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Isolation of Ethanol-tolerant Strains of Yeast in Relation to Their Tolerant Mechanism

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에탄올 내성 효모의 선별과 그의 에탄올 내성 기작

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ABSTRACT: The selection of ethanol-tolerant strains was applied to enrichment culture of YPD broth medium containing various concentrations of ethanol. Isolates were identified to be *Saccharomyces cerevisiae*, the others as *S. dairensis, S. exiguus, S. telluris, Saccharomycodes ludwigii, Schwanniomyces occidentalis* var. *occidentalis* and *Zygosaccharomyces florentinus*. Among isolates *S. cerevisiae* YO-1 was screened as having the highest ethanol tolerance and produced 18% (v/v) ethanol after 4 days fermentation. The change of fatty-acyl residues represents that a progressive decrease in fatty-acyl unsaturation and a proportional increase in saturation in phospholipids of yeast cells during fermentation affected the yeast viability. Supplementation ethanol to the cultures led to an increase of unsaturated fatty-acyl residues, especially C_{16} or C_{18} residues, along with a decrease in the proportion of saturated residues in cellular phospholipids. Increasing the amount of soy flour led to an increase in the maximum number of viable yeast cells and ethanol production. It was possible in 4 days to reach 21%(v/v) ethanol by adding 4% soy flour as source of unsaturated fatty-acyl residues to the fermentation medium. Soy flour not only increased yeast population but also enhanced the physiological properties of yeast cells to be ethanol tolerant in the anaerobic culture.

KEY WORDS □ Ethanol-tolerant yeast, *Saccharomyces cerevisiae*, tolerant mechanism, soy flour

The recent oil crisis encouraged the examination of fermentation as an economic means of ethanol production, particularly in several countries such as Brazil which have large agricultural surpluses and few indigenous sources of useful energy. Yeast are without doubt the most important group of microorganisms commercially exploited by man. At present, only *Saccharomyces cerevisiae* and related species are of major industrial importance for ethanol production.

S. cerevisiae has the ability to take up and ferment a wide range of sugars. However, the use of bacteria for the production of industrial ethanol has only recently been receiving attention. It has been shown that *Zymomonas mobilis* can convert glucose to ethanol with higher specific rates of glucose uptake than *S. cerevisiae*, although it is far less tolerant to high ethanol concentration than yeast, thus rendering its potential use for industrial fermentation is doubtful (D'Amade and Stewart, 1987; Day *et al.*, 1975).

To increase ethanol fermentation, the urgent study is required for the selection of ethanol-tolerant yeast because the ethanol tolerant yeast should produce higher ethanol fermentation. For the selection of ethanol tolerant yeast, there were attempts to isolate ethanol-tolerant mutants by conventional screening (Ismail and Ali, 1971) and continuous selection system(Brown and Oliver, 19 82).

Several reports(Thomas *et al.*, 1978; Beaven *et al.*, 1982) had focused on the importance of cell lipids in ethanol tolerance of *S. cerevisiae*, indicating that cell populations grown in the presence of ergosterol and palmitoleic acid were more resistant to high ethanol concentration. Ethanol produced

during fermentation adversely affects the specific growth and fermentation rates. Several authors reported an improvement in alcoholic fermentation productivity and increase of the final ethanol concentration by broth supplementation such as lipid and soy flour(Viegas *et al.*, 1985a; 1985 b).

MATERIALS AND METHODS

Sampling of materials

For the selection of ethanol-tolerant yeast, 24 samples were taken from brewing companies, foods and soils in Korea.

Isolation of yeast strains

The first selection of ethanol-tolerant strains was applied to enrichment culture of YPD (1% yeast extract, 2% peptone and 2% dextrose) broth medium with various concentrations of ethanol at 30℃ for 24-72 hours. Isolation of yeast strains was monitored on the plate of YPD medium.

Identification of isolates

The yeast strains were identified to morphological observation and the biochemical test including carbohydrate fermentation, assimilation of carbon compounds and nitrate, vitamin free growth, and urease activity (Kreger-van Rij, 1984).

Cell growth and culture condition

Unless otherwise stated, the incubation was carried out at 30℃ with rotary shaker at 180 rpm. Erlenmeyer flasks of 250 m*l* were used for enrichment cultivation of 50 m*l* broth medium. The fermentation culture was performed with 100 m*l* of fermentation broth medium containing 1% yeast extract, 0.5% peptone, 0.3% KH_2PO_4, 0.3% $(NH_4)_2SO_4$, 0.0025% $CaCl_2$, 0.0025% $MgSO_4 \cdot 7H_2O$ and 32% glucose in 250 m*l* Erlenmeyer flask at 30℃ in rotary shaker.

Determination of ethanol concentration

Ethanol concentrations were measured by using gas chromatography on a stainless-steel column. The column packing material was Porapak Q by the methods of Guijarro and Lagunas (1984), Ju *et al.*, (1983), and Nagodawithana *et al.*, (1974).

Assay of glucose concentration

The glucose concentration was determined by the Somogyi-Nelson method (Nelson, 1944).

Extraction of lipids

Lipids were extracted from the cells by the modification of the methods of Thomas *et al.*, (19 78). The yeast cells were harvested from the fermentation medium after 4 days incubation, and lyophilized after being washed several times with water. Dry cells (100 mg) were mixed with 4 m*l* of boron trifluoride methanol, and shaking at 30℃ for 16 hr. This reaction mixture was mixed with 2 m*l* of petroleum ether and lipids was extracted with vortexing 3 times.

Gas-liquid chromotography for fatty acid determination

Table 1. *Ethanol production of yeast strains on aerobic culture condition*

Strains		Percentage (v/v)
Saccharomyces cerevisiae	SE-1	16
	SU-1	17
	KY-1	16
	KY-4	14
	KY-5	17
	YO-1	18
	SH-1	15
	BU-1	16
	JI-1	15
	SW-1	15
	AT	16
	SSY-1	13
	SJR-1	15
	SDJ-1	16
	SJR-396	14
	SIS-1	16
	SJY-1	16
	SGD-1199	16
S. dairensis	IN-1	13
S. exiguus	IN-3	4
S. telluris	BH-4	14
Saccharomycodes ludwigii	BH-2	8
Schwanniomyces occidentalis var. *occidentalis*	SA-4	8
Zygosaccharomyces florentinus	IS-1	14

The methyl ester of fatty acids obtained was analyzed by gas-liquid chromatography. One μ*l* of the solution was injected on to the FFAP stainless column (0.53 mm×25 m) of Hitachi 163 gas-liquid chromatograph fitted with a falame ionization detector. The injection oven temperature was 170℃, the nitrogen gas carrier flow rate 5 m*l*/min and detector at 200℃ for final temperature. Tentative identification of the fatty acids of methyl ester was carried out by comparing their absolute and relative retention time tiem to those of known standards and by triangulation, and the results were expressed as relative percentages.

RESULTS AND DISCUSSION

Identification of yeast strains

Most selection procedures for ethanol-tolerant yeasts was subjected to the exposure of cells to progressively higher ethanol concentrations (D' Amade and Stewart, 1987). The first selection of ethanol-tolerant strains was applied to enrichment culture of YPD broth medium with 6, 8, 10% (v/v) of ethanol concentrations. After incubation with

Table 2. *Ethanol production of yeast strains on the anaerobic fermentation*

(%; v/v)

Strains of *S. cerevisiae*	Culture Time (day)			
	3	4	5	6 day
SU-1	6	9	15	17
KY-5	6	9	16	17
SW-1	6	9	16	16
BU-1	5	8	15	16
SE-1	7	10	14	16
YO-1	6	10	16	17
SDJ-1	4	9	14	16
KY-1	6	9	14	16
SJY-1	6	10	12	15
SIS-1	5	11	15	16

ethanol at 30℃ for 72 hours, ethanol-tolerant yeast strains were on the plate of YPD medium. These yeast strains isolated were identified with the morphological observation and biochemical test including carbohydrate fermantation, assimilation of carbon compounds and nitrate, vitamin free growth, and urease activity according to Kreger-van Rij (1984).

Twenty four isolates, which were screened for high ethanol-tolerant yeast strains, were identified to be 18 strains of *Saccharomyces cerevisiae*, and each strain of *S. diarensis*, *S. exiguus*, *S. telluris*, *Saccharomycodes ludwigii*, *Schwanniomyces occidentalis* var. *occidentalis*, and *Zygosaccharomyces florentious* shown in Table 1.

Ethanol production by yeast strains

We examined the ethanol fermentation of several yeast strains on aerobic and anaerobic culture conditions. Among yeast strains, *S. cerevisiae* YO-1 produced 18% (v/v) of ethanol, and KY-5 and SU-1 strains produced 17%(v/v) of ethanol after 4 days fermentation on the aerobic culture condition as shown in Table 1. From the result of this experiment, it was found that several strains of *S. cerevisiae* showed the higher ethanol production than those of other species in same genus such as *S. dairensis*, *S. exiguus*, *S. telluris*, or other genus *Sacchromycodes*, *Schwanniomyces*, and *Zygosaccharomyces*.

Therefore, the strains of *S. cerevisiae* only were applied the ethanol production on the anaerobic condition without shaking culture. Among the several strains of *S. cerevisiae*, YO-1, KY-5, and SU-1 produced 17%(v/v) of ethanol, SW-1, BU-1, SE-1, SDJ-1, KY-1, and SIS-1 produced 16% of ethanol. Thus it was assumed that these 10 strains were appeared to be ethanol tolerant to high concentrations of ethanol production(Table 2).

However, our result was shown that ethanol production by yeast strain YO-1 was almost low

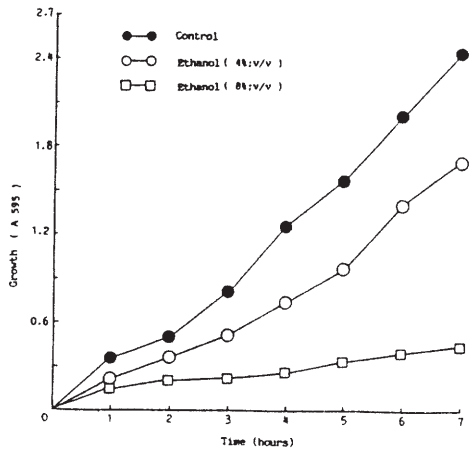

Fig. 1. *Effect of ethanol on the growth of S. cerevisiae YO-1 in YPD broth at 30℃. In YPD broth at 30 ℃. The yeast growth was measured with cell density at A 595.*

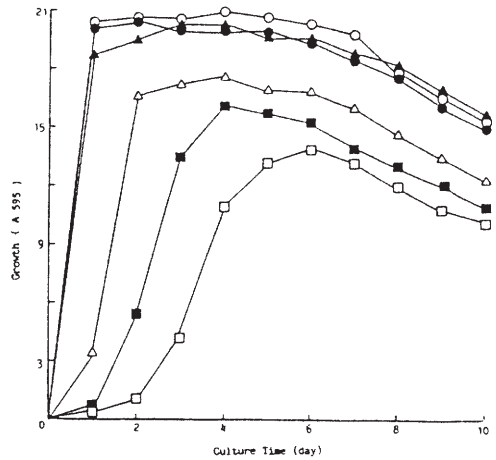

Fig. 2. *Glucose effect on the growth of S. cerevisiae YO-1.*

Fermentation medium was compose of 1% yeast extract, 0.5% peptone, 0.3% KH_2PO_4, 0.3% $(NH_4)_2SO_4$, 0.0025% $CaCl_2$, 0.0025% $MgSO_4 \cdot 7H_2O$ (pH 4.5) with various concentrations of glucose (●—●; 10%, ○—○; 20%, ▲—▲; 30%, △—△; 40%, ■—■; 50%, □—□; 60%).

concentration at anaerobic condition for 4 days incubation, although Yamazaki (1961) and Hayashida *et al.*, (1974), reported that the yeast cells grown on the aerobic condition showed the low fermentation activity than that on anaerobic condition.

Ethanol and glucose effect on the growth of yeast population

Ethanol effect on the growth of *S. cerevisiae*

Table 3. *Changes in the fatty-acyl composition of phospholipids of S. cerevisiae YO-1 during fermentation*

Fatty-acyl residue (% of total phospholipids)	Fatty-acyl composition (% of total) of phospholipids during fermentation period (days)			
	1	2	3	4
$C_{10:0}$	–	–	0.9	1.5
$C_{12:0}$	–	–	0.2	0.8
$C_{14:0}$	1.2	0.9	0.6	0.5
$C_{16:0}$	10.3	14.6	15.4	18.6
$C_{16:1}$	38.4	36.6	34.9	29.4
$C_{18:0}$	8.1	8.1	8.3	9.8
$C_{18:1}$	41.9	39.5	38.1	36.2
unknown	0.1	0.3	1.6	3.2

YO-1 population was examined with YPD broth medium.

Figure 1 showed the result of yeast cell growth on the addition of various concentrations of ethanol. The growth of YO-1 strain was remarkably inhibited by ethanol addition, 4%(v/v) or 8%(v/v) to YPD broth comparing with control group of yeast growth without ethanol addition.

The inhibition of yeast cell growth by substrate was investigated as shown in Fig. 2. The result showed that the growth of yeast cell was increased with 30% of glucose in the fermentation medium, but it was remarkably inhibited with the higher concentrations more than 30% of glucose. It was confirmed that our result was considerably agreed with investigation of Jones *et al.* (1981).

Ethanol tolerance in relation to phospholipids

Hayashida and Hongo (1976) reported that Sake yeast enhanced tolerance to ethanol when grown in the presence of a fraction from the envelope of *Aspergillus oryzae* containing unsaturated fatty-acyl residues. Thomas *et al.* (1978) reported on the manner in which both the sterol and phospholipid fatty-acyl composition of plasma membrane of *S. cerevisiae* influence the yeast viability containing ethanol and ethanol tolerance. Jollow *et al.* (1968) have reported an increase in the proportion of saturated and a decrease in the proportion of unsaturated fatty-acyl residues in total lipids of *S. cerevisiae* during semi-anaerobic fermentation.

Therefore, our experiment is the first to establish the extent to which these changes occur in phospholipids of this yeast. When ethanol was produced during fermentation, the changes were caused to the fatty-acyl composition of phospholipids extracted from organisms harvested. This result showed an increase in the proportion of saturated and a decrease in the proportion of unsaturated fatty-acyl residues in total lipids of *S. cerevisiae* during aerobic and anaerobic fermentation with high alcohol production (Table 3).

Table 4. *Changes in the fatty-acyl composition of phospholipids of S. cerevisiae YO-1 following supplementation of cultures with ethanol.**

Fatty-acyl residue	Fatty-acyl composition of phospholipids (% of total) in yeast cells from cultures supplemented with ethanol at:		
	0.1 M	1.0 M	1.5 M
$C_{14:0}$	1.6	0.6	0.9
$C_{16:0}$	23.2	15.2	14.6
$C_{16:1}$	30.3	35.9	37.8
$C_{18:0}$	8.8	9.1	5.0
$C_{18:1}$	32.6	38.5	40.9
unsaturation	3.5	0.7	0.8

*Cultures were supplemented with ethanol after 8 hrs incubation, and cells were harvested from cultures after a further 10 hrs incubation.

Table 5. *Fatty acid composition during 3 days fermentation by ethanol-tolerant yeast and baker yeast*

(%)

Fatty Acid \ Strain	*S. cerevisiae* YO-1	*S. cerevisiae* KY-5	Baker yeast
10:0	0.7	2.6	–
12:0	0.3	2.2	–
14:0	–	–	–
16:0[a]	12.5	14.2	12.5
16:1[b]	42.4	32.1	6.9
18:0	6.2	11.6	6.3
18:1	36.1	25.0	12.0
18:3	–	–	10.2
20:0	–	–	12.5
>20:0	1.8	12.3	39.6

[a] 16:0 represents $C_{16:0}$, meaning the saturated fatty acid with 16 carbon number; palmitic acid, whereas [b] 16:1 means the unsaturated fatty acid; palmitoleic acid.

It also examined that supplementing ethanol to the cultures (0.1 M, 0.5 M, 1 M) after 8 hr incubation led to increase in the proportion of unsaturated fatty-acyl residues in phospholipids in cellular membrane, especially in C_{16} or C_{18} residues, which was accompanied by a decrease in the proportion of saturated residues as shown in Table 4, which was similar to the result of Jollow *et al.* (1968). In our additional experiment, two strains of ethanol-tolerant yeast and baker yeast were examined to compare the fatty-acyl residues of cellular membrane, showing the amount of unsaturated fatty-acyl composition was higher than that of saturated one (Table 5). This result indicated that ethanol tolerance is involved in a

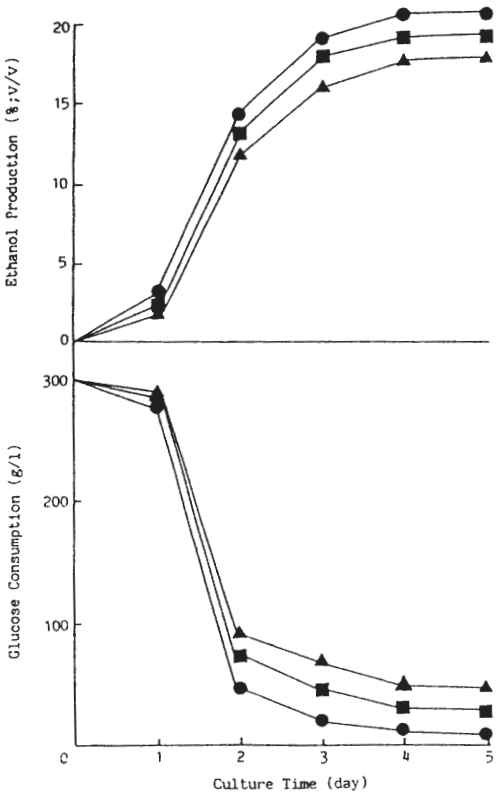

Fig. 3. *Fermentation of S. cerevisiae YO-1 of 300g of glucose per liter in a simple medium (▲) or a medium supplemented with 2% (■) or 4% (●) soy flour on aerobic culture.*

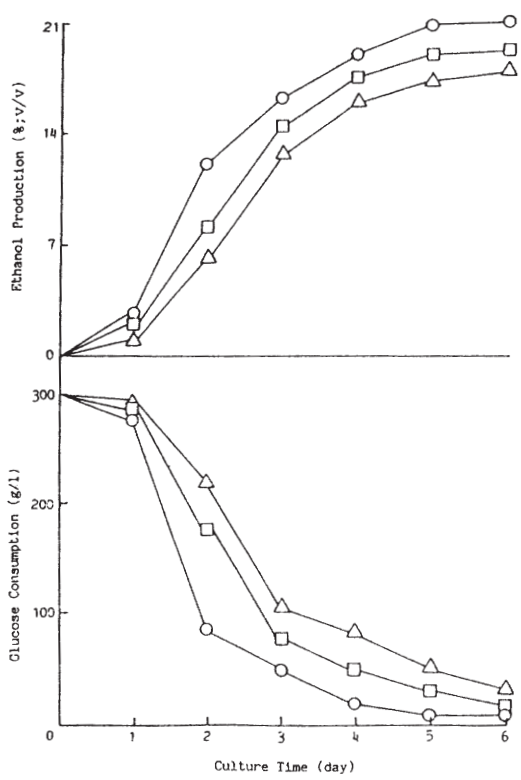

Fig. 4. *Fermentation of S. cerevisiae YO-1 of 300g of glucose per liter in a simple medium (△) or a medium supplemented with 2%(□) or 4%(○) soy flour on anaerobic culture.*

increase of unsaturated fatty-acyl composition. Similar phenomenon was obtained from the result of changes of fatty-acyl residues in phospholipids of cellular membrane during 32 hr growth of yeast (Beavan *et al.*, 1982).

Enhanced ethanol production through soy flour supplementation

The addition of soy flour, an abundant and in expensive source of proteins (38%) and lipids (21 %), has been shown to lead to a significant increase in batch and continuous alcoholic fermentation productivity by both *S. cerevisiae* (Damiano and Wang, 1985), and *Zymomonas mobilis* (Ju *et al.*, 19 83). Thus soy flour supplementation was observed in terms of ethanol production at aerobic or anaerobic fermentation. The addition of 2 or 4% soy flour led to a significant increase in the amounts of glucose fermented and to remarkably high final concentrations of ethanol on aerobic fermentation to reach 19% and 20%, respectively(Fig. 3). And almost similar result was obtained the enhancing ethanol production on anaerobic fermentation to reach 19.8% and 21% with the addition of 2 or 4% soy flour, respectively(Fig. 4).

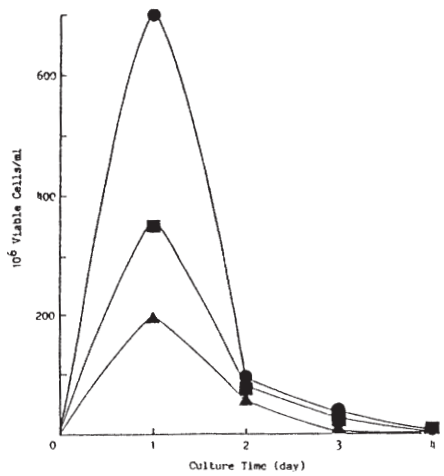

Fig. 5. *Concentration of the viable cells of S. cerevisiae YO-1 during the fermentation of 300g of glucose per liter in a simple fermentation medium (▲) or the medium supplemented with 2% (■) or 4%(●) soy flour.*

Such enhancing effect of ethanol production were obtained after 5 days fermentation. In this results, ethanol production on anaerobic fermentation was appeared to be higher than that on aerobic fermentation.

The presence of soy flour led to an increase in the rate and extent of yeast growth as shown in Fig. 5. After 24 hr of fermentation for both supplemented and unsupplemented media, yeast cell growth was stopped, but the maximum number of viable yeast cell increased with soy flour concentration to be death.

This results are similar to the data obtained from Viegas *et al.* (1985a), representing soy flour supplementation led to an increase in viable cell concentration of yeast cell during fermentation, and increasing the amount of soy flour led to an increase in glucose fermentation to reach maximum percentage of ethanol production. Several authors reported an improvement in alcoholic fermentation and an increase of the final ethanol concentration by broth supplementation. Such additives were basically lipids such as unsaturated fatty acids and sterols, proteins and vitamins(Viegas *et al.*, 1985b; Casey *et al.*, 1983; Day *et al.*, 1975; Hayashida *et al.*, 1974).

적 요

Ethanol-tolerant yeast의 균주개발 및 그의 활용 방안의 일환으로 우수 효모 균주를 분리한 결과, 그 중 우수한 ethanol-tolerant yeast로 동정된 균주는 *Saccharomyces cerevisiae* YO-1, KY-5, SU-1등 이었다. Ethanol의 농도가 높을수록 YO-1 효모 세포의 생장에 저해를 받고 기질로 glucose 농도가 30% 이상 증가하면 현저히 효모의 생장이 억제됨을 알 수 있었다. 4일간 발효를 시킨 결과 4일간 배양후의 ethanol함량은 YO-1이 18%, KY-5와 SU-1은 17%로 고알콜 생성능을 가진 균주로 밝혀졌다. 에탄올 발효에 의한 고에탄올이 생성됨에 따라 인지질 중 불포화 지방산은 감소하나 포화 지방산은 증가하였다. 반면에 배양액에 에탄올을 첨가하면 불포화 지방산은 증가하고 포화 지방산은 감소현상을 보여 주어, 불포화 지방산의 증가는 에탄올 내성과 관련됨을 알 수 있었다. 효모에 의한 ethanol 생성을 높이기 위해 발효액에 protective agent로 soy flour를 사용하였으며, 호기성 발효의 경우 soy flour를 첨가하지 않은 경우(ethanol 18%)보다 4%의 soy flour를 첨가시 2% 더 높은 ethanol 생성을 보여주었으며, 혐기성 발효의 경우에는 호기성 발효시보다 1% 더 높은 ethanol 생성을 보여 주었다.

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