

## Selective Isolation and Characterization of *Schwanniomyces castelli* Mutants with Increased Production of $\alpha$ -Amylase and Glucoamylase

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This study was carried out to isolate and characterize the mutant strains of *Schwanniomyces castelli* NRRL Y-2477. Mutants were prepared with the treatment of ethyl methane sulfonate. 2-deoxy-D-glucose resistant mutants were isolated and two mutants were selected based on their high production of amylolytic enzymes and their ability to ferment starch. The mutants selected had higher  $\alpha$ -amylase and glucoamylase activities than the wild type strain from several other carbon sources. Especially, it was revealed that mutant strain M-9, when cultured in the presence of glucose as a sole carbon source, shows relatively high activities of  $\alpha$ -amylase and glucoamylase compared to those of the wild type strain. In result, this mutant strain can be considered as a constitutive producer of amylolytic enzymes. To compare the ethanol production ability of wild type strain and of mutant strains selected, an alcohol fermentation was carried out using 100 g/l soluble starch. Mutant strain M-9 did not improve the direct alcohol fermentation of starch, despite its excellent amylolytic activities performance. On the other hand, mutant strain M-6 produced 37.9 g/l (4.8%, v/v) ethanol by utilizing about 82% of substrate.

The amylolytic properties of yeast species belonging to the genus *Schwanniomyces* spp. have been studied in several laboratories (1, 3, 9, 10, 12, 15, 19). *Schwanniomyces* spp. can produce extracellular  $\alpha$ -amylase, glucoamylase and debranching enzyme (4, 10, 13, 19), which makes it useful in the direct alcohol fermentation of starch (2, 9, 18). The biosynthesis of amylolytic enzymes in *Schwanniomyces* spp. is subjected to catabolite repression by glucose, and to induction by starch and maltose (6, 12, 13, 15). Recently, several authors (5, 14, 16) have reported that mutants resistant to glucose catabolite repression have been isolated from *Schwanniomyces* spp. by selection for resistance to glucose analogue such as 2-deoxy-D-glucose. These mutants not only increased the production of amylolytic enzymes but also improved the ethanol production from starch (5, 18).

This paper also describes the selection method of mutant of *Schw. castelli* NRRL Y-2477 which has the capability of overproducing the amylolytic enzymes and of direct alcohol fermentation of starch.

## MATERIALS AND METHODS

### Microorganism

The yeast strain used was *Schwanniomyces castelli* NRRL Y-2477. This strain was maintained on slant containing 1% yeast extract, 1% peptone, 2% glucose and 1.5% agar at 4°C.

### Media and Cultures

Inocula were prepared by growing the strain in medium containing 2% glucose, 1% yeast extract and 1% peptone for 16 h with agitation of 150 rpm at 30°C. Fermentation medium consisted of 0.5% yeast extract, 0.5% peptone, 0.5% KH<sub>2</sub>PO<sub>4</sub>, 0.2% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.04% MgSO<sub>4</sub>·7H<sub>2</sub>O and carbon source. The pH of the medium was adjusted by adding 0.1% CaCO<sub>3</sub>.

The enzymes and ethanol were produced in a 500 ml flask containing 200 ml fermentation medium at 30°C with agitation of 150 rpm in a shaking incubator. The culture broth was then centrifuged at 4,000 rpm for 15 min at 4°C and the supernatant containing the extracellular enzymes was used as enzyme solution.

The direct alcohol fermentation of starch was carried out in a 2 l jar fermentor (KFC) by loading 1.5 l fermen-

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tation medium at 30°C with 200 rpm agitation and 0.05 VVM aeration.

#### Analytical Methods

Reducing sugar was measured by DNS method (8) while total sugar concentration was determined by Anthrone method (17). Ethanol was measured by gas chromatography with a flame ionization detector and the cell number was counted by a hemacytometer under microscopy.

#### Assay of Amylolytic Enzymes

$\alpha$ -amylase activity was measured by the iodine method described by Wilson *et al.* (19). One unit of  $\alpha$ -amylase was defined as the amount of enzyme required to degrade one microgram of starch per minute at 40°C and pH 6.0. Glucoamylase activity was measured by using the substrate of 0.3% *p*-nitrophenyl- $\alpha$ -D-glucopyranoside described by Park *et al.* (11). One unit of glucoamylase was defined as the amount of enzyme required to produce one microgram of *p*-nitrophenol per minute at 40°C and pH 5.0.

#### Mutagenesis and Isolation of Mutants

The wild strain was grown in a culture medium containing 5% glucose with shaking at 30°C. In the mid-exponential phase the cells were harvested by centrifugation. The cells were washed three times with 0.9% saline solution and once with 0.1 M potassium phosphate buffer (pH 8.0). The cells were suspended in a solution containing 0.1M potassium phosphate buffer (pH 8.0) and 0.1% EMS, and were incubated at 30°C for 1 hr with occasional shaking. After this period, the same volume of 5% sodium thiosulfate was added and the cells were incubated for 15 min to stop mutagenesis from taking place.

The mutated cells were harvested by centrifugation and washed twice with 0.9% saline solution. The samples of 0.1 ml suspended with 0.9% saline solution were spreaded onto the plates of mutant selective medium containing 2% starch, 0.67% yeast nitrogen base (w/o amino acids), 0.1% 2-deoxy-D-glucose and 2% bacto-agar, and were incubated for 3~5 days at 30°C. Once the colonies appeared, they were selected as 2-deoxy-D-glucose resistant mutants.

## RESULTS AND DISCUSSION

#### Isolation and Selection of Mutants

The wild strain of *Schw. castellii* NRRL Y-2477 failed to grow on the starch medium containing 0.1% 2-deoxy-D-glucose. This was due to the catabolite repression of amylolytic enzymes synthesis by the 2-deoxy-D-glucose, rather than the growth inhibition (14). Indeed, 0.1% 2-

deoxy-D-glucose only slightly inhibited the growth of *Schw. castellii* on the same medium when glucose was substituted for starch. Twenty-two colonies were isolated from the plates of selective medium. These mutant strains were then transferred onto the selective medium, and seventeen mutant strains were isolated as 2-deoxy-D-glucose resistant mutants, based on colony growth and clear zone size.

Among the seventeen mutants, two mutant strains, namely M-6 and M-9, were selected based on the high production of amylolytic enzymes from 5% (w/v) glucose medium, and of ethanol from 10% (w/v) dextrin medium by flask culture.

#### Production of Amylolytic Enzymes by Mutants

Two 2-deoxy-D-glucose resistant mutants were compared to the wild type strain in terms of their ability to produce amylolytic enzymes. In glucose medium, the wild type of *Schw. castellii* NRRL Y-2477 did not produce amylolytic enzymes due to the catabolite repression of glucose (Fig. 1). On the other hand, the mutant strains showed high amylolytic enzyme activities in the presence of glucose, although the cell numbers of the mutants were not higher than that of the wild type (Table 1). In particular, mutant M-9, when cultured in the presence of glucose as a sole carbon source, was found to produce significant quantities of  $\alpha$ -amylase and glucoamylase. Thus, it was concluded that mutant M-9 had become a constitutive producer of amylolytic enzymes. Mutant M-6 was also able to produce the amylolytic enzymes

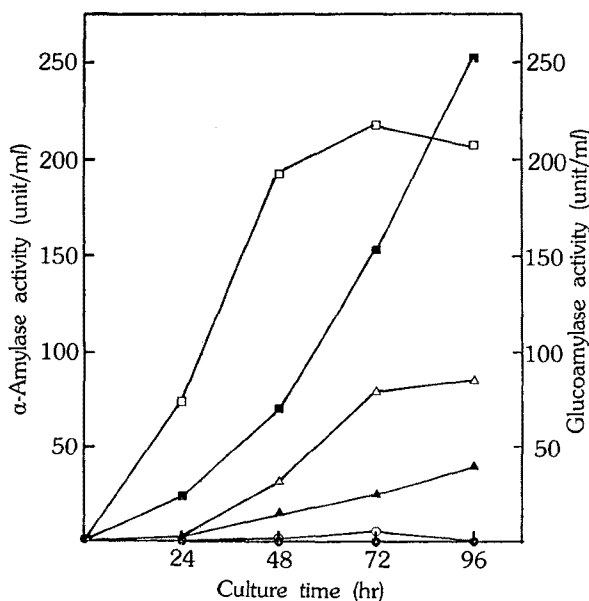


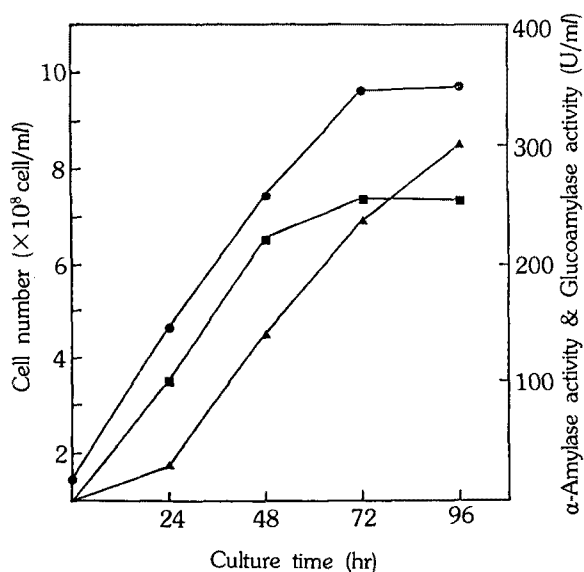
Fig. 1. Production of  $\alpha$ -amylase (○, □, △) and glucoamylase (●, ■, ▲) by the wild type (○, ●), mutant M-6 (△, ▲) and mutant M-9 (□, ■) in 5% (w/v) glucose medium.

**Table 1. Production of cells and amylolytic enzymes in 5% glucose, maltose, dextrin and 2% starch after 72 hr culture.**

Strains	Wild type			Mutant M-6			Mutant M-9		
	Cell No. ( $\times 10^8$ /ml)	$\alpha$ -Amylase (U/ml)	Glucoamylase (U/ml)	Cell No. ( $\times 10^8$ /ml)	$\alpha$ -Amylase (U/ml)	Glucoamylase (U/ml)	Cell No. ( $\times 10^8$ /ml)	$\alpha$ -Amylase (U/ml)	Glucoamylase (U/ml)
Glucose	9.7	5.5	0	9.7	79.1	22.6	9.0	218.3	153.0
Maltose	9.0	204.3	157.8	8.8	197.2	139.3	8.7	225.0	212.1
Dextrin	8.2	201.6	223.0	8.6	236.3	160.0	8.6	256.2	235.9
Starch	6.6	203.8	191.7	7.0	235.8	215.7	6.8	255.1	228.7

in the presence of glucose, although the production level of amylolytic enzymes was about one-third for  $\alpha$ -amylase and one-tenth for glucoamylase, compared to dextrin and starch medium. Therefore, mutant M-6 had become a partially catabolite derepressed mutant strain. In addition, maltose, dextrin and starch were strong inducers for synthesis of amylolytic enzymes of wild type and mutant strains (Table 1) as reported by several authors (6, 12, 13, 15). It was also found out that dextrin is a stronger inducer of amylolytic enzymes production than others.

The profiles of cell growth and amylolytic enzyme production by mutant M-9 are shown in Fig. 2. The biosynthesis of  $\alpha$ -amylase by mutant M-9 appears to be cell growth associated since this enzyme was primarily produced without any induction period during the exponential phase. But  $\alpha$ -amylase production of the wild type was reported to be delayed to some extent in the early exponential phase (6, 7, 14) since it requires time to induce the enzyme. The synthesis of glucoamylase by the

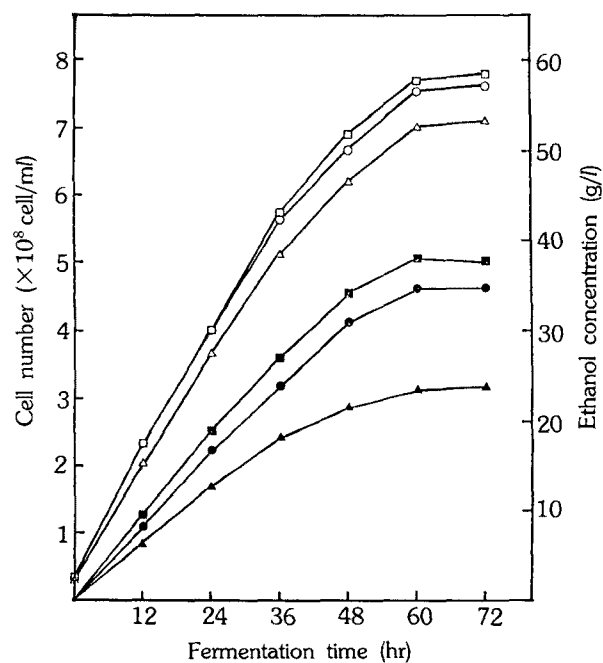
**Fig. 2. Profiles of cell growth (●),  $\alpha$ -amylase activity (■) and glucoamylase activity (▲) by mutant M-9 in 5% (w/v) dextrin medium.**

mutant M-9 started somewhat later than that of  $\alpha$ -amylase whereas the synthesis of this enzyme continued up to the stationary phase.

### Direct Alcohol Fermentation of Starch

In order to compare the ability of the wild type strain and of mutant strains selected to produce ethanol, alcohol fermentations were carried out using 10% (w/v) soluble starch (dextrin). The profiles of cell growth and ethanol production are shown in Fig. 3 and various kinetic parameters are presented in Table 2.

The maximum ethanol concentration and ethanol production rate of mutant M-6 were higher than those of the wild type, whereas the maximum specific growth rate and cell concentration were about the same. The mutant M-6 produced 11.8% more ethanol and showed 10.5% greater ethanol production rate compared with

**Fig. 3. Profiles of cell growth (○, □, △) and ethanol production (●, ■, ▲) during the batch fermentation of 10% (w/v) dextrin by wild type (○, ●), mutant M-6 (△, ▲) and mutant M-9 (□, ■).**

**Table 2. Various kinetic parameters of wild type and mutant M-6, M-9 strains during batch fermentation of 10% (w/v) dextrin.**

Strains	$\mu_{max}$	$X_m$	P	$P_m$	$Y_{p/s}$
Wild type	0.16	7.6	0.57	33.9	0.38
Mutant M-6	0.16	7.8	0.63	37.9	0.42
Mutant M-9	0.16	7.1	0.40	23.7	0.30

$\mu_{max}$ : Maximum specific growth rate ( $h^{-1}$ )

$X_m$ : Maximum cell concentration ( $\times 10^8$  cell/ml)

P: Overall ethanol productivity (g/l-h)

$P_m$ : Maximum ethanol concentration (g/l)

$Y_{p/s}$ : Ethanol yield (g-ethanol/g-dextrin consumed)

the wild type because of higher amylolytic enzymes production. The maximum ethanol concentration by mutant M-6 was 37.9 g/l, and this value was higher than that reported by Dhawale *et al.* (5), which was 33.9 g/l obtained from 9.25% (w/v) soluble starch by the derepressed mutant of *Schw. castellii*. The ethanol yield of mutant M-6 was 0.42 g-ethanol/g-dextrin consumed, which was 82.2% of the theoretical value. This value was slightly higher than that of the wild type. Mutant M-9 did not improve the direct alcohol fermentation of starch despite its relatively high amylolytic activities (Table 1). This result suggested that the mutant M-9's abilities to grow cells and to ferment alcohol had decreased as the result of the decrease in its tolerance to ethanol.

In conclusion, it would be possible to develop a yeast strain that possesses high amylolytic enzyme activities and a great capability of alcohol fermentation, by the protoplast fusion between *Saccharomyces* spp. and mutant M-9 strain in order to produce a high concentration of ethanol from a direct alcohol fermentation of starch.

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