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Heterologous Transformation of *Saccharomyces cerevisiae* by Glucoamylase Gene of *Saccharomyces diastaticus*

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Saccharomyces diastaticus Glucoamylase Gene에 의한 *Saccharomyces cerevisiae*의 Transformation

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To obtain a new yeast strain that is able to efficiently produce ethanol from starch, the glucoamylase gene of *Saccharomyces diastaticus* was transformed into *S. cerevisiae* without a cloning vector. The competent cells of *S. cerevisiae*, induced by the treatment of Li_2SO_4, were transformed with the partial Bam HI-digests of chromosomal DNA of *S. diastaticus*, and the transformants were selected by their abilities to utilize and ferment starch. The transformants, which appeared at a frequency of 8.5×10^{-7}, were able to withstand up to 800 ppm of copper sulfate like the recipient and retained the phenotypic expression of the recipient with the exception of the acquisition of STA gene and MAL gene, as regards fermentation of carbohydrates. The enzymatic properties of glucoamylases produced by transformants were very similar to those produced by *S. diastaticus* as based on optimium pH and temperature.

Saccharomyces cerevisiae, widely used for commercial production of ethanol or alcoholic beverages from starchy raw materials, lacks the amylase enzymes necessary for starch utilization. Thus, in current industrial fermentation processes, raw starch is gelatinized by cooking, liquefied by treatment with alpha amylase, and then saccharified to glucose by glucoamylase hydrolysis. Furthermore, the capital investment for the pretreatment of starchy material is actually one of the main causes to increase the alcohol plant cost.

To eliminate the separate saccharification step, a strain of *S. cerevisiae* was genetically constructed by introducing a vector plasmid, YRp 7 or YEp 13 containing amylase gene of *Bacillus amyloliquefaciens*(1-4). However, this yeast strain was not suited for ethanol production from starch because of low level of amylase expression and instability of the expression vectors.

As an attempt to breed a new brewing yeast, here we demonstrate genetic transformation in *S. cerevisiae* without a cloning vector, using only chromosomal DNA of *S. diastaticus* that is naturally amylolytic. And we compare the performance of the successful transformants with that of parental strains.

Materials and Methods

Strains

In this study, the donor strain was *Saccharomyces diastaticus* IFO 1046, and the recipient strain was *S. cerevisiae* X 2180-1A. The transformants was designated TSD (Table 1).

Media

The yeasts for transformation was cultured aerobically, with shaking, to exponential phase in

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Table 1. List of strains used

Strain	Genotype	Remark
S. cerevisiae X2180-1A	a SUC2 mal mel gal2	recipient
S. diastaticus IFO1046	CUP1 wild type	donor
TSD-11		
TSD-13		heterologous
TSD-14		transformant
TSD-15		

YPD broth containing 0.5% yeast extract, 0.5% peptone, and 1.0% dextrose at 30 °C.

The SM medium containing 2% soluble starch, 0.2% $(NH_4)_2SO_4$, 0.1% Difco yeast nitrogen base, and 1.8% agar was used for selection of glucoamylase producing transformants, and the quantitative assay for glucoamylase production of transformants was carried out with YPS medium containing 0.5% yeast extract, 0.5% peptone, and 1.0% solube starch.

The comparison of carbohydrate fermentation between the transformants and the parental strains was carried out with modified hayduck medium containing 4% each carbohydrates, 0.25% Bacto asparagin, 0.3% $MgSO_4 \cdot 7H_2O$, 0.1% KH_2PO_4, and 0.05% yeast extract.

Preparation of DNA

Total chrosomomal DNA of *S. diastaticus* was prepared by the methods of Rodriguez *et al.*(5) and Miura *et al.*(6). The purified yeast chromosomal DNA was patially digested with restriction endonuclease *Bam*HI according to manufacturer's recommendation, and used for the transformation as donor DNA.

Transformation

The preparation of competent cells and transformation procedure of yeast were performed by a modification of the method of Ito *et al.*(7). The yeast cells grown in YPD medium to the exponential phase were harvested, and then resuspended in 0.1M Li_2SO_4 solution and induced to develop competence at 30 °C for 1 hr with shaking. The competent cells (1×10^8cells/m*l*) were mixed with 25 μg of the donor DNA and incubated at 30 °C for 30 min. An equal volume of prewormed (30 °C) 70% polyethylene glycol (PEG, MW 4,000) solution was added and mixed thoroughly. After standing at 30 °C for 1 hr, the mixture was incubated at 42 °C for 5 min, and then washed twice with water. The cells were properly diluted and spread on SM and YPD agar medium.

The transformation frequency was calculated by dividing the number of colonies per milliliter appearing after 14 days at 30 °C on SM medium by the number of colonies per milliliter on YPD agar medium.

Glucoamylase assay

Quantitave assay for glucoamylase activity was performed by a modification of the method of Somogyi-Nelson(8), which detects reducing sugars released from soluble starch. One milliliter of 1% soluble starch in 0.1M acetate buffer (pH 5.0) was mixed with 0.5 m*l* of enzyme solution. After incubation at 50 °C for 1 hr, the reducing sugar formed was determined. One glucoamylase unit is defined as the amount of glucoamylase which releases one μmol of gluocose per minute under the above conditions.

Fermentation of carbohydrate

Fermentation of carbohydrate was compared between the transformant strains and the recipient yeast *S. cerevisiae* X2180-1A or the amylase gene donor *S. diastaticus* IFO 1046. The fermentation test was performed by the durham tube method.

Results

Transformation frequency

The competent cells of S. *cerevisiae* were transformed by the partially *Bam*HI-digested chromosomal DNA (Fig. 1) of *S. diastaticus* as described in Materials and Methods. As the result that the transformants were selected by their ability to grow on selective minimum (SM) medium containing soluble starch as a sole carbon source after 14 days growth at 30 °C, the frequency of transformation was 8.5×10^{-7} (Table 2).

Selection of yeast transformant

Among the transformant strains, the four strains were finally selected by the results of fermentation test of soluble starch and glucoamylase production. For the quantitative assays of glucoamylase production, the transformants were cul-

Fig. 1. Electrophoretic patterns of *S. diastaticus* chromosomal DNA digested with *Bam*HI (A), *S. diastaticus* chromosomal DNA (B), and Lambda chromosomal DNA digested with *Hind*III (C).

Table 2. Transformation frequency of *S. cerevisiae* by *Bam*HI-digested chromosomal DNA of *S. diastaticus* IFO 1046

Remark	Colony on YPD	Colony on SMM	Transformation frequency
recipient cells alone	2.3×10^{7}	0	0
recipient cells plus donor DNA	2.0×10^{7}	17	8.5×10^{-7}

Table 3. Fermentation of soluble starch and glucoamylase production by transformant

Strain	Fermentation*	Glucoamylase activity (U)**
S. diastaticus IFO 1046	+	18.9
S. cerevisiae X2180-1A	−	0
TSD-11	+	18.6
TSD-13	+	21.1
TSD-14	+	22.6
TSD-15	+	20.9

*Fermentation test was carried out by Durham-tube method. Cells were cultured in a test tube (1.2 × 12cm) containing 5 m*l* of SMM at 30 °C for 1 week.
**One glucoamylase unit is defined as the amount of glucoamylase which releases one μmol of glucose per minute from soluble starch under the condition described in Materials and Methods.

tured in YPS medium with shaking at 30 °C for 4 days. The results are shown in Table 3, and the glucoamylase activity exhibited by the transformants was approximately 10-20% higher than the donor *S. diasaticus*.

Table 4. Copper resistance of transformant in YPD medium.

Strain	Copper sulfate (ppm)				
	0	400	600	800	1000
S. cerevisiae X2180-1A	9.39	9.14	8.54	0.71	−
S. diastaticus IFO 1046	8.70	0.87	−	−	−
TSD-14	8.24	9.03	8.70	0.36	−
TSD-15	7.45	2.19	−	−	−

The yeasts were aerobically cultured at 30 °C for 10days in YPD medium containing various concentrations of copper sulfate, and the cell growth was estimated by measuring optical density at 620nm. Symbols: −, no growth.

Table 5. Fermentation of carbohydrate by transformant

Strain	G	Ga	Su	Ma	Me	St
S. cerevisiae X2180-1A	+	+	+	−	−	−
S. diastaticus IFO 1046	+	−	+	+	−	+
TSD-14	+	+	+	+	−	+
TSD-15	+	+	+	+	−	+

Fermentation of carbohydrate was determined in modified hayduck medium containing various sugars, each as the sole carbohydrate by Durham-tube method, and test was carried out at 30 °C for 4 weeks. Symbols: G, glucose; Ga, galactose; Su, sucrose; Ma, maltose; Me, melibiose; St; soluble starch; +, positive; −, negative.

Characteristics of transformant

In order to confirm the identity between glucoamylase secreting transformants appeared on SM medium and the recipent, the coppor resistance and the ability of carbohydrate fermentation of the transformants were compared with the parental strains.

The property of copper resistance was tested in YPD medium containing various concentration (400 to 1,000 ppm) of $CuSO_4$. As the results shown in Table 4, both the transformant TSD-14 and the recipient were able to withstand up to 800 ppm of copper sulfate, whereas the donor was able to grow below 400 ppm of copper sulfate.

On the other hand, the phenotypic expressions of the parental strains as well as the transformants were investigated. By the genetic transformation, maltose fermentation (MAL) gene cotransferred to the recipient with glucoamylase (STA) gene. With the exception of the acquisition of MAL gene and

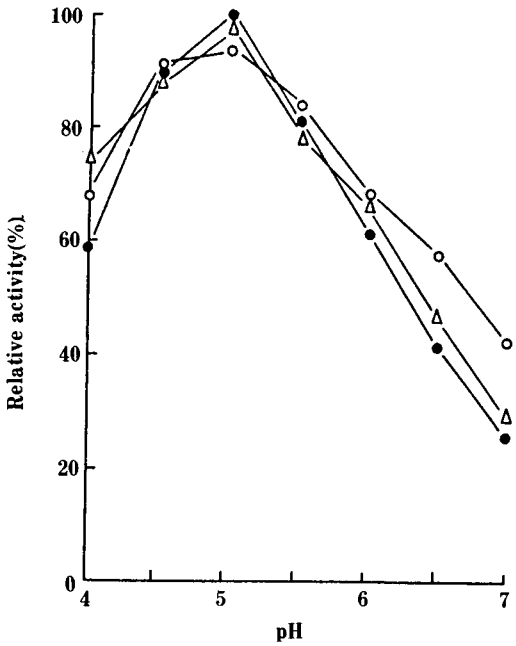

Fig. 2. Effect of pH on glucoamylase activity.
The buffer used was McIlvaine buffer, and the enzyme reaction was carried out at 50 °C for 1 hour. The enzyme activity was determined by Somogyi-Nelson method. symbols: ○, *S. diastaticus* IFO 1046; ●, TSD-14; △, TSD-15.

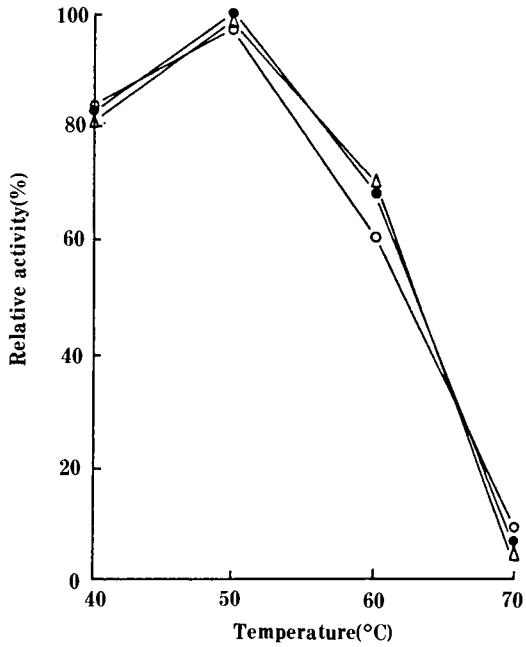

Fig. 3. Effect of temperature on glucoamylase activity.
The enzyme reaction was carried out at each temperature for 1 hour in McIlvaine buffer (pH 5.0), and the enzyme activity was determined by Somogyi-Nelson method. Symbols: ○, *S. diastaticus* IFO 1046; ●, TSD-14; △, TSD-15.

STA gene, the transformants retained the phenotypic expression of the recipient (Table 5).

Glucoamylase produced by transformant

Enzymatic characteristics of glucoamylase was compared between the transformant strains and the donor *S. diastaticus* IFO 1046. As the results shown in Fig. 2 and Fig. 3, the optimum pH and temperature of all the strains were 5.0 and 50 °C, respectively. These results show that the glucoamylases of the transformants are the same with that of the donor strain.

Discussion

With the development of genetic transformation techniques in yeast, a number of new approaches to the study of gene mapping, classification, and gene colning in this organism have become possible. The first successful transformation of intact yeast cells was reported by Oppenoorth(9), but other investigators were unable to confirm the result. Kahn *et al.*(10) also reported similar transformations, and Tuppy *et al.*(11) reported the transformation of spheroplasts of respiratory-deficient *S. cerevisiae,* but these results also were not confirmed. Using these expriments as a basis, however, Hinnen *et al.*(12) unequivocally demonstrated the transformation of yeast by introducing a chimeric Col E1 plasmid carring yeast DNA into spheroplasted yeast recipients. Recently, Barney *et al.*(13, 14) demonstrated transformation of yeast spheroplasts to introduce dextrin utilization or flocculence into *Saccharomeces* strains, and Ito *et al.*(7) reported the transformation of intact yeast cells treated with alkali cations by plasmid YRp 7.

In this study, we attempted transformation of intact cells of *S. cerevisiae,* treated with alkali cation for inducing competence, by partially *Bam* HI-digested chromosomal DNA of *S. diastaticus* in order to develop a new brewing yeast capable of fermenting liquefied starch to ethanol directly. Actually, *S. diastaticus* does secret glucoamylase and is naturally amylolytic, however, it is not as well suited as *S. cerevisiae* is for commercial production of ethanol because of production phenolic off-fla-

vors(15).

As the results, we obtained the starch-fermenting yeasts as the successful transformants, and the transformation frequency was 8.5×10^{-7}. The transformants retained the phenotypic expression of the recipient with the exception of the acquisition of STA gene and MAL gene. This result may be due to the cotransformation of Mal gene with STA gene in the recipient.

The results, presented in Fig. 2 and Fig. 3, do show the identity of glucoamylases produced by both the transformants and the donor *S. diastaticus*. The optimum pH and temperature were 5.0 and 50 °C, respectively. And these results were consistent with the characeristics of glucoamylase described by Fukui *et al.*(16).

요 약

Starch로부터 ethanol을 직접적으로 발효 생산할 수 있는 새로운 효모 균주를 개발하고자 glucoamylase 생성균으로 알려진 *Saccharomyces diastaticus*의 glucoamylase gene을 cloning vector를 사용하지 않고 *S. cerevisiae*에 transformation시켰다. Li_2SO_4 처리로써 competent화 한 *S. cerevisiae*의 intact cells을 recipient로 하여 BamHI으로 partial digestion한 *S. diastaticus*의 chromosomal DNA를 transformation시키고 starch를 유일한 탄소원으로 함유한 최소 배지상에서 starch 자화능을 marker로 하여 transformant를 선별한 결과, 8.5×10^{-7} 빈도로 transformant를 얻었다. Transformant의 특성을 recipient 및 donor와 비교하기 위해 copper resistance와 당 발효능을 조사한 결과, donor인 *S. diastaticus*와 동일한 성질로서 표현된 maltose와 starch 발효능을 제외하고는 800 ppm 농도까지 생육 가능한 copper resistance와 galactose 발효능 등에 있어서는 recipient와 동일하게 나타났다. 또한 transformant가 생성하는 glucoamylase의 그 작용에 있어서의 최적온도와 최적pH를 조사하여 본 바 각각 pH5.0, 50C로서 donor의 glucoamylase와 동일함을 알 수 있었다.

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