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Effect of Plasmid Stability on the Glucoamylase Productivity of *Saccharomyces diastaticus* Harboring Recombinant Plasmid Containing Glucoamylase Gene *STA* 1

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Glucoamylase 유전자 *STA*1 이 포함된 재조합 Plasmid 를 갖는 *Saccharomyces diastaticus* 의 Glucoamylase 생산성에 미치는 Plasmid 안정성의 영향

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For the purpose of improving glucoamylase productivity of *Saccharomyces diastaticus*, useful yeast in direct ethanol fermentation of starch, the effects of growth rate on the plasmid stability and glucoamylase productivity of *S. diastaticus* harboring recombinant plasmid pYES 18 containing glucoamylase gene *STA* 1 were investigated. In a selective medium, the recombinant plasmids were maintained stably at constant level but glucoamylase productivity was very low. On the other hand, in the complex medium containing starch, growth rate of the cell was stimulated by the supplementation of glucose and plasmid stability was improved by growth stimulation. We can conclude that glucoamylase productivity of *S. diastaticus* harboring the recombinant plasmid was increased as the maintaining of high plasmid stability in the cell.

Saccharomyces diastaticus can ferment starch to ethanol directly by the excretion of glucoamylase, and is very similar to *S. cerevisiae,* physiologically and genetically (1-3). The glucoamylase productivity of *S. diastaticus* is very important in direct ethanol fermentation from starch using this yeast. After succeeding in the cloning of glucoamylase gene of *S. diastaticus* by Yamashita *et al.* (4, 5) and other researchers (6, 7), there have been many efforts to introduce the glucoamylase gene of *S. diastaticus* into *S. cerevisiae* and to increase the glucoamylase productivity of *S. diastaticus* through recombinant DNA technique. But the glucoamylase productivities of the transformed strains were not so high as those expected from gene dosage effect by plasmid copy number.

In the attempts to increase the products of foreign gene introduced into plasmid, plasmid stability is one of the most important and critical consideration factor. There have been many reports about the factors affecting chimeric plasmid stability in *S. cerevisae* (8). As one of those factors, the relationship between growth rate and plasmid stability have studied with various chimeric plasmids in *S. cervisiae* (9,-12).

We also cloned the glucoamylase gene (*STA* 1) of *S. diastaticus* and constructed hybrid YEp plasmid (pYES 18) containing this gene (13). Thereafter we have tried to improve the glucoamylase productivity of *S. diastaticus* transformed by this hybrid plasmid. In this paper, we demonstrate the effect of growth rate on the plasmid stability and the glucoamylase prodctivity of *S. diastaticus* harboring

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Table 1. Genotypes and source of yeast strains used.

Strains	Genotype	Remarks	Source
S. diastaticus 2-11A	a, *leu*2-3, 112, *his*2, *STA*1, *inh*°	gene source	Yamashita *et al.* (3)
S. diastaticus YIY345	a, *ura*3, *leu*2-1, 112, *his*4, *sta*°, *inh*°	recipient	Yamashita *et al.* (3)
S. diastaticus GMT 18		transformant of YIY345	this work

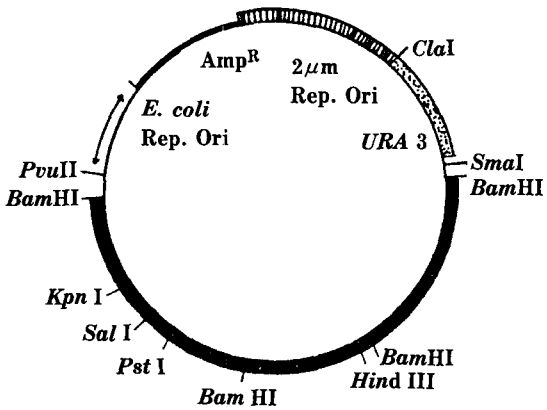

Fig. 1. Schematic diagram of recombinant plasmid pYES 18 containing glucoamylase gene *STA* 1.
Total plasmid size was 17 kb and the cloned *STA* 1 was 9.4 kb.

recombinant plasmid pYES 18.

Materials and Methods

Yeast strains and plasmid

The yeast strains used in this experiments are listed in Table 1. *S. diastaticus* GMT 18 is the transformed strain of *S. diastaticus* YIY 345 with the recombinant plasmid pYES 18. Recombinant plasmid pYES 18 was constructed from YEp 24 and the cloned *STA* 1 gene of *S. diastaticus* 2-11A by ourselves (13). The restriction enzyme map and structure of pYES 18 is diagrammed in Fig. 1.

Media and culture conditions

As the minimal and selective medium, YNBD medium (Difco, yeast nitrogen base without amino acid 0.67%, dextrose 2.0%) was used and, if necessary, histidine, leucine and uracil were added at appropriate concentrations (14). As the complex media, YEPD (yeast extract 1.0%, peptone 2.0%, dextrose 2.0%), YPS (yeast extract 1.0%, peptone 2.0%, soluble starch 2.0%) and YPDS (YPS medium added with the various concentration of dextrose) medium were used.

Yeast cells were grown on a rotary shaker (rpm 110) at 30°C and all cultures were inoculated with 2.0% of seed culture grown in a minimal medium. Cell growth was monitored by counting of cell number using a haemacytometer.

Determination of glucoamylase activity

The glucoamylase activty of culture supernant was measured by the method of Yamashita, *et al.* (15) with slight modification. Reaction mixture contained 200 μl of 1.0% soluble starch, 250 μl of 0.1 M sodium acetate buffer (pH 5.0), and 50 μl of culture supernatant. The reaction mixture was incubated at 50°C for 30 minutes and the amounts of glucose produced were determined by PGO enzyme kit (Sigma Chemical Co., Kit No. 510-DA). One unit of enzyme activity was defined as the amount of enzyme to be able to produce 1.0 μg of glucose per minute under the above condition.

Determination of plasmid stability

Plasmid stabilities of transformed cells were determined as the ratio of number of plasmid harboring cells to total viable cells. The number of plasmid harboring cells was measured by the counting the number of colonies formed on a selective medium (omitting uracil), and the number of total viable cells by counting the colonies formed on the complete medium (containing uracil).

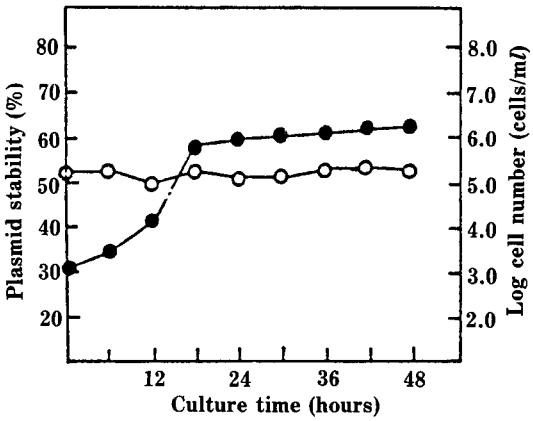

Fig. 2. Cell growth and plasmid stability of the transformed strain GMT-18 in a selective medium.
●-●; log cell number, ○-○; plasmid stability

Results

Plasmid stability in selective medium

The transformant *S. diastaticus* GMT 18, could stably maintain the recombinant plasmid pYES 18 in a selective medium. The plasmid stability in a selective medium was constant at about 55% from the time of inoculation to stationary phase during the batch cultivation (Fig. 2).

In a selective medium, the glucoamylase activity was not nearly detected with the transformed cells or donor strain 2-11A (data not shown), and the final cell concentration was very low as compared with the complex medium.

Glucoamylase productivity and plasmid stability in complex medium

As shown in Fig 3-A, the glucoamylase productivities of the transformant strain GMT-18 in YEPD and YPS media were different as compared with those of dornor strain 2-11A in the same media. The glucoamylase productivity of dornor strain was higher in YPS medium than in YEPD medium. But the glucoamylase productivity of transformant strain was higher in YEPD medium than in YPS medium. The growth of dornor cell and transformed cell in YEPD medium were very similar, but the growth of transformed cell in YPS medium was different from other cases (Fig. 3-B). Although, the final cell concentrations of all cases were almost same, the transformed strain in YPS grew very slowly, compared with other cases, namely had low specific

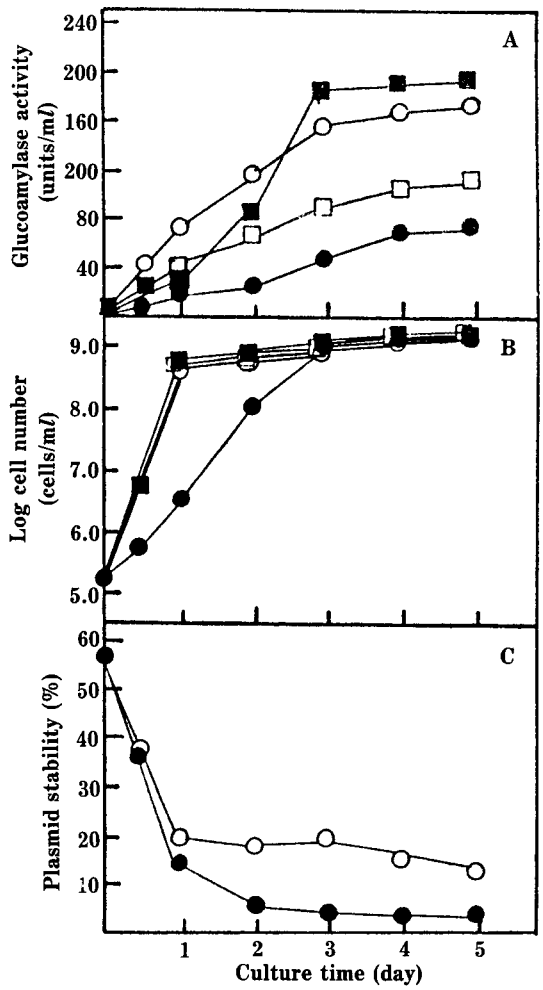

Fig. 3. Comparison of the glucoamylase productivity, cell growth and plasmid stability in YEPD and YPS medium between the transformed strain GMT-18 and gene source strain YIY2-11A.
■-■; YIY2-11A in YPS □-□; YIY2-11A in YEPD
●-●; GMT-18 in YPS ○-○; GMT-18 in YEPD

growth rate in exponential growth phase and relatively long lag phase (The specific growth rates of each case were not calculated).

In addition to enzyme productivity and growth we monitored the plasmid stability of transformed cells in YEPD and YPS medium for clarifying the differences of enzyme productivities and growth in these medium (Fig. 3-C). The plasmid stabilites in YEPD and YPS medium decreased dramatically during growing phase, but the final plasmid stabilities in each medium were very different. The decreasing rate of plasmid stability in YPS medium was more rapid

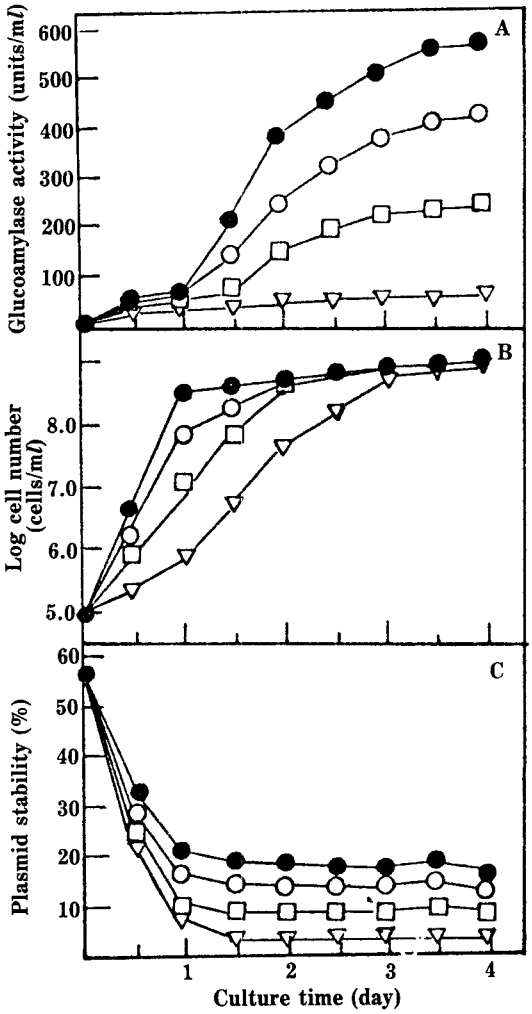

Fig. 4. Effect of glucose supplementation on the glucoamylase productivity, cell growth and plasmid stability of the transformed strain GMT-18 in YPS medium.

●-●; 2% glucose addition ○-○; 1.5% glucose addition □-□; 0.5% glucose addition △-△; no glucose addition

than in YEPD medium.

Effect of growth rate on the plasmid stability and glucoamylase productivity

To examine the influence of growth rate on the plasmid stability and enzyme productivity of transformed strain GMT-18, we used YPDS medium, glucose supplemented YPS medium, with the variation of glucose concentration. As shown in Fig. 4-A, the growth rate was significantly increased by glucose supplementation to YPS medium. As glucose concentration increased, growth were more stimulated and stationary growth phases were reached more rapidly. Glucoamylase productivities in each medium were increased with the cell growth and continuously increased after reaching stationary growth phase. After 3 days, glucoamylase activities were increased no more in each case. Although the final cell concentrations were almost the same, but glucoamylase productivities were significantly different (Fig. 4-B).

Plasmid stabilities in each medium were decreasing rapidly during growing phase, but the decreasing rates were different with the glucose concentrations. So the final plasmid stabilities were different. Consequently, the population of plasmid-harboring cells in stationary phase, although cell concentrations were the same, was different (Fig. 4-C).

Discussion

S. diastaticus is the most promisible yeast to produce ethanol from starch directly. Glucoamylase productivity of *S. diastaticus* is very important for ethanol fermentation from starch. For the purpose of increasing glucoamylase productivity, we cloned glucoamylase gene *STA* 1 of *S. diastaticus* and introduced *STA* 1 gene into recipient *S. diastaticus* with recombinant plasmid pYES 18. In this paper, we showed that glucoamylase productivity of transformed cells harboring recombinant plasmid containing *STA* 1 gene intimately was affected by the growth rate of transformed cell.

The plasmid stability of pYES 18 in a selective medium was relatively high to about 55% and maintained stably, but the glucoamylase activity was not able to detect quantitatively. It was assumed that this result was derived from the low cell density in a selective medium. Comparison of glucoamylase productivities in the complex media, YEPD and YPS medium, showed that the medium composition, being of starch as carbon source, intimately affected the glucoamylase productivity. The glucoamylase productivity of gene source strain YIY2-11A in YPS medium was higher than that in YEPD medium (Fig. 3-A). This result was well coincided with the fact that glucoamylase synthesis of *S. diastaticus* is stimulated by starch (16). But with the transformed cells, strain GMT-18, the enzyme productivity in YEPD medium was higher than in YPS medium. This result could

be explained by that the populations of plasmid harboring cells were lower in YPS medium because of the low plasmid stability (Fig. 3-C). And the difference of plasmid stability in these two media caused by the difference of growth rate could be suggested from the results in Fig. 3-B (9, 10).

To confirm that the glucoamylase productivity was affected by the plasmid stability which was influenced by the growth rate, we could make a variation of growth rate of the transformant GMT-18, by the use of glucose added YPS medium (YPDS medium). As glucose concentration was high and as growth rate was increased (Fig. 4-A), plasmid stability was maintained more highly (Fig. 4-C). Although the final cell concentrations, after entering in stationary growth phase, were almost the same in all YPDS medium and YPS medium, but the glucoamylase productivity was increased proportionally to the added glucose concentration (Fig. 4-B). This result was well explained in Fig. 4-C. The final plasmid stabilities, after rapid decline during the growing phase, were higher in the glucose added media. Therefore populations of plasmid harboring cells were higher and consequently the glucoamylase productivities increased. From these results, we could explain why glucoamylase productivity was increased in the glucose added YPS medium.

In this study, we preliminary confirmed the effect of growth rate on plasmid stability in batch culture system but we did not show the exact specific growth rate in exponential growth phase. After this we will try to confirm this relationship in chemostatic continous cultivation systems, with the variation of specific growth rate controlled by dilution rate. And also we will apply these results for the direct ethanol fermentation of starch with this transformed *S. diastaticus* (GMT-18).

요 약

전분질의 에탄올 직접발효에 유용한 *Saccharomyces diastaticus* 의 glucoamylase 생산성 향상을 위하여 glucoamylase 유전자 *STA1* 을 함유한 재조합 plasmid pYES 18을 갖는 *S. diastaticus* 의 형질전환 균주에서 성장속도가 plasmid 의 안정성에 미치는 영향과 plasmid 안정성에 따른 glucoamylase 생산성을 조사하였다. 최소 선택배지에서는 plasmid 가 일정한 수준에서 안정하게 유지되었으나, 균체의 증식과 glucoamylase 생산성은 매우 미약하였다. Starch 가 포함된 완전배지에서는 glucose 를 첨가해 줌으로써 생육속도를 촉진시킬 수 있었으며 생육속도의 촉진에 의해 plasmid 안정성이 증가되었고 이에 따라 glucoamylase 생산성이 향상됨을 알았다.

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