under nonselective conditions (data not shown).

The superior genetic stability and production efficiency of the chromosome-integrated production system enable a higher final production level than the plasmid-harboring system, in which genetic stability and gene production level progressively decrease. In a continuous culture, genetic stability is an extremely important factor. Therefore, the chromosome-integrated recombinant yeast MMY2SUCSTA-I, with much higher genetic stability and production efficiency, can be applied successively to a continuous culture, allowing continuous protein production and ethanol production by simultaneous saccharification and fermentation of starch.

The localizations of cloned glucoamylase in the plasmid-harboring recombinant yeast MMY2SUCSTA and the chromosome-integrated recombinant yeast MMY2SUCSTA-I were investigated (Fig. 2). Protein localization can be divided into cytoplasm, periplasm, and culture broth. When secretion efficiency was defined as a ratio of glucoamylase activity in the culture broth per total glucoamylase activity, the secretion efficiencies of both the recombinant yeast strains increased proportional to the culture time. In the case of MMY2SUCSTA, the increase in secretion efficiency was stopped at 72 h of culture, and the efficiency was maintained at about 74% (Fig. 2A). However, in the case of MMY2SUCSTA-I, the secretion efficiency was increased linearly up to about 65% at 96 h of culture (Fig. 2B). In the case of recombinant yeast that has a high plasmid copy number, generally an adverse effect caused by the accumulation of product in the secretory apparatus may occur when the production rate is faster than the secretion rate *(6,17)*. This means that a capacity limitation of the secretory apparatus exists. When the adverse effect occurs, secretion efficiency decreases. Some efforts have been taken to increase secretion efficiency without causing the adverse effect when integrating desired genes *(6,18)*. However, in the present study, the plasmid-harboring recombinant yeast MMY2SUCSTA had a higher secretion efficiency level than the chromosome-integrated recombinant yeast MMY2SUCSTA-I, probably because the production level of MMY2SUCSTA did not reach the capacity of the secretory apparatus of the host MMY2. This indicated that the adverse effect might not occur or be small in this case. Thus, the plasmid-harboring recombinant yeast that had relatively higher glucoamylase production had higher secretion efficiency compared to the chromosome-integrated recombinant yeast. It is expected that the chromosome-integrated yeast MMY2SUCSTA-I (which has relatively slower expression of the cloned glucoamylase than the plasmid-harboring MMY2SUCSTA) requires longer culture time to reach maximal glucoamylase secretion efficiency. However, even though the plasmid-harboring system had higher secretion efficiency, the chromo-

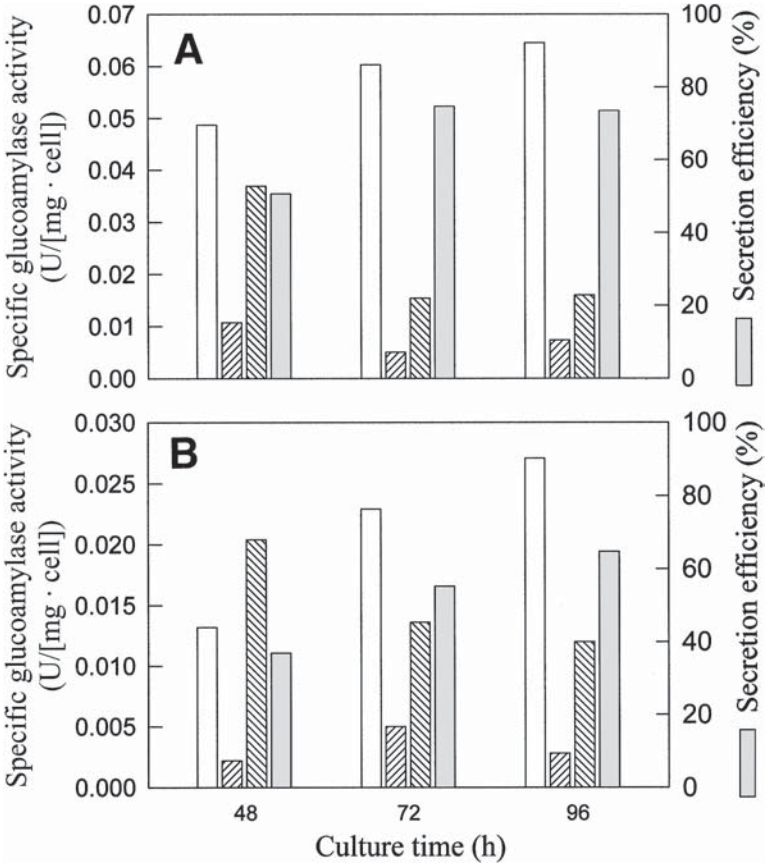

Fig. 2. Glucoamylase localization in the flask cultures of **(A)** the plasmid-harboring recombinant yeast MMY2SUCSTA and **(B)** the chromosome-integrated recombinant yeast MMY2SUCSTA-I. □, Culture broth glucoamylase; ▨, periplasmic glucoamylase; ▧, cytoplasmic glucoamylase; ■, secretion efficiency.

some-integrated system had a higher specific glucoamylase secretion rate (discussed later).

In both the yeasts, intracellular glucoamylase was mostly detected in the cytoplasm, and a small portion of glucoamylase was located in the periplasm (*see* Fig. 2). Initially, the large amounts of cytoplasmic glucoamylase were detected until 72 h of culture. Then the cytoplasmic glucoamylase decreased with culture time and its portion was about 18% in the plasmid-harboring recombinant yeast and about 28% in the chromosome-integrated recombinant yeast. The portion of glucoamylase in the periplasm of both yeasts remained below 10% during the entire culture period. From the results of glucoamylase localization, it was thought that the rate-limiting step of the cloned glucoamylase secretion pathway existed prior to transfer through the cell wall. This suggested that the transport of glucoamylase through the cell wall was not a rate-limiting step in cloned

glucoamylase secretion. Secretion efficiency is affected by several factors *(8)*. Table 1 summarizes the previously reported results for secretion efficiency of cloned protein from recombinant yeast. Most yeast glycoproteins (such as invertase and heterogeneous secreted proteins) are accumulated in the periplasm or cell wall. However, both the recombinant yeast strains used in the present study (MMY2SUCSTA and MMY2SUCSTA-I) secreted glucoamylase mainly into the culture broth even though glucoamylase is a very large protein (420 amino acids and glycosylated). Therefore, the *STA* signal sequence we used seems to be quite effective in the secretion of recombinant proteins.

Comparison of Production and Secretion Patterns in Fermentations

Several batch fermentations were carried out in the semisynthetic minimal media containing 50 m*M* succinate (Fig. 3). Because this medium was starch free, fermentations were finished when glucose in the medium was depleted. Therefore, we could study production and secretion patterns of the two recombinant yeasts regarding only the effects of glucose. Since the pH changes during the fermentation were very small and production of glucoamylase occurs normally in this pH range, the control of pH was not necessary in the fermentation experiments. The profiles of cell growth and glucose consumption were similar in both the recombinant yeast fermentations. The pH profiles were similar to the pattern of glucose consumption in both the recombinant yeasts. Although the pH in the culture broth decreased depending on the cell growth, this decrease was small (data not shown) because the addition of succinate to the medium played a role in buffering (protecting the decrease in pH) *(22)*.

In the case of the plasmid-harboring recombinant yeast, genetic stability (plasmid stability in this case) depended on glucoamylase production. As the production of glucoamylase increased, the genetic stability decreased. Note, that since the fermentation medium contained yeast extract, this caused some portion of genetic instability in the data. By contrast, the genetic stability of the chromosome-integrated recombinant yeast remained good throughout the entire culture time (Fig. 3A). Glucoamylase was produced late in both the recombinant yeasts because the *SUC2* promoter was regulated by glucose concentration in the culture broth, as previously reported *(5,12,15)*. In the plasmid-harboring recombinant yeast, glucoamylase synthesis began below a glucose concentration of 7 g/L, and secretion occurred when the glucose concentration decreased below 5 g/L (Fig. 3B). The production of glucoamylase then increased rapidly, and the secretion increased slowly as the glucose concentration decreased. In the chromosome-integrated recombinant yeast, glucoamylase synthesis began when the glucose concentration decreased to 4 g/L, and secretion began when the glucose concentration decreased below 2 g/L (Fig. 3B). The production of glucoamylase then rapidly increased, and the secretion of glucoamylase increased slowly, as with the plasmid-harboring recombinant yeast. The behavior of the two recombinant yeasts with respect to

Table 1
Comparison of Secretion Efficiencies of Cloned Proteins from Recombinant Yeast Cultures

Protein	Signal sequence	Promoter	Secretion efficiency[a]	Reference
Bacillus α-amylase	*MFα1*	*MFα1*	55% at d 4; 67% at d 8	*19*
Yeast invertase	*MFα1*	*MFα1*	21.6%	*5*
Yeast invertase	*SUC2*	*SUC2*	12.4%	*5*
Human α-antitrypsin	*SUC2*	*TPI*	20%	*20*
E. coli β-galactosidase	*STA2*	N/R[b]	No secretion; 76% in periplasm	*9*
Bacillus endoglucanase	*STA1*	N/R[b]	93%	*10*
Yeast glucoamylase	*STA*	*STA*	30–40%	*21*
Yeast glucoamylase	*STA*	*GAL1–10*	50%	*21*
Yeast glucoamylase	*STA*	*SUC2*	74% in MMY2SUCSTA; 65% in MMY2SUCSTA-I	This study

[a]All data of secretion efficiency were recalculated by definition of secretion efficiency used in this research.
[b]N/R, not reported.

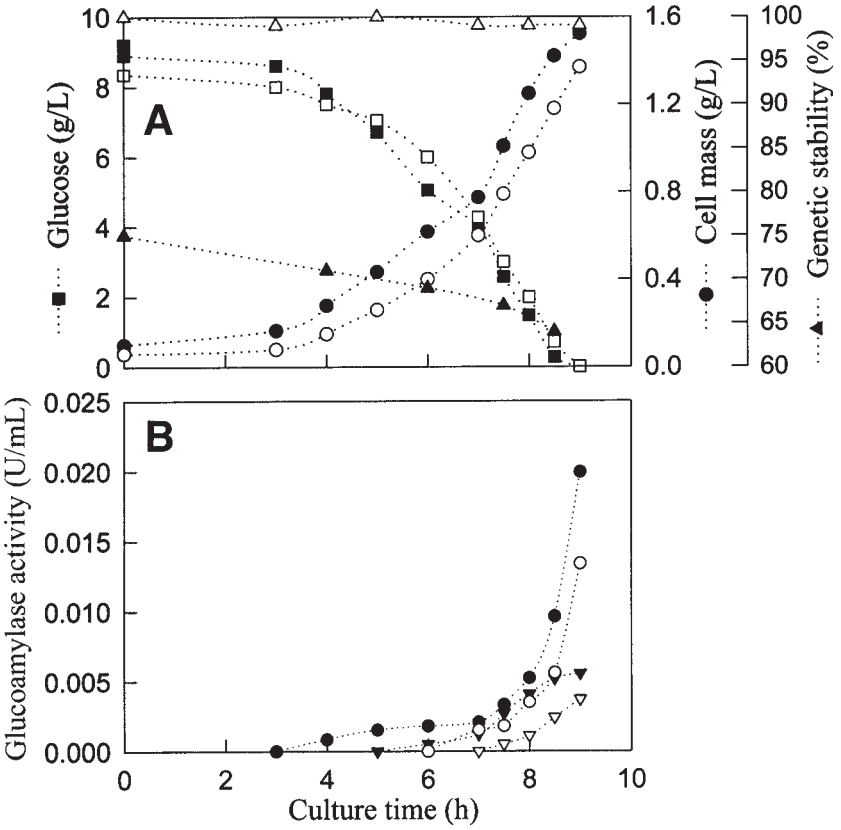

Fig. 3. Profiles of batch fermentations using the plasmid-harboring recombinant yeast MMY2SUCSTA (closed symbols) and the chromosome-integrated recombinant yeast MMY2SUCSTA-I (open symbols). **(A)** Circles, cell density; squares, glucose concentration; triangles, genetic stability. **(B)** Circles, intracellular glucoamylase activity; triangles, extracellular glucoamylase activity.

glucose concentration clearly differed. This difference might be owing to a gene dosage (the number of copies of a given gene present in a cell) effect *(23)*. Because the plasmid-harboring recombinant yeast had a high number of copies *(20–40)*, the transcription regulation of the *SUC2* promoter by glucose in the recombinant plasmid was not close. By contrast, because the chromosome-integrated recombinant yeast had three copy numbers of the desired gene, the expression of glucoamylase was closely regulated by glucose. Therefore, the optimal glucose concentration for maximum production of glucoamylase in the chromosome-integrated recombinant yeast MMY2SUCSTA-I would be lower than that in the plasmid-harboring recombinant yeast MMY2SUCSTA.

Specific growth rate, specific glucoamylase production rate, and specific glucoamylase secretion rate were calculated using the least square regression based on the model of Cha and Yoo *(24)* and plotted as a function of glucose concentration (Fig. 4). The specific growth rate of the plasmid-

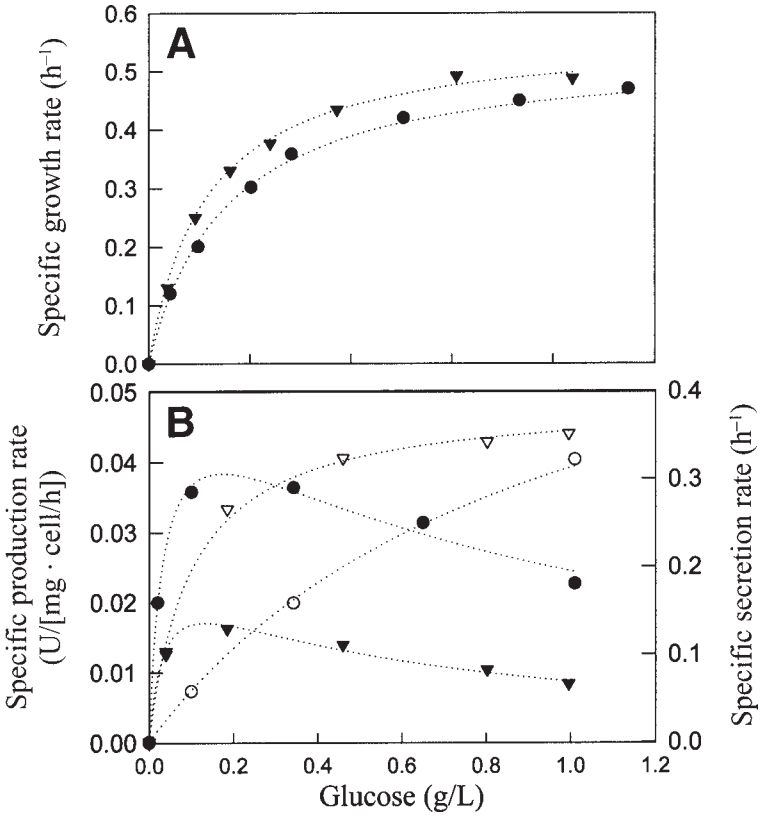

Fig. 4. **(A)** Specific growth rate and **(B)** specific expression rate and specific secretion rate in the batch fermentations of the plasmid-harboring recombinant yeast MMY2SUCSTA (●) and the chromosome-integrated recombinant yeast MMY2-SUCSTA-I (▼). (B) Closed symbols: specific production rate; open symbols: specific secretion rate.

harboring recombinant yeast including plasmid-free cells is indicated in Fig. 4A, and its maximum value was 0.536/h. The maximum specific growth rate of the chromosome-integrated recombinant yeast was 0.564/h. The specific glucoamylase production rate *(24)* of the plasmid-harboring recombinant yeast is indicated in Fig. 4B, and its maximum value was 0.054 U/(mg – cell)/h. The maximal production of the plasmid-harboring recombinant yeast occurred at a glucose concentration of 0.17 g/L in the culture broth. Also, the maximum specific glucoamylase production rate *(24)* of the chromosome-integrated recombinant yeast was 0.026 U/(mg – cell)/h. In this case, the maximal production occurred at a glucose concentration of 0.13 g/L. This result confirmed that the optimal glucose concentration for the production of glucoamylase in the chromosome-integrated MMY2-SUCSTA-I was lower than that in the plasmid-harboring MMY2SUCSTA. The specific glucoamylase secretion rate was related to the cell growth condition (data not shown), which has been indicated by other researches *(17,24)*. In Fig. 4B, the specific secretion rate was described as a function of

glucose concentration. In both the recombinant yeasts, the specific secretion rate increased with an increase in glucose concentration and finally reached a saturation value. Interestingly, in the case of the chromosome-integrated recombinant yeast, the specific secretion rate more rapidly reached a saturation and was higher. Therefore, from this result, we understood that secretion rate would be higher in the chromosome-integrated system even though the final secretion efficiency is lower (recall the previous result; *see* Fig. 2). This lower secretion rate in the plasmid-harboring system might be explained by an adverse effect caused by their higher production rate (*see* Fig. 4B).

The determination of an optimal glucose concentration for protein production is important from a biotechnological point of view. As shown in the plots of specific production and secretion rates (Fig. 4B), production and secretion must be considered together for determination of an optimal glucose concentration for protein production. In fed-batch and/or continuous cultures for high-cloned glucoamylase production, the glucose concentration for the chromosome-integrated recombinant yeast should be maintained at a lower level than that for the plasmid-harboring recombinant yeast.

In summary, the chromosome-integrated expression system would be quite useful for cloned gene production thanks to superior genetic stability, higher production efficiency (production per copy number), and higher secretion rate. Despite several of these advantages, desired gene-integrated strains have their own limitations for use owing to low gene copy number. Therefore, obtaining a chromosome-integrated recombinant strain of high gene copy number is a prerequisite.

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