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CHARACTERISTICS COMPARISON BETWEEN PLASMID-HARBORING AND CHROMOSOME-INTEGRATED RECOMBINANT *SACCHAROMYCES CEREVISIAE* CULTURES

Hyung Joon Cha* and Young Je Yoo†

Department of Chemical Engineering and The Institute for Molecular Biology and Genetics, Seoul National University, Seoul 151-742, Korea

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Abstract—It is important to understand the differences in characteristics between plasmid-harboring recombinant yeast and chromosome-integrated recombinant yeast for the design and optimization of bioprocess employing recombinant yeast. In the present study, heterologous glucoamylase gene was inserted into yeast. The glucoamylase activity per gene copy number of chromosome-integrated recombinant yeast MMY2SUCSTA-I-5 was 3 to 6 folds higher than that of plasmid-harboring recombinant yeast MMY2SUCSTA. And the genetic stability of chromosome-integrated recombinant yeast (99%) was far better compared to plasmid-harboring recombinant yeast (65%). Better genetic stability and glucoamylase activity per gene copy number of chromosome-integrated recombinant yeast can provide advantages in higher final expression level, especially in continuous culture, compared to the plasmid-harboring recombinant yeast. The optimal glucose concentration for maximum expression of glucoamylase in chromosome-integrated recombinant yeast was lower than that in plasmid-harboring recombinant yeast.

Key words: *Saccharomyces cerevisiae, Plasmid-harboring Recombinant Yeast, Chromosome-integrated Recombinant Yeast, Glucoamylase, Culture Characteristics*

INTRODUCTION

One of the principal tools in the new biotechnology may be the recombinant DNA technique, which allows us to manipulate directly the genetic material of individual cells. By inserting foreign genetic information into fast-growing microorganisms, we can produce foreign gene products such as proteins with higher rates and yields that have not been possible with any other cellular system. However, one of the severe problems encountered in the use of recombinant cells is their inability to retain the cloned genetic information and compete with the host cells devoid of this information [Kingsman et al., 1987; Seo and Bailey, 1985; Siegel and Ryu, 1985]. These cause the decrease of overall productivity. The cell growth and biosynthesis of genetic product depend on the plasmid copy number and the plasmid stability. Therefore, a method of overcoming plasmid instability would be needed and there have been several studies for this purpose, such as the introduction of *par* sequence [Meacock and Cohen, 1980], *cer* sequence of ColE1 plasmid [Summers and Sherratt, 1984], yeast centromere sequence [Rose, 1987], dominant selectable marker [Fogel and Welch, 1982], and cell immobilization [Barbotin et al., 1990] etc. One of the commonly used methods is the usage of regulated promoters which control gene expression by change of medium composition, addition of chemicals, or change of cultural conditions [Siegel and Ryu, 1985; Da Silva and Bailey, 1991]. By this manner, the competitive interaction between plasmids and host cells can be minimized. However, one of the most efficient and ideal method for cloned gene stabilization is a chromosomal integration of desired genes. The pioneering work of Hinnen demonstrated that heterologous DNA sequences could be stably integrated into the yeast chromosome [Hinnen et al., 1978]. Using the δ sequence as a recombination site, mouse α-amylase gene was integrated into the yeast chromosomal DNA and copy number was estimated as three to five [Sakai et al., 1990]. The high copy number integration of a heterologous gene into chromosome could be obtained using a δ sequence. But the possibility that δ-integrative plasmid is relatively unstable can not be ruled out. Using the *PGK1* or *ADH1* locus, two bacterial α-acetolactate decarboxylase genes were introduced into the brewer's yeast and the copy number was one [Blomqvist et al., 1991]. The present actual fermentation processes employing recombinant microorganisms are almost batch type and their productivities are very low. It is very important to decide what types of recombinant yeast (chromosomal-integrated or recombinant plasmid-harboring) can be used for the cultures. To achieve this, characteristics of two different type recombinant yeasts are to be studied for engineering purpose.

The objective of this work is the comparison between plasmid-harboring recombinant yeast culture and chromosome-integrated recombinant yeast culture. For achieve this, we first constructed chromosome-integrated recombinant yeast using homologous recombination method and compared the culture characteristics employing two different recombinant yeasts.

MATERIALS AND METHODS

1. Choice of the System

Yeast *Saccharomyces cerevisiae* is often used as a recombinant host that expresses foreign genes and secretes proteins because

†To whom all correspondences should be addressed.
*Present address: Bio-Pilot Plant, Korea Research Institute of Bioscience and Biotechnology, KIST, P.O. Box 115, Taedok Science Town, Taejon 305-600.

of their advantages [Kingsman et al., 1987]. Glucoamylase (EC 3.2.1.3) which is used to saccharify starchy feed stocks in commercial processes for glucose and ethanol productions, is not produced in *S. cerevisiae*. Having this in mind, the *STA* gene (glucoamylase gene of *Saccharomyces diastaticus*) was chosen as a glucoamylase gene source for this research [Yamashita et al., 1985]. The *STA* gene was cloned from a wild type *S. diastaticus* DS101 [Ahn, 199-]. Since the restriction maps of three *STA* genes were known to be identical, it could not be determined whether the *STA* gene in this study was *STA1* or *STA2* or *STA3*. In this research, the *SUC2* promoter that is regulated by glucose in the culture broth was used as a promoter [Carlson and Botstein, 1982; Chu and Maley, 1980]. The *STA* signal sequence is a sequence of yeast *S. diastaticus* glucoamylase that is secreted into the culture broth and was used in this work for glucoamylase secretion. In our previous study, the multi-copy recombinant plasmid contained fused *SUC2-STA* gene was constructed [Cha et al., 1992].

2. Strains, Plasmids, and Media

MMY2 (a, ura3-52, sta10) strain was used as a host. *Escherichia coli* HB101 was used for genetic manipulation. Recombinant plasmid YEpSUCSTA [Cha et al., 1992] and YIp5 were used for construction of recombinant chromosomal integration plasmid YIpSUCSTA. Yeast was grown at 30℃ in complex rich medium containing 1% yeast extract (Difco), 2% bacto-peptone (Difco), 1% glucose, and 1% potato starch (Sigma), in semi-synthetic minimal medium containing 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, 1% glucose, 1% starch, and 50 mM succinate, or in minimal medium containing 0.67% yeast nitrogen base without amino acids (Difco), 0.6% casamino acid (Sigma), and 2% glucose.

3. Construction of the Recombinant Plasmid YIpSUCSTA

For the insertion of the *STA* glucoamylase gene into chromosome of *S. cerevisiae*, chromosomal integrating recombinant plasmid was constructed as shown in Figure 1. The DNA fragment that contained the *SUC2* promoter, the *STA* signal sequence, and the *STA* structural gene, was separated from recombinant plasmid YEpSUCSTA that had been previously constructed [Cha et al., 1992]. And this fragment was subcloned in chromosomal integration vector YIp5. The resulting recombinant plasmid constructed was named YIpSUCSTA. To insert the YIpSUCSTA into chromosome of host strain MMY2, YIpSUCSTA was linearized by StuI digestion of the *URA3* gene region and transformed into MMY2. Transformants were named MMY2SUCSTA-I.

4. Genetic Manipulation

The alkaline lysis method [Birnboim and Doly, 1979] was used for the extraction of plasmids DNA from *E. coli*. For transformation of *E. coli*, the calcium chloride method [Kushner, 1978] was used. And the alkaline cation method using lithium acetate [Ito et al., 1983] was used for transformations of yeast.

5. Genomic Southern Blot Analysis

In Southern blot analysis, the desired DNA bands were detected by ECL kit (Enhanced chemiluminescent; Amersham, RPN 3000). The DNA bands densities on Southern blotting membranes were scanned by densitometer (Ultroscan XL laser densitometer; Pharmacia) at 633 nm wavelength.

6. Glucoamylase Activity

The 0.7 mL culture supernatants were incubated in 0.1 mL 1 M sodium acetate buffer (pH 5.0) and 0.2 mL 8% soluble starch (Junsei) at 50℃ for 30 minutes and boiled at 100℃ for 5 minutes for inactivating glucoamylase. Glucose produced by the action of glucoamylase on soluble starch was assayed by using a glucose-diagnostic kit (Sigma, No. 510). Glucoamylase activity was expressed as unit and one unit is one micromole of glucose released per one minute at above condition.

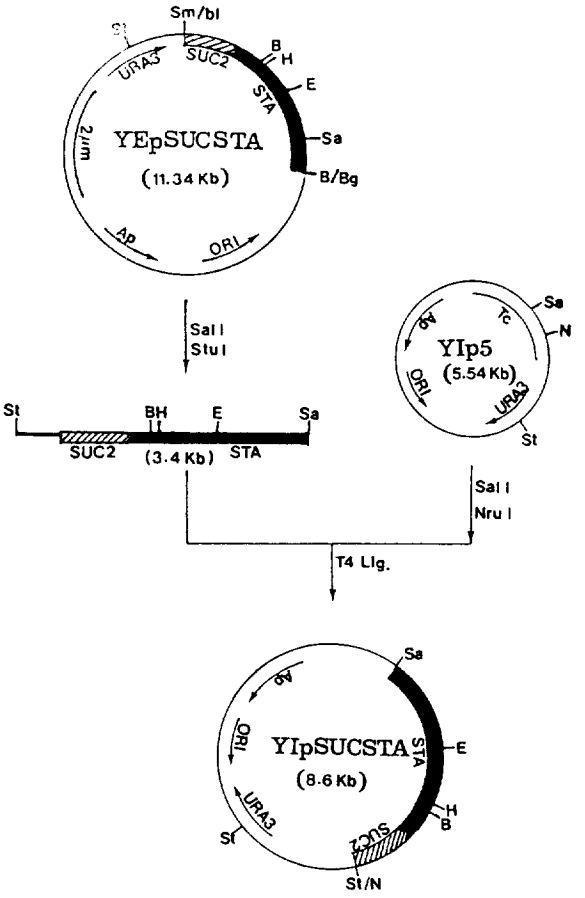

Fig. 1. Schematic diagram for construction of chromosome integrating recombinant plasmid YIpSUCSTA.
Abbreviations of restriction and modification enzymes and the sites; B: BamHI, Bg: BglI, E: EcoRI, H: HindIII, N: NruI, Sa: SalI, Sm: SmaI, St: StuI, T4 Lig.: T4 Ligase, bl: blunt end.

RESULTS AND DISCUSSION

1. Identification of Chromosomal Integration of Recombinant Plasmid YIpSUCSTA

The halos around colonies were observed when five typical transformants were picked on YPS media as shown in Figure 2. These halos indicated that MMY2SUCSTA-I secreted glucoamylase and was able to hydrolyze starch to glucose. These five transformants were named MMY2SUCSTA-I-1, -I-2, -I-3, -I-4, and -I-5, respectively.

Southern blot analysis was performed to identify the chromosomal integration of YIpSUCSTA into the host chromosome. The *URA3* gene or the *STA* gene fragment was used as a probe. In the case of using the *STA* gene fragment as a probe, 4.3 kb DNA band appeared in the host strain MMY2 [lane 1 in Fig. 3(A)]. However, two DNA bands of 4.3 kb and 1.77 kb were shown in transformants (lane 2-6). 4.3 kb DNA band was the fragment of sporulation-specific glucoamylase (*SGA*) gene that exists in *S. cerevisiae* and 1.77 kb band was the fragment containing the chro-

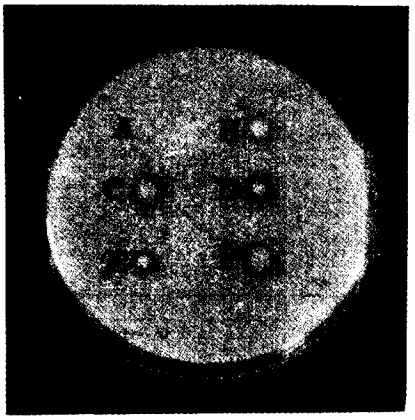

Fig. 2. Identification of transformed cells for secretion of glucoamylase.
A: host yeast MMY2, B: transformant MMY2SUCSTA-I-1, C: transformant MMY2SUCSTA-I-2, D: transformant MMY2SUCSTA-I-3, E: transformant MMY2SUCSTA-I-4, F: transformant MMY2SUCSTA-I-5.

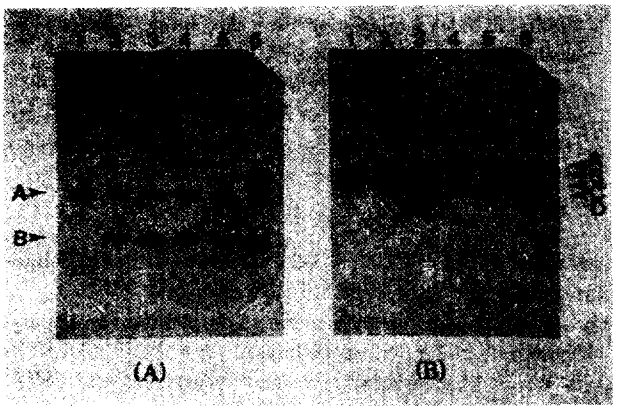

Fig. 3. Southern blot analysis of EcoRI digested chromosomal DNA from recipient strain MMY2 and its transformants.
(A) Using 1.1 kb fragment of *STA* as a probe. A: Chromosomal *SGA*-4.3 kb, B: Integrated *STA*-1.77 kb (B) Using 0.8 kb fragment of *URA3* as probe. A: Integrated *URA3*-10 kb, B: Chromosomal *URA*-8 kb, C: Integrated *URA3*-7 kb, D: Integrated *URA3*-5.2 kb, lane 1: host yeast MMY2, lane 2: transformant MMY2SUCSTA-I-1, lane 3: transformant MMY2SUCSTA-I-2, lane 4: transformant MMY2SUCSTA-I-3, lane 5: transformant MMY2SUCSTA-I-4, lane 6: transformant MMY2SUCSTA-I-5.

mosome-integrated *STA* gene. This 1.77 kb DNA band was the same in size with the digested fragment of YIpSUCSTA by EcoRI. As shown in Fig. 3(B), when the *URA3* gene fragment was used as a probe, 8 kb DNA band was only detected in the host MMY2 (lane 1). Therefore, 8 kb band was the fragment of original chromosomal *URA* gene. However, two types of several DNA bands appeared in the transformants. 10 kb, 8 kb, and 5.2 kb DNA bands were detected in the transformants MMY2SUCSTA-I-1, MMY2 SUCSTA-I-3 and MMY2SUCSTA-I-4, respectively. On the other hand, all bands of 10 kb, 8 kb, 7 kb, and 5.2 kb were detected in the transformants MMY2SUCSTA-I-2 and MMY2SUCSTA-I-5. Here, 7 kb DNA band might indicate that recombinant plasmid YIpSUCSTA was inserted into MMY2 chromosome by tandemly

Table 1. Densitometer scanning of DNA bands of Southern blotting membrane from the *STA* probe analysis

Strains	Chromosomal *SGA* area (AU×mm)	Integrated *STA* area (AU×mm)	Area ratio
MMY2	1.687	-	-
MMY2SUCSTA-I-1	0.612	0.437	1.4
MMY2SUCSTA-I-2	0.615	1.907	3.1
MMY2SUCSTA-I-3	0.727	0.766	1.1
MMY2SUCSTA-I-4	1.370	0.920	1.5
MMY2SUCSTA-I-5	0.795	2.613	3.3

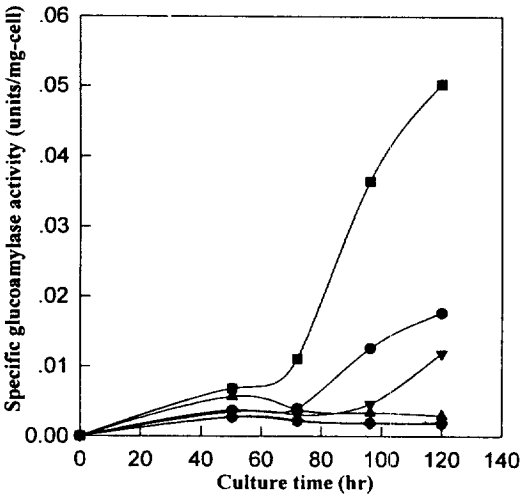

Fig. 4. Glucoamylase expression of plasmid-harboring yeast MMY2 SUC STA and chromosome-integrated yeast MMY2SUCSTA -I.
Symbols; ■: MMY2SUCSTA, ●: MMY2SUCSTA-I-1, ▼: MMY 2SUCSTA-I-2, ▲: MMY2SUCSTA-I-3, ◆: MMY2SUCSTA-I-4, ●: MMY2SUCSTA-I-5.

repeated multi-copy. Because the chromosomal integration of YIp-SUCSTA occurred by repeated multi-copy recombination, the densities of 10 kb and 5.2 kb DNA band were not changed. However, density of 7 kb band was increased.

As shown in Fig. 3(A), 1.77 kb and 3.4 kb DNA bands in transformants had different densities. Increase of relative density of 1.77 kb DNA band per 3.4 kb band indicated that multi-copy integration of recombinant plasmid YIpSUCSTA had occurred. These relative densities of bands were measured by densitometer as shown in Table 1. From the results of densitometer scanning, it could be concluded that MMY2SUCSTA-I-1, MMY2SUCSTA-I-3, and MMY2SUCSTA-I-4 had one copy and MMY2SUCSTA-I-2 and MMY2SUCSTA-I-5 had three copies of integrated fused *SUC 2-STA* gene. These results were consistent with the supposition derived from the *URA3* probe analysis.

2. Biosynthesis of Glucoamylase from Chromosome-Integrated Yeast MMY2SUCSTA-Is and Plasmid-harboring Yeast MMY2SUCSTA

To investigate the specific glucoamylase activity of five MMY2 SUCSTA-I transformants and the recombinant yeast MMY2SUCSTA harboring the plasmid YEpSUCSTA, flask cultures were performed in complex YPDS medium. As shown in Fig. 4, MMY2SUCSTA-I-2 (0.012 units/mg-cell) and MMY2SUCSTA-I-5 (0.018

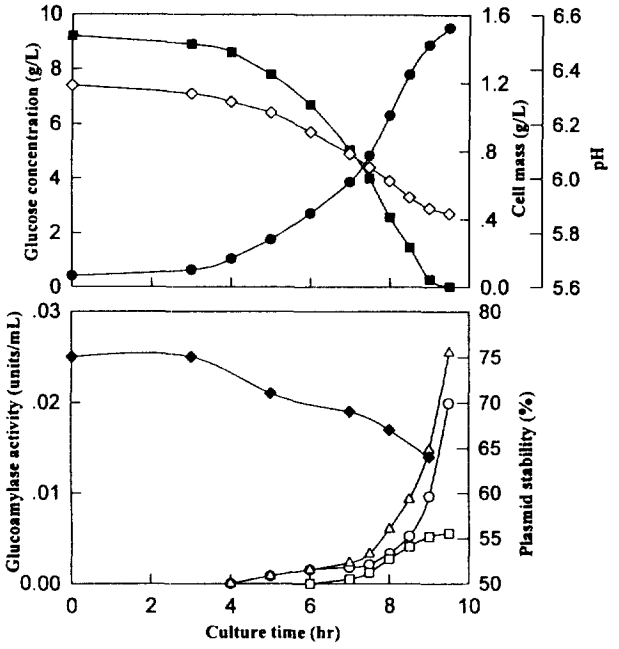

Fig. 5. Profiles of batch fermentation using plasmid-harboring recombinant yeast MMY2SUCSTA.
Symbols; ●: cell mass, ■: glucose concentration, ◇: pH, □: extracellular glucoamylase activity, ○: intracellular glucoamylase activity, and △: total glucoamylase activity, ◆: plasmid stability.

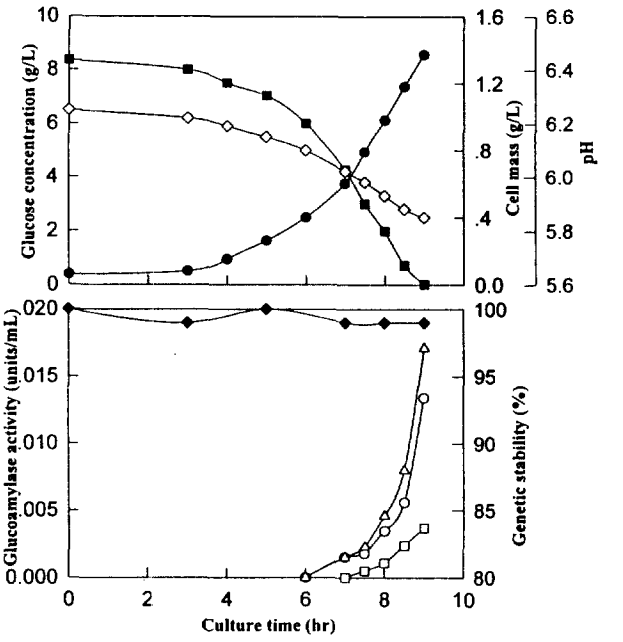

Fig. 6. Profiles of batch fermentation using chromosome-integrated recombinant yeast MMY2SUCSTA-I-5.
Symbols; ●: cell mass, ■: glucose concentration, ◇: pH, □: extracellular glucoamylase activity, ○: intracellular glucoamylase activity, and △: total glucoamylase activity, ◆: genetic stability.

units/mg-cell) that had three copies of integrated gene, had higher glucoamylase activities than the transformants with single copy. But, they had relatively lower glucoamylase activities (one half times in the culture broth) than MMY2SUCSTA (0.050 units/mg-cell) that had the higher copy number of plasmid (20 to 40). Thus, multi-copy chromosomal integration techniques are needed for high level expression of desired gene. However, the glucoamylase activity per copy number of MMY2SUCSTA-I-5 (0.006 units /mg-cell/gene-copy) was 3 to 6 times higher than that of MMY2 SUCSTA (0.002-0.001 units/mg-cell/gene-copy). This result indicated that chromosome-integrated recombinant yeast was more effective in expression of foreign glucoamylase than plasmid-harboring recombinant yeast. However, the reason for superior glucoamylase activity per copy number of chromosome-integrated recombinant yeast was not understood exactly and further study for this result will be necessary. All the integrated transformants had about 99 % genetic stability during a period of 50 generations under non-selective conditions.

Superior genetic stability and glucoamylase activity per gene copy number of chromosome-integrated recombinant yeast strains enable higher final expression level than plasmid-harboring recombinant yeast which plasmid number and gene expression level are in general progressively decreased. In continuous culture, the genetic stability is an important factor. Therefore, chromosome-integrated recombinant yeast MMY2SUCSTA-I with high genetic stability and high glucoamylase activity per copy number can be applied successively to the continuous culture, allowing the continuous protein production and ethanol production by simultaneous saccharification and fermentation of starch.

3. Characteristics Comparison between Chromosome-Integrated Yeast MMY2SUCSTA-I-5 Culture and Plasmid-harboring Yeast MMY2SUCSTA Culture

In order to compare the characteristics of the cultures employing chromosome-integrated recombinant yeast MMY2SUCSTA-I-5 with plasmid-harboring recombinant yeast MMY2SUCSTA, cultivations were performed in semi-synthetic minimal media containing 50 mM succinate as shown in Fig. 5 and Fig. 6. Because change of pH was very small and expression of glucoamylase occurred normally in this pH range, control of pH was not necessary in the fermentation experiments. The patterns of cell growth and glucose consumption were not largely different in both two types of the recombinant yeast cultures. The profiles of pH were similar to the pattern of glucose consumption in both recombinant yeasts. pH in the culture broth was decreased depending on the cell growth. However, the decrease of pH was small because succinate that was added in the medium played a role in protecting the decrease of pH. In the case of plasmid-harboring recombinant yeast MMY2SUCSTA, the plasmid stability was decreased depending on the glucoamylase expression. As the expression of glucoamylase became larger, the plasmid stability was more decreased. However, the genetic stability of the chromosome-integrated recombinant yeast MMY2SUCSTA-I-5 maintained nearly perfect. The glucoamylase was produced from the late stage in both recombinant yeast cultures because the *SUC2* promoter that was used in this work, was regulated by glucose concentration in the culture broth as previously reported [Chu and Maley, 1980; Marten and Seo, 1989; Cha et al., 1992]. In the case of MMY2SUCSTA, the glucoamylase was began to be biosynthesized at about below 7 g/L glucose concentration and secreted at about below 5 g/L glucose concentration as shown in Fig. 5. After then, the

expression of glucoamylase was rapidly increased. However, the secretion of glucoamylase was slowly increased as the glucose concentration in the culture broth was decreased. In the case of MMY2SUCSTA-I-5, the glucoamylase was began to be biosynthesized at about below 4 g/L glucose concentration and secreted at about below 2 g/L glucose concentration as shown in Fig. 6. After then, the expression of glucoamylase was rapidly increased and the secretion of glucoamylase was slowly increased as the above case of MMY2SUCSTA. In the case of chromosome-integrated recombinant yeast culture, the glucose concentration that affected the expression and secretion of glucoamylase, was lower than that of plasmid-harboring recombinant yeast culture. These might be resulted by gene dosage (the number of copies of a given gene present in a cell) effect [Stockhaus et al., 1987]. Since MMY2SUCSTA had the high copy number of plasmid (20 to 40), transcription regulation of *SUC2* promoter by glucose in recombinant plasmid was not tightly worked. On the contrary, because MMY2SUCSTA-I-5 had three copy number of the desired gene, the expression of glucoamylase was tightly regulated by glucose. This meant that the optimal glucose concentration for maximum expression of glucoamylase in chromosome-integrated yeast culture was lower than that in plasmid-harboring yeast culture. Determination of optimal glucose concentration for desired protein production is important from a biotechnological point of view. In the case of chromosome-integrated recombinant yeast MMY2SUCSTA-I-5, the glucose concentration in the culture broth should be maintained at lower level than that of plasmid-harboring recombinant yeast MMY2SUCSTA for high glucoamylase production using fed-batch culture or continuous culture.

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