Genetically Engineered Yeast by Heterologous Transformation and Intergeneric Two-Step Protoplast Fusion for Ethanol Fermentation

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A strain of yeast which can convert starch directly to ethanol was developed by the intergeneric protoplast fusion between *Schwanniomyces alluvius* possessing α amylase as well as glucoamylase with debranching activity and FSC-14-75 which previously had been formed from a heterologous transformation and subsequent intergeneric protoplast fusion. Fusants were selected on minimal medium after protoplasts of auxotrophic mutant of *S. alluvius* fused with heat-treated protoplasts of FSC-14-75 in the presence of 30%(w/v) PEG and 20 mM $CaCl_2$. The fusion frequency was in the range of 10^{-6} order. All fusants tested were intermediate types of parental strains for carbon compound assimilation, and their cell volumes were approximately 1.1 times larger than FSC-14-75 and 1.8 times larger than *S. alluvius*. The fusants were unable to sporulate like FSC-14-75, while *S. alluvius* could sporulate. In flask scale the most promising fusant, FSCSa-R10-6, produced 7.83%(v/v) and 10.17%(v/v) ethanol from 15% and 20% of liquefied potato starch, respectively, indicating that the fermetation efficiency of each case increased 1.2 times and 1.6 times than that of FSC-14-75. The elution pattern on DEAE-cellulose chromatography showed that FSCSa-R10-6 has four distinct amylase peaks of which two peaks originated from *S. alluvius* and the other two from FSC-14-75. These results suggest that the enhanced fermentation efficiency of the fusant might be due to almost-complemented parental amylases.

Since *Saccharomyces cerevisiae* widely used for commercial production of ethanol or alcoholic beverages from starchy raw materials lacks amylase, starch should be hydrolysed to simple fermentable sugars by diastatic enzymes obtained from malt, molds, or bacteria. The production of industrial and fuel ethanol from starchy biomass commonly involves a three-step pretreatment such as gelatinization by cooking, liquefaction by α amylase, and enzymatic saccharification to glucose, before the onset of fermentation. Commercial enzymes being used for liquefaction and saccharification actually cause a significant expense in the ethanol production process. As an attempt to reduce the capital investment for the enzymatic pretreatment of starchy materials, several efforts have been made in the past decade to genetically construct a new yeast strain capable of fermenting starch directly to ethanol (1, 7, 8, 11-15).

In a previous study, we attempted a transformation of yeast intact cells which had been treated with alkali cation to induce competence by partially *Bam* H1-digested chromosomal DNA of *Saccharomyces diastaticus* in order to introduce the ability to ferment starch into *S. cerevisiae* (7). Despite the successful transformation, the conversion from starch to glucose was still the most limiting step in the fermentation employing the transformant because the glucoamylase of *S. diastaticus* did not hydrolyze α 1,6-glucosidic linkage of starch. Subsequently the intergeneric protoplast fusion between the transformant and *Candida tropicalis* capable of hydrolyzing α 1,6-linkage was also performed (13). The fusant FSC-14-75 was genetically stable, and in a 300 liter-pilot scale it produced 6.6%(v/v) of ethanol from 13.3% of liquefied sweet potato starch (total sugar, 14.8%) in 8 days with efficiency of 70.0% to the theoretical maximum, which is comparable to that from the conventional industrial process (15). However, it was highly likely that FSC-14-75 should be improved further to reduce the fermentation period and residual sugar and to enhance

the ethanol productivity.

In the present study, to improve the ethanol productivity of FSC-14-75 from liquefied starch, we performed another intergeneric protoplast fusion between FSC-14-75 and Schwanniomyces alluvius that has been known to secret α amylase as well as glucoamylase having strong debranching activity. FSCSa-R10-6, the most promising fusant, was genetically stable, and possessed traits complementary to parental strains in terms of both the carbon source assimilation and the amylase production.

MATERIALS AND METHODS

Strains

The fusant FSC-14-75 and the auxotrophic mutants of Schwanniomyces alluvius IFO 1839 were used as parental strains for the intergeneric protoplast fusion. Their origin and genetic marker are listed in Table 1.

Media

Both complete medium (CM) and minimal medium (MM) were prepared as previously described (13). Yeasts for protoplast formation were cultured aerobically with shaking to exponential phase in production medium containing 4% dextrose, 0.3% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% KCl and 0.02% yeast extract. For the regeneration of protoplast, 0.6 M KCl was added to both CM and MM. YPS medium for producing amylase and estimating specific growth rate contained 1% yeast extract, 1% polypeptone and 2% soluble starch, and cultivation was carried out at 30°C with shaking. The basal medium used to quantitate ethanol fermentation from either soluble starch or liquefied potato starch was 0.3% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.2% $MgSO_4 \cdot 7H_2O$ and 0.1% yeast extract.

Protoplast Formation and Fusion

Protoplast formation was performed essentially as previously reported (13). To prepare protoplasts of FSC-14-75, the log-phase cells (4×10^8) grown in production medium were suspended in 4 ml of 0.6 M KCl (pH

Table 1. List of strains used

Strain	Genotype	Remark
S. cerevisiae X2180-1B	α, SUC2, mal, mel, gal2, CUP1	
S. alluvius IFO 1839	wild type	
S. alluvius RSA-2	arg⁻	
S. alluvius RSA-5	thr⁻	NTG mutant of
S. alluvius RSA-8	leu⁻	IFO1839
S. alluvius RSA-9	cys⁻, met⁻	
FSC-14-75	wild type	intergeneric fusant (13)

Auxotrophic mutants of S. alluvius were screened after the wild type IFO1839 was treated with N-methyl-N'-nitro-N-nitrosoguanidine (NTG) as previously descrived (6).

8.0) containing 1 mg of zymolyase 20,000 (Kirin Brewery, Japan) and 50 mM 2-mercaptoethanol. For protoplasts of S. alluvius auxotrophs, 20 mg of cellulase (Onozuka R-10, Yakult Honsha, Japan) was added with zymolyase to the reaction mixture to enhance protoplast formation. After incubation at 30°C for 60 min, protoplasts were washed and stored in a solution of 1.2 M KCl containing 20 mM $CaCl_2$.

The protoplast fusion was done as described by Fournier et al. (4) and the fusant was selected by the method of Foder et al. (3) with some modifications. Briefly, the protoplasts of FSC-14-75 were heated at 52°C for 50 min and employed in combination with those of the auxotrophic mutant of S. alluvius IFO 1839, which after treatment with 30%(w/v) polyethylene glycol (PEG, MW 4,000) containing 20 mM $CaCl_2$ were plated onto MM forcing the selection of fused protoplasts by nutritional complementation.

Characteristics of Fusant

Since it is essential to determine that the fusants selected on MM are hybrids derived from the protoplast fusion, the physiological and morphological characteristics of the fusants were compared with those of the parental strains. As for the physiological characteristics, the ability to assimilate carbon source , and sporulation were investigated as described by Lodder et al. (9). The specific growth rate was determined by using the growth curve of each strain, which was completed after shaking culture for 36 hr in YPS medium. To determine the morphological characteristics, parental strains and fusants were cultured in CM at 30°C for 2 days with shaking. The cell size was measured with micrometer.

Genetic Stability of Fusant

The existence of heterokaryons among fusants may result in significant genetic instability. To investigate whether fusants are genetically stable hybrids, the fusants appeared on MM were cultured in CM with shaking for 2 days and then spread onto solid CM. 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.

Ethanol Fermentation Test

Ethanol fermentation was carried out in a 100 ml flask with 80 ml medium per flask as previously reported (13). The flask was equipped with air restrictor containing sulfuric acid. To quantitate the ethanol fermentation, the flask was inoculated with one loopful of cells and incubated at 30°C for up to 2 weeks. The loss in weight resulting from carbon dioxide production was measured and the result was converted to ethanol equivalent.

Liquefaction of Potato Starch

The suspension of potato starch liquefied by 0.08% (v/w, to starch content) of Thermamyl (α amylase, Novo) as previously described (7) was used to prepare the fer-

mentation medium.

Amylase Assay

The quantitative assay of amylase activity was performed by the method of Somogyi-Nelson with some modifications as previously reported (7). After the reaction mixture was incubated at 50°C for 1 hr, the reducing sugar formed was determined. One amylase unit is defined as the amount of amylase which releases one μM of glucose under the above conditions.

RESULTS

Heat Inactivation of Protoplast Regeneration

Although the procedure of protoplast fusion commonly requires complementary auxotrophic strains as parents to select fusants on MM, we employed dead donor technique using heat treatment (3) because FSC-14-75, one of the parents, was not an auxotroph. To investigate regeneration response of the protoplasts of FSC-14-75 to heat treatment, the protoplasts were treated at 52°C for different time intervals. The regeneration was completely inhibited by 50 min treatment at 52°C (Fig. 1).

Fusion Frequency and Fusant Selection

The frequency of protoplast fusion expressed as the ratio of the number of the colonies on MM to that on CM was shown in Table 2. The frequency of intergeneric fusion between protoplasts of FSC-14-75 and *S. alluvius*
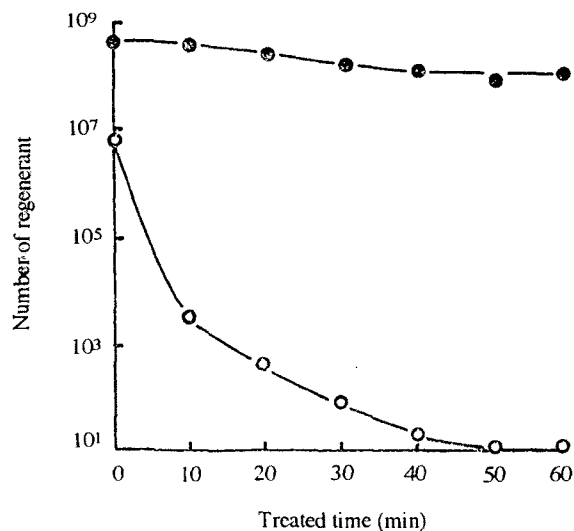
Fig. 1. Regeneration response of protoplast of FSC-14-75 to heat treatment.
The protoplast suspended in a solution of 1.2 M KCl containing 20 mM CaCl$_2$ were treated at 52°C for various intervals, and then induced regeneration on hypertonic CM. Symbols: O, Treated; ●, Not treated.

Table 2. Frequency of intergeneric protoplast fusion

Parental strains	Colony on MM	Colony on CM	Fusion frequency
FSC-14-75×RSA-2	$2.7×10^1$	$8.5×10^6$	$3.2×10^{-6}$
FSC-14-75×RSA-5	$5.2×10^1$	$9.0×10^6$	$5.8×10^{-6}$
FSC-14-75×RSA-8	$3.8×10^1$	$7.6×10^6$	$5.0×10^{-6}$
FSC-14-75×RSA-9	$1.8×10^1$	$8.4×10^6$	$2.1×10^{-6}$

Table 3. Screening of fusant from different combination of parental strains

Strain	Parental strains	Ethanol productivity* % (v/v)
FSC-14-75		4.85
S.alluvius IFO 1839		0.58
FSCSa-R10-6	FSC-14-75×RSA-2	5.02
FSCSa-F-1	FSC-14-75×RSA-5	4.92
FSCSa-F-2	FSC-14-75×RSA-8	4.71
FSCSa-F-10	FSC-14-75×RSA-9	4.96

*Ethanol fermentation was carried out in a 100 m*l* flask with 80 m*l* medium which is composed of 15% soluble starch, 0.3% (NH$_4$)$_2$SO$_4$, 0.1% KH$_2$PO$_4$, 0.2% MgSO$_4$·7H$_2$O, 0.1% yeast extract.

auxotrophs was in the range of 10^{-6} order, which was similiar to the other cases of intergeneric protoplast fusion. To select the most promising fusant for direct fermentation of starch, the ethanol productivity of each fusant was examined as compared with the parental strains, FSCSa-R10-6 showed the highest ethanol productivity (Table 3).

Genetic Stability

Since the genetic stability of fusant which acquired desirable characters of the parental strains is a prerequisite for the practical application, the genetic stability of each fusant was examined. As shown in Table 4, the fusants were genetically stable.

Table 4. Genetic stability of fusant

Strain	Colony		Auxotroph (%)
	on MM	on CM	
FSCSa-R10-6	500	500	0
FSCSa-F-1	496	500	0.80
FSCSa-F-2	493	500	1.40
FSCSa-F-10	500	500	0

Percentage of auxotrophic cells recovered from the intergeneric fusion products between auxotrophic mutants of *S. alluvius* and FSC-14-75 after cultivation in CM for 2 days at 30°C.

Table 5. Physiological characteristics of fusant

Strain	Assimilation of carbon compound						Sporulation			Specific growth rate (hr^{-1})
	G	Rham	C	Mel	Sal	St	V_8	YL	PA	
S. alluvius IFO 1839	+	−	+	+	+	+	+	+	+	0.36
FSC-14-75	+	−	−	−	−	+	−	−	−	0.33
FSCSa-R10-6	+	−	+	+	−	+	−	−	−	0.30
FSCSa-F-1	+	−	−	+	−	+	−	−	−	0.27
FSCSa-F-2	+	−	+	+	−	+	−	−	−	0.22
FSCSa-F-10	+	−	+	−	−	−	−	−	−	0.21

*Assimilation of carbon compound was determined in MM containing various sugars, each as the sole carbon source, and the test was carried out at 30℃ for 4 days. Sporulation test was done as described in Materials and Methods. Specific growth rate was estimated by using the growth curve of each strain, which was completed after shaking culture for 36 hr at 30℃ in YPS medium containing 1% yeast extract, 1% polypeptone, and 2% soluble starch. Symbols: G, D-glucose; Rham, L-rhamnose; C, cellobiose; Mel, melibiose; Sal, salicin; St, soluble starch; V_8, vegetable juice agar; YL, yeast lysate medium; PA, potassium acetate medium.

Physiological Characteristics

In order to confirm that the fusants which appeared on MM were obtained from the protoplast fusion between FSC-14-75 and S. alluvius auxotrophs, the physiological characteristics, such as carbon source assimilation, sporulation, and specific growth rate of the fusants were investigated (Table 5). Each fusant showed a mixed pattern of carbon compound assimilation derived from characteristics of both parental strains, indicating the intermediate type. In regarding sporulation, three different media i.e., V_8, Yeast lysate, and Potassium acetate were employed according to Lodder et al. (9). Different from S. alluvius IFO 1839 sporulating in each media, FSC-14-75 as well as all of the fusants was unable to sporulate. The specific growth rates of the fusants were slightly slower than those of parents.

Morphological Characteristics

The results in Table 6 show the morphological characteristics of the fusants and the parental strains. Whereas the cell volumes of FSC-14-75 and S. alluvius IFO 1839 were 130.3 μm^3 and 80.7 μm^3, respectively, those of the fusants varied between 139 μm^3 and 149 μm^3.

Ethanol Productivity

Since FSCSa-R10-6 was the most promising fusant with the best ethanol productivity from soluble starch, the possibility of applying it to eliminate saccharification step from the conventional ethanol fermentation process was investigated. The fusant was able to produce 7.87% (v/v) and 10.17%(v/v) from 15% and 20% liquefied potato starch , respectively. The corresponding fermentation efficiencies were 72.8% and 71.0% to the theoretical maximum. Under these conditions, the ethanol productivities of FSC-14-75 were 6.5%(v/v) and 6.6%(v/v), respectively representing 60.7% and 44.6% efficiency. Regardless of the substrate used, S. alluvius produced no more than 0.7%(v/v) of ethanol (Table 7). These results

Table 6. Morphology of fusant

Strain	Cell size (μm)	Cell volume (μm^3)*
S. alluvius IFO1839	5.7×5.2	80.7
FSC-14-75	6.9×6.0	130.3
FSCSa-R10-6	7.0×6.2	140.8
FSCSa-F-1	7.2×6.3	149.6
FSCSa-F-2	7.4×6.0	139.4
FSCSa-F-10	7.1×6.2	142.8

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

Table 7. Ethanol productivity of fusant

Liquefied potato starch	Ethanol productivity % (v/v)		
	Parental strain		Fusant
	S. alluvius IFO1839	FSC-14-75	FSCSa-R10-6
15%	0.5	6.5	7.8
20%	0.6	6.6	10.2

*In fermentation, the basal medium containing either 15% or 20% liquefied potato starch, 0.3% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.2% $MgSO_4 \cdot 7H_2O$, and 0.1% yeast extract was used.

showed that the ethanol productivity of FSCSa-R10-6 from 15% and 20% liquefied potato starch was enhanced 1.2 times and 1.6 times, respectively, as compared to those of FSC-14-75.

Amylase Activity of Fusant

The amylase of FSCSa-R10-6 was characterized by DEAE-cellulose column chromatography and its elution pattern was compared with those of the parental strains (Fig. 2). After each strain was cultured in YPS medium at 30℃ with shaking for 4 days, the cells were removed by centrifugation and the supernatant was 80% saturated with ammonium sulfate to precipitate the amylase. The precipitate was dissolved in an aliquot of 50 mM phos-
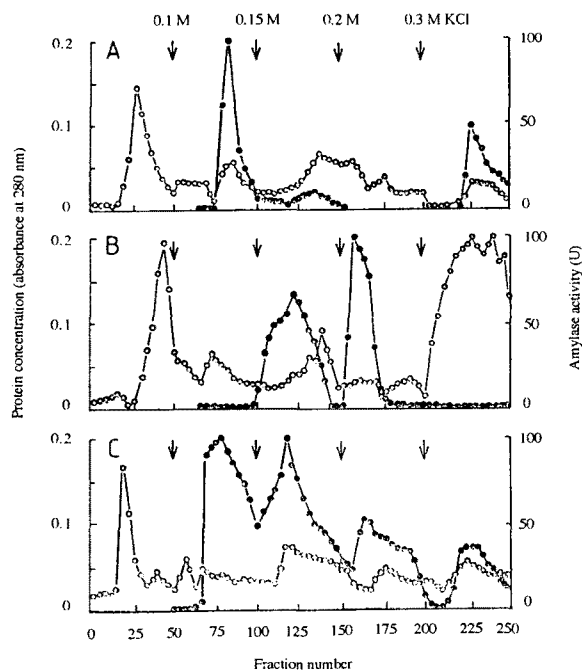
Fig. 2. Elution pattern of amylase on DEAE-cellulose.
Elution pattern of the amylase produced by fusant FSCSa-R10-6 was compared with those of parental strains, FSC-14-75 and S. alluvius. The column (0.9 cm×25 cm) was eluted with 50 mM phosphate, pH 7.5, with increasing KCl, stepwisely. Symbols: ○, Protein; ●, Amylase activity; A, FSC-14-75; B, S. alluvius; C, FSCSa-R10-6.

phate buffer (pH 7.5) and dialyzed against the same buffer. The enzyme solution was subsequently applied on a DEAE-cellulose column (0.9 cm×25 cm) and eluted stepwisely by 0.05 M, 0.15 M, 0.2 M, and 0.3 M KCl. As might be expected from its ethanol productivity and both physiological and morphological characteristics, FSCSa-R10-6 exhibited the amylases originated from its parental strains.

DISCUSSION

When the main substrate for industrial ethanol fermentation by yeast is starch, the fermentation process should employ the hydrolysis process of starch in order to obtain fermentable sugars. In the previous studies, we performed the heterologous transformation of S. cerevisiae by Bam H1-digested chromosomal DNA of S. diastaticus (7) and the subsequent intergeneric protoplast fusion with C. tropicalis (13), as an attempt to develop a new yeast strain capable of fermenting starch directly to ethanol. In mini-jar fermentor scale adopting a desirable fusant FSC-14-75, we were able to show a

possibility that the saccharification step could be eliminated from the current fermentation process of ethanol (14). However, when typical raw starchy materials such as sweet potato and barley powder were used as substrate after liquefaction in a pilot scale of 300 liters, the efficiency of ethanol productivity of FSC-14-75 was 70% to theoretical maximum, indicating further improvement still remained (15).

To improve the fermentation efficiency of FSC-14-75, another intergeneric protoplast fusion between FSC-14-75 and S. alluvius was undertaken. Since it is generally known that complementary auxotrophic mutants of parental strains are useful for the selection of fusant on MM, auxotrophic mutants obtained from the NTG-treatment of S. alluvius were employed. However, the protoplast of FSC-14-75 were heat-inactivated according to the method of dead donor techniques (3) to prevent not only its own regeneration on MM but also the disturbance of its genetic characteristics during the NTG-mutagenesis to select auxotrophs. The fusion frequency was in the range of 10^{-6}, which was consistent with other cases (2), but 10 times lower than the frequency between S. cerevisiae and C. tropicalis (6). The most desirable fusant of each of the four different combinations of the parental strains was screened based on the specific growth rate and its ability to produce ethanol from soluble starch, and designated as FSCSa-R10-6, FSCSa-F-1, FSCSa-F-2, and FSCSa-F-10.

These fusants appeared to be the intermediate type of the parental strains in terms of carbon source assimilation, and the cell volumes were 1.1 times larger than FSC-14-75 and 1.8 times larger than that of S. alluvius. Consistently with the size, the DNA content of fusants was 1.3 times higher than FSC-14-75 , 1.7 times higher than S. alluvius, and 3.5 times higher than that of haploid strain, S. cerevisiae X2180-1B(α) (data not shown). Not like S. alluvius, the fusants were similar to FSC-14-75 in that they could not sporulate. These results indicate that both the fusants and FSC-14-75 are polyploids. However, a routine test of genetic stability showed that the fusants were genetically stable. It is generally accepted that industrial yeast strains are often polyploids and that the polyploidity enable their multiple gene structure to be less susceptible to mutational forces (16).

When 15% and 20% liquefied potato starch were employed as substrates, the most promising fusant, FSCSa-R10-6, produced 7.83%(v/v) and 10.17%(v/v) of ethanol, respectively, whereas the parental strain FSC-14-75 produced 6.52%(v/v) and 6.54%(v/v) under the same conditions, indicating that the fermentation efficiency of FSCSa-R10-6 was 1.2 times and 1.6 times higher than those of FSC-14-75. These results suggested that the most promising fusant FSCSa-R10-6 could be applied

to direct ethanol fermentation from liquefied starch, since under the optimal condition in mini-jar fermentor scale the parental strain FSC-14-75 could produce 8.7%(v/v) ethanol from 15% liquefied potato starch with an efficiency of 80.6% to the theoretical maximum (14). To confirm the enhanced fermentation efficiency of FSCSa-R 10-6 was due to coexistence of the amylases originating from the parental strains, the characteristics of the amylases synthesized by both FSCSa-R10-6 and the parental strains were compared in terms of the elution pattern of DEAE-cellulose column chromatography. FSC-14-75 had three different amylase peaks which contained two major and one minor peak. Since S. diastaticus produces only one glucoamylase (16), one of the amylase peaks may be from S. diastaticus and the other two peaks may be from C. tropicalis. Schwanniomyces alluvius is known to synthesize α amylase as well as glucoamylase (16). This suggests that the two amylase peaks produced by S. alluvius may correspond to each amylase. Interestingly, FSCSa-R10-6 had four distinct amylase peaks of which two peaks from S. alluvius and the other two from FSC-14-75, demonstrating the coexistance of the parental amylases in the fusant.

Taken together, although the precise results concerning ethanol productivity under the optimum condition were not yet contained, these results indicate that the fusant FSCSa-R10-6 may have enough potential to be applied to direct ethanol fermentation from liquefied starch.

Acknowledgements

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(Received July 30, 1993)