Intergeneric Protoplast Fusion of Heterologous Transformant of Saccharomyces cerevisiae and Candida tropicalis

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Saccharomyces cerevisiae의 Transformant와 Candida tropicalis간의 Intergeneric Protoplast Fusion

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To enhance the capability of starch fermentation of the transformant TSD-14, the heat treated protoplasts of TSD-14 were fused with the protoplasts of C. tropicalis (lys$^-$) in the presence of 30% (w/v) PEG and 20 mM CaCl$_2$. Fusants were selected by nutritional complementation on minium medium and the fusion frequency was 4.4×10^{-5}. All fusants tested were possessed of complemented traits concerning carbon compound assimilation, and the cell volumes of the fusants were approximately 1.5 times larger than the parental strains. The fusants were genetically very stable, and were able to hydrolyze alpha 1,4-glucosidic linkage as well as alpha 1,6-linkage of starch contrary to one of parents TSD-14. The most promising fusant FSC-14-75 produced 8.7% (v/v) of ethanol from 15% liquefied potato starch medium, but the result was enhanced to 9.3% (v/v) by addition of 0.3% peptone. The corresponding fermentation efficiency was 86.0%.

The starch is not directly fermentable material by yeast and must be hydrolyzed to simple sugars by diastatic enzymes obtained from malt, molds, or bacteria. Thus, the production of industrial and fuel ethanol from starchy biomass commonly involves a four-steps process such as gelatinization of starch by cooking, liquefaction by alpha-amylase, enzymatic saccharification to glucose, and fermentation of glucose. Commercial enzymes are used for liquefaction and saccharification, and represent a significant expense in the ethanol production process. In order to reduce capital investment for the pretreatment of starch material, great efforts have been made to genetically construct a new yeast strain capable of fermenting starch directly to ethanol during the past decade(1-9). Fukui et al.(2) cloned the glucoamylase gene of Saccharomyces diastaticus in S. cerevisiae and found that it was not functionally expressed. We also cloned the alpha-amylase gene from Bacillus amyloliquefaciens into S. cerevisiae(6,7). However, the new strain was not suited for ethanol fermentation because of relatively low levels of glucoamylase expression, and instability of the expression vector.

As an another way of improving ethanol production from starch, we did the yeast transformation without a cloning vector, using only partially BamHI-digested chromosomal DNA of S. diastaticus to introduce the capability of starch fermentation into S. cerevisiae(10). Despite the successful transformation, the conversion from starch to glucose was the most limiting step in the fermentation because the glucoamylase of S. diastaticus can not hydrolyze alpha 1,6-glucosidic linkage of starch.

Key words: Transformant, C. tropicalis, intergeneric fusant, alpha-1,6-glucosidase, ethanol productivity, liquefied starch
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For these reasons, it might be desirable to introduce the capability of hydrolyzing alpha 1,6-linkage into the transformant TSD-14 obtained in our previous work(10) in order to improve the ethanol productivity from starch. Hence, we have undertaken the intergeneric protoplast fusion between TSD-14 and *Candida tropicalis* possessing debranching activity in an attempt to introduce alpha 1,6-glucosidase activity.

Materials and Methods

Strains

The transformant TSD-14 and *Candida tropicalis* RCT-40 were used as the parental strains for protoplast fusion. Their origin and genetic marker are listed in Table 1.

Media

YPD medium containing 0.5% yeast extract, 0.5% peptone and 2% dextrose was used as the complete medium, and the synthetic medium containing 2% dextrose, 0.2% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% KCl and 0.2% yeast nitrogen base without amino acids was used as the minimal medium. If necessary, the media were solidified by addition of 1.8% agar.

The yeasts for protoplast fusion were cultured aerobically, with shaking, to exponential phase in production medium containing 4% dextrose, 0.3% $(NH_4)_2 SO_4$, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% KCl and 0.2% yeast extract. For the regeneration of protoplast, the solid CM and MM including 0.6 M KCl were used. Culture medium for

glucoamylase production of fusants was 1% yeast extract, 1% peptone and 2% soluble starch, and cultivation was carried out at 30 °C for 4 days.

Protoplast formation and fusion

Yeast cells grown in production medium to log phase were harvested and washed twice with sterile saline solution. In order to prepare protoplasts, the yeasts (4×10^8 cells) were suspended in 4 m*l* of 0.6M KCl (pH 8.0) containing 1 mg of zymolyase 20,000 and 50mM 2-mercaptoethanol. After incubation at 30 °C for 60min, protoplasts obtained were washed and stored in a solution of 1.2M KCl containing 20mM $CaCl_2$.

The protoplast fusion of TSD-14 and *C. tropicalis* RCT-40 was performed by the method of Fournier *et al.*(11) and the selection of fused protoplasts was followed by a modification of the method of Foder *et al.*(12). The protoplasts of TSD-14 were heat treated at 52 °C for 60 min and employed in combination with those of the auxotropic mutant of *C. tropicalis* IFO 0589, which after treatment with 30%(w/v) polyethylene glycol (PEG, MW 4,000) containing 20mM $CaCl_2$ were plated onto minimal medium forcing the selection of fused protoplasts by nutritional complementation.

Characteristics of fusant

In order to determine that the fusants were hybrids derived from TSD-14 and *C. tropicalis* RCT-40 by protoplast fusion, the physiological and morphological characteristics of the fusants were compared with those of the parental strains. As a physiological characteristic, the ability of carbon source assimilation was investigated. The cells of

Table 1. List of strains used

Strain	Genotype	Remark
C. tropicalis IFO 0589	wild type	
C. tropicalis RCT-40	lys⁻	NTG mutant of IFO 0589
S. cerevisiae X2180-1A	a SUC2 mal mel gal2 CUP1	
S. diastaticus IFO 1046	wild type	transformant of X2180-1A by IFO 1046
TSD-14		
FSC-14-75		
FSC-14-76		intergeneric fusant between RCT-40 and TSD-14
FSC-14-78		
FSC-14-80		

parents and fusants were inoculated into the minimum medium containing various carbohydrates as a sole carbon source, and cultured at 30 °C for 4 days with shaking. The cell growth was determined by measuring optical density at 570nm with a spectrophotometer. No growth was designated($-$), growth above O.D: 0.15 was designated ($+$), and intermediate growth was designated (v).

On the other hand, the determination of morphological characteristics was performed by the method of Lodder(13). The parental strains and fusants were cultured in YPD medium at 30 °C for 2 days with shaking. The cell size was measured with micrometer.

Genetic stability of fusant

The existence of heterokaryons among the fusants resulted in the great instability of the latter. To establish whether the fusants are genetically stable hybrids or not, the fusant cells grown on minimal medium were cultured on fresh CM for 2 days and spread onto YPD solid medium. After incubation at 30 °C for 4 days, the colonies grown were then replica plated on CM and MM in order to measure the percentage of auxotrophic cells in the CM medium of transfer.

Assay for glucoamylase activity

Quantitative assay for glucoamylase activity was performed by a modification of the method of Somogyi-Nelson as reported previously(10). 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 release one μmol of glucose per minute under the above conditions. To prepare the enzyme solution, the parental strains and fusant were cultured with shaking at 30 °C for 4 days in YPS medium. Culture fluid was obtained by centrifugation. The 5 ml of fluid was brought to 70% (v/v) saturation by addition of cold acetone. The precipitate was collected by centrifugation, dissolved in 5 ml of 10 mM phosphate buffer (pH 6.0), and dialyzed overnight against 200 ml of the same buffer. After centrifugation, the supernant was used as the enzyme solution.

Ethanol fermentation test

Ethanol fermentation was carried out in 250 ml flask with 200 ml medium per flask by the method

of Kim *et al.*(14). For quantitative assay of ethanol productivity, the flask was incubated at 30 °C for up to 2 weeks and measure the loss in weight resulting from carbon dioxide production. The result was converted to ethanol equivalent.

Liquefaction of potato starch

A 15% (w/v) suspension of potato starch liquefied by 0.08% (v/w, to starch content) of Thermamyl (alpha-amylase, Novo) as described previously (14) was used for preparing the fermentation medium.

Results

Heat inactivation of protoplast regeneration

Since the transformant TSD-14 was not a amino acid auxotroph in contrast to *C. tropicalis* RCT-40 (lys⁻), we could not do the general fusion experiments utilizing auxotrophic mutant strains as parents. Thus we employed dead donor techniques using heat treatment in order to select fusants on MM by nutritional complementation.

To investigate the regeneration response of the protoplasts of TSD-14 to heat treatment, the proto-
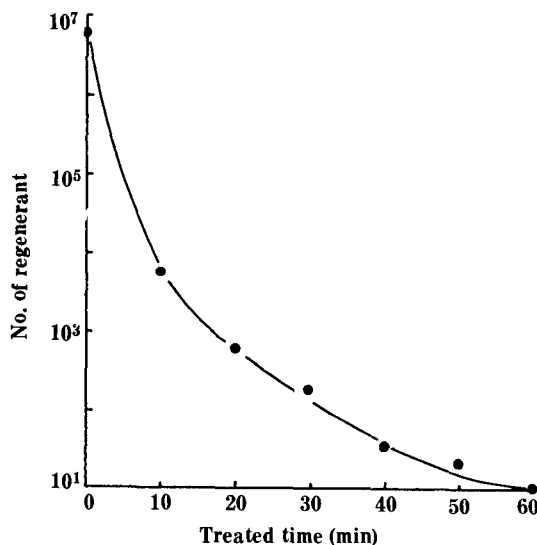

Fig. 1. Regeneration response of protoplast of TSD-14 to heat treatment.

The protoplasts suspended in a solution of 1.2M KCl containing 20 mM CaCl₂ were treated at 52 °C for various intervals, and then induced regeneration on hypertonic CM.

Table 2. Frequency of intergeneric protoplast fusion

Parenal strains	Colony on CM	Colony on MM	Fusion ferquency
TSD-14 × RCT-40	1.4×10^6	6.2×10^1	4.4×10^{-5}

The frequency of protoplast fusion expressed as the ratio of the number of colonies on the MM to that on the CM.

Table 3. Assimilation of carbon compound

Strain	G	Ra	Ri	St	Suc	X
C. tropicalis RCT-40	+	−	−	+	+	+
TSD-14	+	+	+	+	−	−
FSC-14-75	+	+	+	+	+	+
FSC-14-76	+	+	+	+	+	+
FSC-14-78	+	+	+	+	+	+
FSC-14-80	+	+	+	+	v	+

Assimilation of carbon compound was determined in MM containing various sugars, each as the sole carbon compound, and the test was carried out at 30 °C for 4 days. Symbols: G, glucose; Ra, raffinose; Ri, ribose; St, soluble starch; Suc, succinate; X, xylose; +, positive; −, negative; v, very week.

Table 4. Morphology of fusant

Strain	Cell size(μm)	Cell volume(μm^3)*
C. tropicalis RCT-40	5.8 × 4.9	72.8
TSD-14	6.1 × 5.5	96.5
FSC-14-75	7.1 × 6.0	133.8
FSC-14-75	6.7 × 6.2	142.8
FSC-14-78	6.8 × 5.9	123.8
FSC-14-80	6.7 × 5.8	118.0

*Cell volume $= \frac{4}{3}\pi \cdot \frac{a}{2} \cdot (\frac{b}{2})^2$: where a, length; b, width

Table 5. Genetic stability of fusant

Strain	Colony on CM	on MM	Auxotroph(%)
FSC-14-75	500	500	0
FSC-14-76	500	500	0
FSC-14-78	500	498	0.4
FSC-14-80	500	495	1.0

Percentage of auxotrophic cells recovered from the intergeneric fusion products between *C. tropicalis* RCT-40 and TSD-14 after cultivation on fresh CM for 2 days at 30 °C.

plasts were treated at 52 °C for various intervals. As the result shown in Fig. 1, regeneration of the protoplasts was completely inhibited by 60 min treatment at 52 °C.

Fusion frequency

The frequency of protoplast fusion expressed as the ratio of the number of colonies on the MM to that on the CM, was shown in Table 2. The intergeneric fusion frequency between protoplasts of TSD-14 and *C. tropicalis* RCT-40 was 4.4×10^{-5}, which was somewhat high in comparison with that of other intergeneric protoplast fusion.

Assimilation of carbon compounds

In order to confirm that the fusants appeared on MM were obtained from protoplast fusion between TSD-14 and *C. tropicalis* RCT-40, the assimilation of carbon compounds of fusants were compared with those of the parental strains. The assimilation patterns of both the fusants and parents are shown in Table 3. While the transformant TSD-14 was unable to assimilate D-xylose and succinate, the fusants could assimilate as satisfactory as *C. tropicalis* RCT-40. Similarly, *C. tropicalis* RCT-40 could not

assimilate D-ribose and raffinose but the fusants could assimilate like TSD-14. Each of the fusants, showed mixed assimilation pattern of carbon compounds of parental strains, was considered intermediate type.

Morphological characteristics

The results appeared in Table 4 shows the morphological characteristics of the fusants and parental strains. Whereas the cell volumes of TSD-14 and *C. tropicalis* RCT-40 were 96.5 um³ and 72.8 um³, respectively, those of the fusants were varied from 118.0 um³ to 142.8 um³ and generally 1.5 times larger than parental cells.

Genetic stability

The genetic stability of fusant acquiring desirable properties of parental strains is a prerequisite factor for the practical application. To examine the genetic stability, the prototrophic fusants growing on selection MM were transferred on fresh CM. After 2 days on this medium, a loop of cells was suspended in sterile water, diluted and spread on solid CM to obtain about 100-150 colonies per plate. Plates were replica plated on MM and CM in

order to measure the percentage of auxotrophic cells in the CM medium of transfer. As shown in Table 5, the fusants except FSC-14-80 were genetically stable.

Amylase activity of fusant.

The object of this study that we have undertaken was to introduce debranching activity of *C. tropicalis* into the transformant TSD-14 possessing the glucoamylase originated from *S. diastaticus*. Since it was thought that the successful fusant could produce the glucoamylase of *S. diastaticus* as well as alpha 1,6-glucosidase of *C. tropicalis,* the amylase activities of fusants for various substrates were detected compared with the parental strains. The

Table 6. Glucoamylase activity on various substrates

Strain	Glucoamylase activity (U)*		
	soluble starch	pullulan	isomaltose
C. tropicalis RCT-40	4.4	2.8	0.6
TSD-14	21.5	2.4	0
FSC-14-75	25.0	17.8	1.8
FSC-14-76	23.2	11.7	0.8
FSC-14-78	22.4	14.3	1.5
FSC-14-80	21.3	9.6	0

One glucoamylase unit is defined as the amount of glucoamylase which releases one μmol of glucose per minute from various substrates under the condition described in Materials and Methods.

results, summarized in Table 6, illustrate that the fusants produce glucoamylase and alpha 1,6-glucosidase, simultaneously. And among the fusants, FSC-14-75 was the most promising strain for direct fermentation of starch.

Ethanol productivity

In order to assess the application possibility of the successful fusants to direct one-step process for
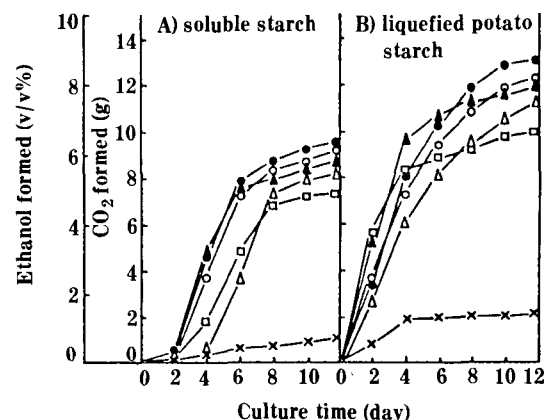

Fig. 2. Ethanol productivity from 15% soluble starch, and liquefied potato starch.

Ethanol fermentation was carried out in 250 ml flask with 200 ml medium per flask, which was equipped with air restrictor containing sulfuric acid, as described in Materials and Methods. Symbols: x, *C. tropicalis* IFO 0589; □, TSD-14; ●, FSC-14-75; ○, FSC-14-76; ▲, FSC-14-78; △, FSC-14-80.

Table 7. Effect of peptone on ethanol fermentation

Medium	Strain	Alcohol (v/v%)		Yield(%)	
		1st*	2nd**		
	S. diastaticus IFO 1046	5.0	5.2	46.7	48.5
	C. tropicalis IFO 0589	0.9	0.9	8.6	8.0
Soluble starch	TSD-14	5.0	5.2	46.7	48.5
	FSC-14-75	6.5	6.5	60.2	60.2
	FSC-14-76	6.3	6.4	58.4	59.0
	S. diastaticus IFO 1046	6.8	8.1	62.7	74.9
	C. tropicalis IFO 0589	2.0	2.1	18.4	19.5
Liquefied potato starch	TSD-14	6.9	8.3	63.9	76.8
	FSC-14-75	8.7	9.3	80.5	86.0
	FSC-14-76	8.2	8.9	76.2	82.9

*In first test of fermentation, the basal medium containing 15% carbohydrate, 0.3% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.2% $MgSO_4 \cdot 7H_2O$, and 0.2% yeast extract was used.
**In second test of fermentation, 0.3% peptone was added to the basal medium.

starch fermentation, ethanol productivities of the fusants from soluble starch or liquefied potato starch were examined compared with the parental strains.

Fig. 2(A) shows the results of ethanol yields of the strains in 15% soluble starch medium. As a rule, the ethanol yields of the fusants were preferable to those of the parents. After 12 days incubation at 30 °C, the parental strains that *C. tropicalis* and TSD-14 produced 0.9%(v/v) and 5.0%(v/v) of ethanol, respectively, whereas the fusant FSC-14-75 produced 6.5%(v/v) of ethanol.

Fig. 2(B) shows ethanol productivities of the strains from 15% liquefied potato starch medium. While the parents that *C. tropicalis* and TSD-14 produced 1.7%(v/v) and 6.9%(v/v) of ethanol, respectively, the most promising fusant FSC-14-75 produced 8.7%(v/v) of ethanol after 12 days incubation.

Effect of peptone on ethanol fermentation

To examine the influence of peptone on ethanol fermentation of the fusants and parental strains, 0.3% peptone was added to the fermentation medium containing 0.3%$(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.2% $MgSO_4 \cdot 7H_2O$, 0.2% yeast extract, and 15% carbon source such as soluble starch or liquefied potato starch.

As the results shown in Table 7, the ethanol productivities of the strains from 15% soluble starch were not enhanced, but those from 15% liquefied potato starch were comparatively enhanced. Especially, the ethanol productivity of FSC-14-75 was enhanced to 9.3%(v/v) and the corresponding fermentation efficiency was 86.0%.

Discussion

Although *Saccharomyces cerevisiae* is widely used for commercial production of ethanol or alcoholic beverages from starchy biomass, it lacks the amylase necessary for starch utilization. Thus the starchy materials must be hydrolyzed to fermentable simple sugars by the treatment of commercial enzymes, which is one of the main causes to increase the alcohol plant cost.

In previous work, we attempted transformation of intact cells of *S. cerevisiae*, without a foreign vector, by partially BamHI-digested chromosomal

DNA of *S. diastaticus* to develop a new yeast capable of converting starch to ethanol directly. However, the results were not favorable because the glucoamylase of transformant originated from *S. diastaticus* did not possess debranching activity.

The object of present investigation was to enhance the capability of starch fermentation of the transformant TSD-14, obtained in our previous study(10), by intergeneric protoplast fusion with *Candida tropicalis* possessing debranching activity. The heat treated protoplasts of TSD-14 were fused with the protoplasts of *C. tropicalis* RCT-40 which was lysine auxotrophic mutant of *C. tropicalis* IFO 0589 in the presence of 30% (w/v) PEG and 20 mM $CaCl_2$. The fusion frequency was 4.4×10^{-5} and consistent with the result described in our previous study(15).

The carbon assimilation pattern and morphology of fusants were investigated. All fusants tested were possessed of complemented traits concerning assimilation of carbon compound, and the cell volumes of fusants were 1.5 times larger than the parental strains. These findings suggested that the fusants were obtained from the intergeneric protoplast fusion between TSD-14 and *C. tropicalis*.

According to other investigators(16,17), the fusant of intergeneric protoplast fusion was genetically very unstable and rapidly reverted to parent-like segregants during subculture. However, the fusants obtained in this study were very stable.

On the other hand, the amylase activity and ethanol productivity of the fusants were examined to assess the possibility of practical application to ethanol fermentation employing starch-fermenting yeast. As the results shown in Table 6, the fusants could hydrolyze alpha 1,4-glucosidic linkage of pullulan and starch as well as alpha 1,6-glucosidic linkage of isomaltose, pullulan, and starch. This indicates that the fusants have both glucoamylase of *S. diasticus* and debranching enzyme of *C. tropicalis*, simultaneously. In fact the ethanol productivities of the fusants from 15% soluble starch and 15% liquefied potato starch were comparatively enhanced in comparison with those of parental strains. Although the *C. tropicalis* and TSD-14 produced 0.9% (v/v) and 5.0% (v/v) of ethanol from 15% soluble starch medium, respectively, the fusant FSC-14-75 produced 6.5% (v/v) of ethanol. This result was more favorable than that of Fukui *et al.*(2), which obtain-

ed 0.5% (v/v) of ethanol from 3% soluble starch medium by fusant. When the ethanol productivities of fusants from 15% liquefied potato starch medium were examined compared with the parents, *C. tropicalis* and TSD-14 produced 2.0% (v/v) and 6.9% (v/v) of ethanol, respectively, whereas the fusant FSC-14-75 produced 8.7% (v/v) of ethanol corresponding to 80.6% efficiency. The ethanol productivity of FSC-14-75 from 15% liquefied potato starch medium was enhanced to 9.3% (v/v) by addition of0.3% peptone, and the corresponding fermentation efficiency was 86.0%. This result also was more preferable to that of Mot *et al.*(4), which obtained 8.6-9.6% (v/v) of ethanol from 22.5% liquefied dextrin.

Although the precise results about ethanol productivity were not yet contained, the above results showed that the successful fusant FSC-14-75 might have the possibility for practical application to the ethanol production from liquefied potato starch.

요 약

Transformant TSD-14의 starch 발효능을 향상시키기 위해, 52℃에서 60분간의 열처리로써 regeneration능을 제거한 TSD-14의 protoplast와 *C. tropicalis* RCT-40(lys⁻)의 protoplast를 20 mM CaCl₂를 함유한 30% PEG를 fusogenic agent로 하여 융합시키고 최소배지상에서 fusant를 선별한 결과 4.4×10^{-5} 빈도로 fusant를 얻었다. Fusant들의 탄소원 자화능을 조사한 결과 parental strains의 성질이 동시에 존재함을 알 수 있었으며 cell volume은 parental strains에 비해 약 1.5배 정도 크게 나타났다.

또한 fusant는 유전적으로 매우 안정하였으며, parent인 TSD-14와는 달리 α-1,6-glucosidic linkage를 가수분해할 수 있었다. Fusant중 가장 우수한 FSC-14-75 균주는 15%의 liquefied potato starch로부터 8.7%(v/v)의 ethanol을 생성하였고 또한 이때의 fermentation broth에 0.3%의 peptone을 첨가한 경우, ethanol 생성은 9.3%(v/v) 수준으로 증가하여 총당에 대해 86.0%의 발효율을 나타냈으며 산업적 이용가능성을 시사하였다.

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