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Ethanol Fermentation of Corn Starch by a Recombinant *Saccharomyces cerevisiae* Having Glucoamylase and α-Amylase Activities

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Abstract

Starch is an abundant resource in plant biomass, and it should be hydrolyzed enzymatically into fermentable sugars for ethanol fermentation. A genetic recombinant yeast, *Saccharomyces cerevisiae* GA7458, was constructed by integrating the structural gene of both α-amylase from *Bacillus stearothermophilus* and the gene (*STA1*) encoding glucoamylase from *S. diastaticus* into the chromosome of *S. cerevisiae* SH7458. The recombinant yeast showed active enzymatic activities of α-amylase and glucoamylase. The productivity of ethanol fermentation from the pH-controlled batch culture (pH 5.5) was 2.6 times greater than that of the pH-uncontrolled batch culture. Moreover, in a fed-batch culture, more ethanol was produced (13.2 g/L), and the production yield was 0.38 with 2% of corn starch. Importantly, the integrated plasmids were fully maintained during ethanol fermentation.

Key words: ethanol fermentation, glucoamylase, α-amylase, fed-batch culture

INTRODUCTION

Industrial ethanol fermentation derived from raw starch consists of three steps : liquefaction of starch by α-amylase, saccharification of the liquefaction starch, and fermentation of ethanol from fermentable sugars by yeast. The enzymatic conversion of starch to fermentable sugars is needed because most yeasts are genetically deficient in degrading starch to sugars (1,2). To make it suitable for yeast growth, raw starch is treated over in 100°C for 2 to 4 hours, which converts the carbohydrate into the gelatinizing form that is hydrolyzed to fermentable sugars with bacterial α-amylase and fungal glucoamylase. Thus, using enzymes and heating for the pretreatment, as well as plant, and labor costs, and time commitment, is costly in the total processing for fermentation of ethanol. To reduce the cost of ethanol fermentation, either simplification of the process, or development of recombinant yeast that can utilize starch to produce alcohol has been studied (3,4). *Saccharomyces diastaticus*, which is close to *S. cerevisiae* physiologically and genetically, secretes an extracellular glucoamylase that can converts starch to glucose (5-7). However, fermentation of ethanol using *S. diastaticus* (8) has some disadvantages including having poor exo-amylase activity and low productivity of ethanol from glucose. On the other hand, use of recombinant yeast has the drawback of maintaining the plasmid, which is unstable mostly in long-term ethanol fermentation (9,10).

Using homologous recombination, we developed a recombinant yeast that could directly ferment corn starch to ethanol. To obtain the recombinant yeast that was genetically stable and performed high ethanol production, the glucoamylase gene (*STA1*) from *S. diastaticus* and the α-amylase gene from *Bacillus stearothermophilus* were introduced into *S. cerevisiae* SH7458. In a fed-batch culture, more ethanol was produced (13.2 g/L), and the production yield was 0.38 with 2% of corn starch. Importantly, the integrated plasmids were fully maintained during ethanol fermentation.

MATERIALS AND METHODS

Strains and plasmids

Strains and plasmids used in this study are listed in Table 1. *S. cerevisiae* SH7458 was kindly provided by Dr. S. Harashima from Osaka university in Osaka, Japan. *E. coli* JM109 was used for plasmid preparation. YIp-STA (11) and pGAR17 (12) were used for respective integration of either genes of glucoamylase, or α-amylase into *S. cerevisiae* SH7458.

Media and culture conditions

E. coli was grown in LB medium (13). For selecting transformants, ampicillin (100 g/mL) was added in the LB medium. Yeast was grown in YPD medium (1.0% yeast extract, 2.0% peptone, and 2.0% glucose). Transformants of yeast were grown on the YNBDS agar (0.67% yeast nitrogen base without amino acids, 2.0% glucose, 2.0% soluble starch, and 2.0% agar). Amino acids of necessity were added in the YN BSD medium. For 3 L jar fermentation, yeast transformants were grown at 30°C in the YPS medium (1.0% yeast extract, 2.0% peptone, and 2.0% raw starch). The inoculum size was 2% (v/v) of the working volume, and the transformant was in the exponential growth phase. During growth, air flow rate was 1 vvm and stirring rate was 250 rpm. The oxygen

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Table 1. Microbial strains and plasmids used in this study

Strains or plasmids	Relevant genotype or property	Source (reference)
Strains		
S. cerevisiae SH7458	α *leu2*, *trp1*, *ura3*, *his3*	S. Harashima (Osaka Univ.)
S. cerevisiae STA7458	Integrative transformant of SH7458 with YIp-STA	This work
S. cerevisiae GA7458	Integrative transformant of STA7458 with pGAR17	This work
E. coli JM109	F-, *rec*A1, *sup*E44, *end*A1, L*sd*R17, *gyr*A96, *rel*A1, thi△ (Lac-proAB) F-[traD36 proAB+lacI^q lacZ△M15]	(13)
Plasmids		
pGAR17	α-Amylase gene of *B. stearothermophilus*	(12)
YIp-STA	*STA1* of *S. diastaticus*	(11)

concentration in the fermentor sparged with air was controlled at 30% air saturation. Dissolved oxygen (DO) was measured by using a DO sensor (Mettler-Toledo GmbH, Switzerland), and pH was adjusted at 5.0 with 5 M of NaOH and 28% of ammonia solution.

Transformation and selection of transformants

E. coli was transformed according to manufacturers manual of Invitrogen Electroporator II (San Diego, CA, USA). Transformation of yeast was conducted as described in Ito et al. (14). The transformants which produced glucoamylase and α-amylase were obtained from the sequential transformation of YIp-STA and pGAR17 (Fig. 1). First, YIp-STA, which contained a structural gene of glucoamylase, was treated with *Kpn*I, and integrated into the host chromosomal DNA. In the absence of uracil, the transformants were selectively grown at 30°C on YNBDS agar that was supplemented with leucine, histidine and tryptophan. Subsequently, the agar plate was placed at 4°C for one day. Clones which formed clearing zones were cultured in a liquid medium (YPS) for obtaining a clone that showed the highest glucoamylase activity. The clone was named STA7458. Second, pGAR17, which contained a structural gene of α-amylase (12), was treated with *Apa*I. The linear plasmid was transformed into *S. cerevisiae* STA7458. The pGAR17 was integrated into the *URA3* gene of the chromosomal DNA. Transformants that did not require tryptophan for their growth were selectively grown in YNBDS with addition of leucine and histidine. By measuring glucoamylase and α-amylase activities, a transformant that showed the highest activities was named GA7458.

Enzymatic assays

α-Amylase activity was measured by the formation of reducing sugars from starch (15). Glucoamylase activity was determined by the formation of glucose from starch, coupled with glucose oxidase to peroxidase in the presence of paramisidine (16). The oxidised paramisidine was determined spectrophotometrically at 520 nm using UV-VIS spectrophotometer (JASCO International Co. Ltd., Tokyo, Japan). One unit of enzyme activity is defined as one mole of product formed min^{-1}.

Analytical procedures

Growth of each culture was measured spectrophotometrically at 660 nm. The dry cell mass was calculated from the correlation between optical density and cell mass. The concentration of ethanol produced was measured by using a biochemical analyzer (model 2700 SELECT Yellow Spring Instruments, Co., Yellow Springs, USA). Total reducing sugars were formed from starch hydrolysis by treatment of phenol-H_2SO_4 (15). The sugars were determined by the DNS method as described by Miller (17). For clearing zone assay of glucoamylase and α-amylase produced in *S. cerevisiae*, the recombinant cells grown in YNBDS medium were trans-

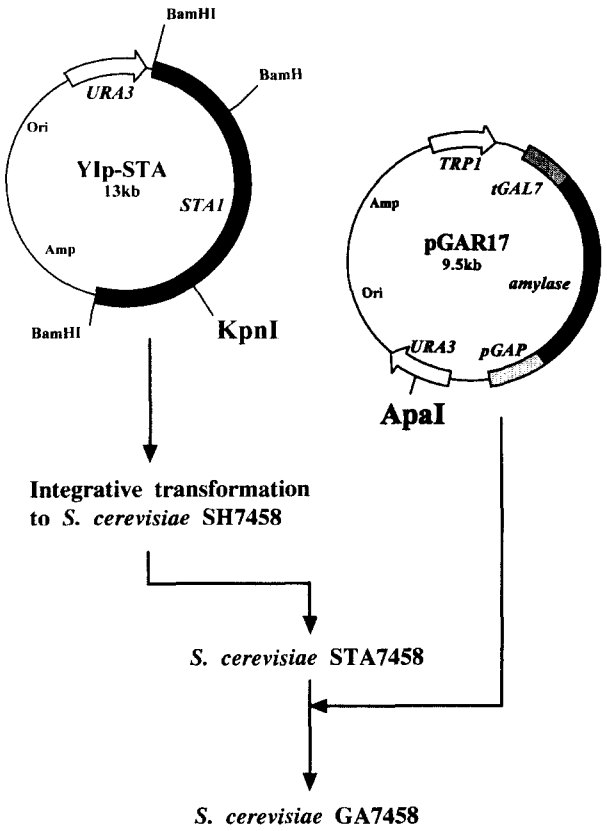

Fig. 1. Integrative transformation of glucoamylase (*STA1*) and α-amylase genes into the chromosomal DNA of *S. cerevisiae* SH7458. The plasmid YIp-STA containing *STA1* gene was linearized with *Kpn*I and introduced into *S. cerevisiae* SH7458. Transformant cells with glucoamylase activity were screened by the halo formation on appropriate selecting plate media. The plasmid pGAR17 containing α-amylase gene was linearized with *Apa*I and introduced into *S. cerevisiae* *STA*7458.

ferred to agar plates containing 2% of soluble starch. The plate was then stained with iodine solution containing 0.02% (w/v) of I2 and 2%(w/v) of KI.

Enzymes and reagents

Restriction and modifying enzymes were purchased from Takara Shuzo Co. Ltd. (Osaka, Japan). Media was bought from Difco Laboratories Co. (Detroit, USA). Raw starch was obtained from Shin Dong-Bang Co. (Seoul, Korea). Other chemicals were of analytical grades or better.

RESULTS AND DISCUSSION

Production of α-amylase and glucoamylase in *S. cerevisiae* GA7458

Although the production of glucoamylase and α-amylase in *S. cerevisiae* GA7458 resulted in formation of clearing zones around clones on the starch-contained agar plate, the nontransformant of *S. cerevisiae* SH7458 did not form the clearing zone (Fig. 2). Apparently, the size of clearing zones of *S. cerevisiae* GA7458 consisting of the genes of glucoamylase and α-amylase were larger than those of *S. cerevisiae* STA7458 having the gene of glucoamylase. This result implied that both enzymes were efficiently expressed from the genes and secreted from the cell.

Effect of initial starch concentration for ethanol fermentation

Ethanol production by *S. cerevisiae* GA7458 at different concentration levels of starch (1,2% and 3%) was determined in flask culture (Fig. 3). In general, ethanol production by the transformant was saturated in 5 days, regardless of the concentration of starch tested. However, the highest ethanol production (6.3 g/L) was obtained at 2% of starch. The low production of ethanol at 3% of starch might be a result from an excessively produced glucose that entered many different pathways to ferment some other organic acids such as citric acid, isocitric acid, etc (18). Decrease of ethanol production in the end of culture possibly came from consumption of nutrients, and inactive metabolism to ethanol. Thus, an initial starch concentration was determined to reach its optimum at 2%. Importantly, both genes of glucoamylase and α-amylase were stable through the fermentation process (Fig. 3).

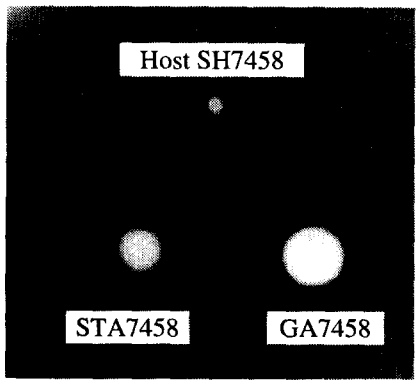

Fig. 2. α-Amylase and glucoamylase production of *S. cerevisiae* GA7458 on YPS plate containing 2% of soluble starch. The clearing zone indicates starch hydrolysis of glucoamylase activity (STA 7458), or α-amylase and glucoamylase activities (GA7458).

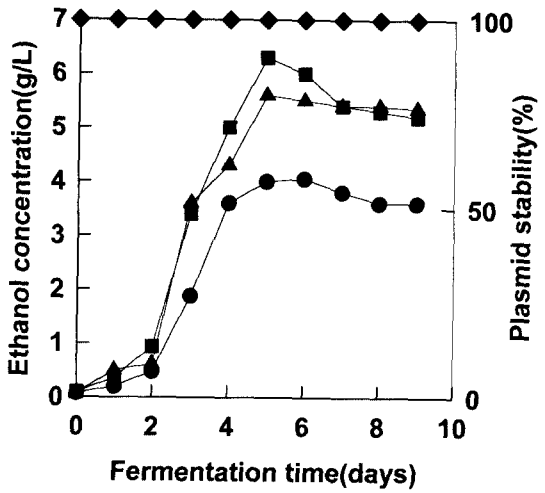

Fig. 3. Time course of ethanol production by *S. cerevisiae* GA7458 with different starch concentrations in the flask fermentation. (●), 1% starch; (■), 2% starch; (▲), 3% starch; (◆), plasmid stability.

Ethanol fermentation of pH-uncontrolled batch culture

In a pH-uncontrolled batch culture of *S. cerevisiae* GA 7458, changes of cell mass, pH, ethanol production, and utilization of starch, along with changes in activities of glucoamylase and α-amylase were fully determined (Fig. 4). When growth of the transformant entered the stationary phase, the glucoamylase activity increased to 0.49 U/mL, and then, the activity slowly decreased throughout the phase. Similarly, the α-amylase activity reached 0.6 U/mL when the cell density and ethanol production were the highest. The activity of α-amylase continued to increase up to 0.97 U/mL in the

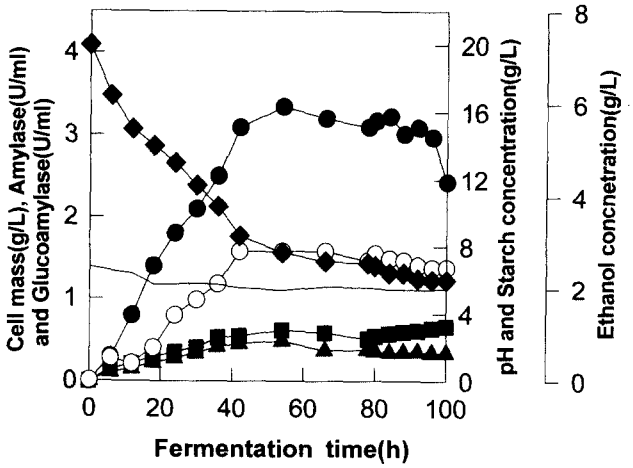

Fig. 4. Ethanol fermentation by *S. cerevisiae* GA7458 in pH-uncontrolled batch culture. (◆), starch concentration; (○), ethanol concentration; (●), cell concentration; (▲), glucoamylase activity; (■), α-amylase activity; (—), pH.

late stationary phase. Before the stationary phase, starch was proportionally consumed and cell mass and ethanol increased. Nonetheless, at the end of fermentation, 24.5% of starch was not utilized. Toward the stationary phase, the cell mass of *S. cerevisiae* GA7458 continued to increase up to 3.3 g/L with concomitant increase of ethanol production to 2.8 g/L. Without a control system, pH of the medium decreased from 6.8 to 5.4. At a pH level of 5.5, the maximal production of ethanol was measured (2.8 g/L). Also, the glucoamylase activity at pH 5.5 was 0.46 U/mL, which was close to the maximal activity (0.49 U/mL). The optimal pH of the glucoamylase from *S. diastaticus* is 5.4 (19). Thus, pH level of 5.5 would be the optimum for the production of glucoamylase in this experiment.

Ethanol fermentation in pH-controlled batch culture

In adjustment at pH 5.5, ethanol fermentation by *S. cerevisiae* GA7458 was significantly different from the pH-uncontrolled fermentation (Fig. 5). First, the glucoamylase activity increased 5-fold (2.5 U/mL) at the early stationary phase, and then, the activity decreased during the phase. Thus, the increase of glucoamylase activity appeared to accelerate ethanol production that reached its highest activity in the early stationary phase (Fig. 5). In contrast, the activity of α-amylase did not change significantly, which increased 1.4-fold (0.85 U/mL) in the stationary phase. At pH 5.5, cell mass decreased to 2.7 g/L, but ethanol production increased 2.6-fold (7.34 g/L). Most importantly, the highest production

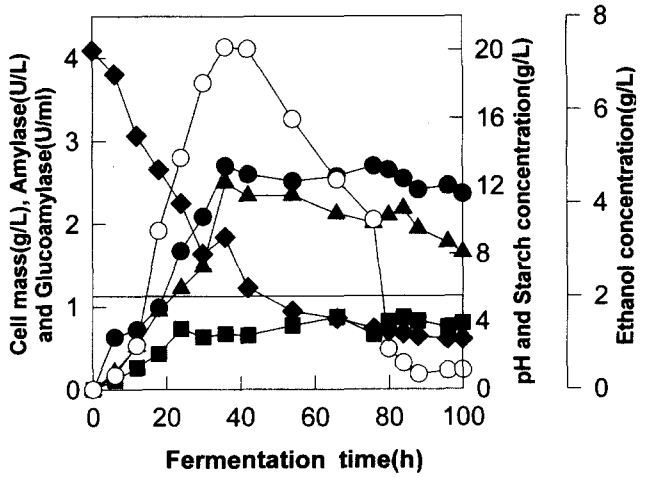

Fig. 5. Ethanol fermentation by *S. cerevisiae* GA7458 in pH-controlled (5.0) batch culture. (◆), starch concentration; (○), ethanol concentration; (●), cell concentration; (▲), glucoamylase activity; (■), α-amylase activity; (–), pH.

of ethanol in the early stationary phase implied that ethanol fermentation was most favorable at acidic pH and greatly affected by the production of glucoamylase. Thus, pH adjustment of the medium in the batch culture of *S. cerevisiae* GA7458 appeared to be important for ethanol productivity.

In Table 2, various ethanol fermentations of starch in mixed cultures of two different wild types, or in single cell cultures of yeast transformants were compared. Interestingly, the highest yield of ethanol production was found in the batch culture of *S. cerevisiae* GA7458. This result implied that due to the efficient expression and excretion of the glucoamylase and α-amylase in *S. cerevisiae* GA7458, more starch would be utilized for ethanol production. This suggestion was supported by the lower yield of ethanol production from *S. cerevisiae* SR93, which harbors the glucoamylase gene alone (22). Thus, *S. cerevisiae* GA7458 could be of a better choice for ethanol fermentation from starch.

Ethanol fermentation in fed-batch culture

To maximize the production of ethanol from the *S. cerevisiae* GA7458, regular feeding of starch into the batch culture seemed to be effective. The amount of starch in the culture was maintained between 6 and 20 g/L for the maximal yield of ethanol (Fig. 6). Addition of starch at 42 and 88h caused the increment of cell mass and ethanol; however, the rates of cell growth and ethanol production continuously declined (Fig. 6). The highest cell mass and ethanol production were 4.4 and 13.2 g/L, respectively, and the genes of *STA1* and α-amylase were maintained steadily (data not shown). Glucoamylase activity increased to 2.7 U/mL at 150 hr; α-amylase activity reached 0.84 U/mL at 24 hr. After 150 hr, cell mass was increasing although glucoamylase and ethanol production were decreasing because the recombinant yeast might utilize the produced ethanol (23,24). However, a further study to prove this possibility has not been conducted. In short, the ethanol productions from pH-uncontrolled and pH-controlled batch cultures, and from the pH-controlled fed-batch culture were 2.7, 7.34, and 13.2 g/L, respectively. Thus, the pH-controlled (5.5) fed-batch culture seemed to be more useful for the production (13.2 g/L) and yield (0.38) of ethanol compared to the other cultures (Fig. 6). The use of fed-batch culture also takes advantage of the fact that ethanol continuously produced during fermentation time of 150 hr, avoiding the repeated batch culture with fermentation intervals of 40 hr. In conclusion, ethanol production by the recombinant yeast increased according to the supply of substrate, and the fed-batch culture may have some advantages in the industry for ethanol production.

Table 2. Comparison of ethanol concentration produced and ethanol yield from starch under several incubation systems

Microorganism Saccharification fermentation	Initial starch concentration (g/L)	Ethanol produced (g/L)	Ethanol yield	References
S. fibuliger Z. mobilis	30	9.7	0.32	(20)
A. awamori Z. mobilis	100	22.0	0.22	(21)
S. cerevisiae SR93	100	24.9	0.25	(22)
S. cerevisiae GA7458	20	7.34	0.37	This work

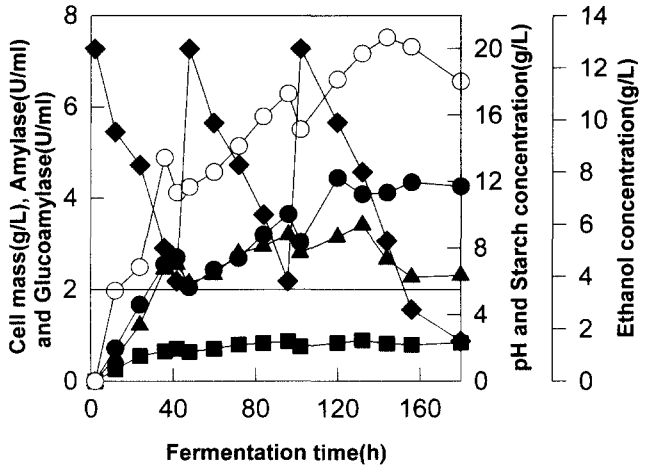

Fig. 6. Time course of ethanol production by *S. cerevisiae* GA7458 in fed-batch fermentation. Starch concentration ranged from 6 to 20 g/L. (◆), starch concentration; (○), ethanol concentration; (●), cell concentration; (▲), glucoamylase activity; (■), α-amylase activity; (–), pH.

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