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Enhancement of Ethanol Tolerance of Lactose Assimilating Yeast Strain by Protoplast Fusion

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In order to construct a yeast strain having high ethanol tolerance together with good lactose fermentation ability, the protoplast fusion using *Saccharomyces cerevisiae* STV 89 and *Kluyveromyces fragilis* CBS 397 was carried out. Auxotrophic mutants of *K. fragilis* were obtained as a selection marker by treatment of ethylmethane sulfonate. The best mutant for protoplast fusion was selected based on the capabilities of β-galactosidase production and lactose fermentation. The protoplast fusion using polyethylene glycol and calcium chloride solution led to the fusion frequence of 3×10^{-6} and a number of fusants were obtained. Among these fusants, a fusant F-3-19 showed the best results in terms of ethanol tolerance, β-galactosidase activity and lactose fermentation. The performance of lactose fermentation and ethanol tolerance by this fusant were better than those of *K. fragilis*. Study on the ethanol tolerance having relation to fatty acid composition and intracellular ethanol concentration revealed that the fusant F-3-19 had a higher unsaturated fatty acids content and accumulated less amount of intracellular ethanol compared with a parent of *K. fragilis*.

Although many attempts have been made to utilized lactose, the major carbohydrate of whey, for alcohol production since 1940 (16) with little success due to the facts that *S. cerevisiae* is unable to ferment lactose and relatively few yeasts are able to ferment lactose. *K. fragilis* was known as a most efficient lactose fermenting yeast strain (14). However, it has been shown that only a fraction of the available lactose was converted to ethanol, possibly due to an ethanol inhibition (15). In advanced studies on ethanol production from lactose by *K. fragilis*, it was found that 11% (v/v) ethanol was produced from 20% (w/v) lactose in around 90 h fermentation (2, 8, 13). This prolonged fermentation time was mainely attributed to end-product inhibition, in other words, the ehtanol adversely affects cellular physiology, resulting in reduced cell growth and ethanol production rate.

The ethanol tolerance of yeasts depends on the fermentation conditions and cellular characterization of yeasts (3). Among yeasts, *Saccharomyces* spp. appear to be the most ethanol-resistant organisms, although their tolerance to ethanol is strain-dependent (25).

The toxic effects of ethanol on yeast are manifold and there are many possible target sites for the action of ethanol. Especially, alcohols are soluble in lipid and, for the reason, much attention has been paid to the plasma membrane as a target site for the toxic action of ethanol (4). A major effect of ethanol on the plasma membrane is a decrease of membrane fluidity (5), which inhibits the transport of nutrients across a plasma membrane (9, 10, 16). Thus, the common trend associated with ethanol tolerance of yeasts is an increase in the proportion of unsaturated fatty acids and sterol found in membrane (6, 20, 27). This results in increased membrane fluidity, which enhances the ethanol leakage from the cell (17) and the transport of nutrients into the cell (9, 10, 26). Intracellular ethanol concentration was consistently lower when linoleic acid content was increased, suggesting that the ethanol efflux rate might be higher in linoleyl-residue enriched cells (17, 20, 26). Recently it has been suggested that the protein components of the cell membrane also play an important role in the ethanol tolerance of yeast strains (7).

Protoplast fusion has been widely used for genetic improvement of industrial yeasts (2, 21, 22, 24, 28, 29).

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Because this technique has a total disregard for ploidy and mating type and consequently has great advantages for improving the ethanol tolerance of yeast strains (2, 21). Since protoplast fusion technique was used to develop a genetically constructed yeast strain which is capable of high ethanol tolerance and good lactose fermentation ability for producing the high ethanol concentation. We have also studied on the factors affecting the ethanol tolerance by measuring fatty acid composition and intracellurar ethanol concentration of fusants.

MATERIALS AND METHODS

Microorganisms and Media

The yeast strains used in this study were *K. fragilis* CBS 397 obtaining from Centraalbureau voor Schimmel Cultures (Netherland) and *S. cerevisiae* STV 89 reported to produce high ethanol content (19). Both yeast strains were grown and maintained on YPD medium, which consists of 1% yeast extract, 2% Bacto-peptone, 2% glucose and 2% Bacto-Agar. The YNB medium containing 0.67% yeast nitrogen base (w/o amino acids) and 2% glucose was used as the minimal medium. The completely synthetic minimal medium (SMM) of Bacto yeast nitrogen base w/o amino acids described in a Difco manual (1) was used to confirm an auxotrophic mutants and to select a fusant.

Spores Formation and Isolation

K. fragilis sporulated after 5 days of incubation at 30℃ on sporulation medium containing 1% potassium acetate, 0.5% glucose, 0.1% yeast extract and 2% argar. The isolation of spores was carried out by the method of Farahnak *et al.* (2).

Mutagenesis and Auxotrophic Mutant Selection

Ethylmethane sulfonate (EMS) was used as a mutagenizing agent. Spores were washed three times with sterile distilled water, suspended in a solution containing 0.1 M potassium phosphate buffer (pH 8.0) and EMS, and incubated at 30℃ for 1 h with occasional shaking. After this period, spores were washed with sterile distilled water and treated with 5% sodium thiosulfate for 15 min to stop a mutagenesis. To increase the population of mutants, the enrichment culture was used in the nystatin procedure as reported by Snow (23). The cells harvested from enrichment culture were washed three times with sterile distilled water and 0.1 m*l* was spreaded onto YPD plastes and incubated for 3 days at 30℃. After the colonies appeared, they were replica plated onto YNB medium to screen for auxthotrophic mutants. Colonies which grew on YPD medium but were unable to grow on YNB medium were further transferred to SM medium containing different pools of amino acids in order to elucidate their auxotrophic requirments.

Protoplast Preparation

S. cerevisiae and *K. fragilis* (Cys^-, Met^-) were incubated aerobically at 30℃ to early stationary phase in 250 m*l* flasks containing 20 m*l* of YPD medium. The yeasts (5×10^8 cells) of each culture were collected and centrifuged at 500×g for 5 min. Cells were then washed three times with sterile distilled water and resuspended in the pretreatment buffer containing 0.1 M sodium phosphate buffer (pH 7.0), 0.5 M dithiothreitol and 0.06 M EDTA. After each cells incubated at 30℃ for 30 min, cells were washed two times with sterile distilled water and then resuspended in 1 m*l* of protoplasting solution containing 0.1 M sodium phosphate buffer (pH 7.0), 0.6 M manitol, 0.01 M EDTA, 3 mg of β-glucuronidase (Type H-1, Sigma) and Novozyme 234 (Novo), respectively. The suspension was incubated at 30℃ for 60 min with shaking (75 rpm). Protoplasts were collected by centrifugation at 1,000×g for 10 min and washed three times with osmotic stabilizer (0.1 M phosphate buffer, pH 7.0 and 0.6 M manitol).

Protoplast Fusion and Fusant Selection

The protoplasts from both strains of yeast were mixed and suspended carefully in polyethylene glycol solution (40% PEG, 10 mM $CaCl_2$ and 0.8 M sucrose, pH 8.0) and the suspension was incubated for 10 min at 30℃. The fused cells were washed with osmotic stabilizer, and resuspended with osmotic stabilizer. The suspension was mixed with regeneration medium (SM medium contained 1% lactose, 0.8 M sorbitol, 0.04% IPTG, 0.08% X-gal and 0.8% agar) and poured onto plate containing a thin bottom layer of medium (SM medium contained 1% lactose and 1.5% agar). Plates were sealed and incubated for a period of 3 to 7 days at 30℃. The fusants were selected from blue colonies appeared on plates and the fusion frequency was determined by counting the blue colonies on the plates over the number of cells inoculated.

Alcohol Fermentation

The fermentations were carried out in a 20 liter jar fermenter (Bioengineering Co., Swiss) by loading 14 liter fermentation medium (20% lactose, 0.5% yeast extract, 0.5% peptone, 0.5% KH_2PO_4, 0.2% $(NH_4)_2SO_4$ and 0.04 % $MgSO_4\cdot7H_2O$) at 30℃ with 200 rpm agitation and 0.2 vvm aeration.

Analytical Methods

Cell mass, ethanol concentration, cell viability, intracellular ethanol concentration, and fatty acid content were determined according to the methods described previously (20). Lactose concentration was measured with respect to reducing sugar using DNS method (12). β-galactosidase activity was measured by the method of Mahoney *et al.* (11) using ρ-nitrophenyl-α-D-galactopyranoside

as substrate. One unit of enzyme was defined as the amount of enzyme required to produce one mmole of p-nitrophenol per one hour at 30℃ and pH 6.6.

RESULTS AND DISCUSSION

Sensitivity to EMS

The effect of EMS on the viability of vegetative cell and spores of *K. fragilis* was studied. When the vegetative cell and spore were treated with various concentrations of EMS for 60 min at 30℃, vegetative cells were far more susceptible than were ascospores. At an EMS concentration of 7%, all the vegetative cells well killed, while about 22% of spores were viable. The same result was reported by Farahanck *et al.* (2). Therefore, the concentration of 7% (w/v) EMS was used to expose the spores to the mutagen for 60 min and to ensure that a large fraction of spores could germinate and give rise to a vegetative population.

Isolation and Selection of Auxotrophs

Six class (Met^-, Met^-Cys^-, Met^-Ura^-, $Met^-Cys^-Ura^-$, Arg^-, $Arg^-Asp^-Glu^-$) of auxotrophs were isolated. The mutants isolated were mostly found to be methione and cysteine requiring strains. Among the sixty auxotrophic mutants, the best mutant for protoplast fusion were selected based on the high β-galactosidase activity, low ethanol-assimilating and high lactose fermenting abilites. A methionine and cysteine auxotroph of M-29 selected maintained the almost same abilities of ethanol production and β-galactosidase activity as *K. fragilis*, although the capacity of ethanol assimilation of M-29 was lower than that of parent. *K. fragilis* utilized the ethanol for its growth at late stage of fermentation (18). Thus, the elimination of this character is favorable for alcohol fermentation. The mutant M-29 (Met^-, Cys^-) was used in the subsequent protoplast fusion to provide genetic marker for the selection of fusants.

Selection of Fusants

The blue colonies (Fig. 1) which grew on plate of SM medium containing X-gal, IPTG and lactose were isolated as the fused hydrids between *S. cerevisiae* and *K. fragilis* (Met^-Cys^-). Complementation must have occurred in these fusants. Since *K. fragilis* was auxotroph it is incapable of growing on SM medium. Likewise, *S. cerevisiae* is incapable of growing on lactose carbon source. The fusion frequency was found to be about 3×10^{-6}. To select the best fusant among 189 fusants isolated, experiments of lactose fermentation, β-galactosidase production and ethanol assimilation were carried out for fusants in a shaking incubator at 30℃ for 48 h. The results were shown that the best fusant selected was F-3-19 as the abilities of lactose fermentation and β-galactosidase production were improved compared with

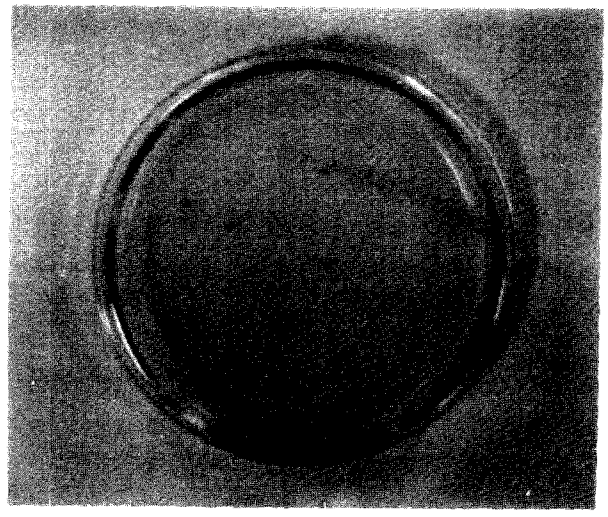

Fig. 1. Photograph of fusants on the selection medium (blue dot: fusants).

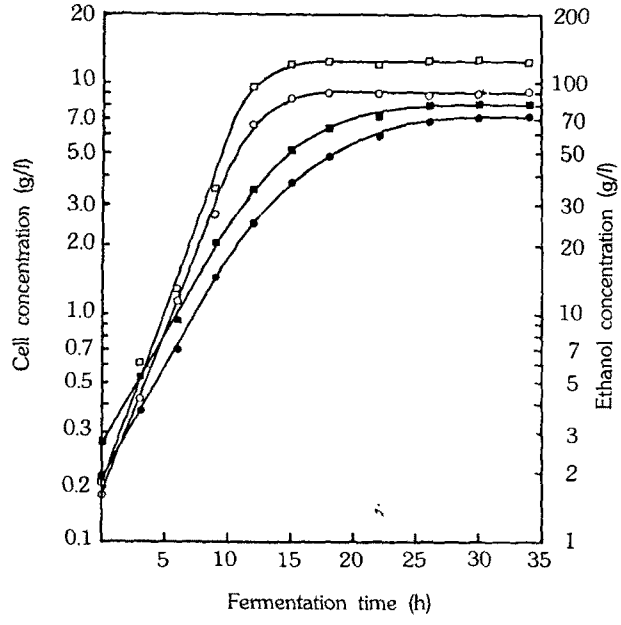

Fig. 2. Cell growth (○, □) and ethanol production (●, ■) during the batch fermentation of lactose by *K. fragilis* (○, ●) and fusant (□, ■).

parent strain of *K. fragilis*, whereas the ability of ethanol assimilation in the fusant was decreased up to 50% of that in *K. fragilis*.

Alcohol Fermentation of Lactose

To compare the alcohol fermentation preformence of parent (*K. fragilis* CBS 397) and fusant (F-3-19), the alcohol fermentations were carried out using 20% (w/v) lactose. The profile of cell growth and ethanol production were presented in Fig. 2 and the various kinetic parame-

Table 1. Various kinetic parameters of fusant (F-3-19) and *K. fragilis*

Stranis	μ_{max}	X_m	P	P_m	$Y_{p/s}$
K. fragilis	0.326	9.1	2.12	72.1	0.434
F-3-19	0.346	12.6	2.42	82.4	0.459

μ_{max}: Maximum specific growth rate (h^{-1})
X_m : Maximum cell concentration (g/l)
P : Overall ethanol productivity (g/l·h)
P_m : Maximum ethanol concentration (g/l)
$Y_{p/s}$: Ethanol yield (g-ethanol/g-lactose consumed)

ters were shown in Table 1. The maximum specific growth and ethanol production rates of the fusant were higher than those of parent strain. The fusant produced 14.3% more ethanol and 38.5% greater cell concentration as compared with the parent strain. The ethanol yield of the fusant was 0.459 g-ethanol/g-lactose consumed, which was 89.8% of theoretical value. This value was slightly higher than that of the parent strain, because the fusant had lower ethanol assimilation ability than the parent strain. The cell viability of the fusant improved as compared with the parent strain (Fig. 3). Thus, it is shown that the fusant is more tolerant to ethanol than the parent strain. The *S. cerevisae* strain used was a high ethanol tolerant strain which produced about 18% (w/v) ethanol when fermented on 30% (w/v) glucose medium (19). Therefore, the fusant was at least as tolerant to ethanol as the parent strain of *S. cerevisiae*. The same results were also reproted by Farahnak *et al.* (2) and Seki *et al.* (21). Thus, there is a good possibility of getting a hybridized fusant with higher ethanol tolerance when other strain of *S. cerevisiae* with higher ethanol tolerance becomes available. The profile of extracellular β-galactosidase activity during the alcohol fermentation was presented in Fig. 4. The fusant showed higher enzyme production as compared with *K. fragilis*. It was suggested that the fusant secreted more enzyme into medium by the modification of cell membrane.

Ethanol Tolerance

To investigate the factors affecting ethanol tolerance, qualitative fatty acid analysis was performed from the fusant (F-3-19) and *K. fragilis* cultures harvested during the lactose fermentation. The fatty acid compositions of the fusant and *K. fragilis* were shown in Table 2. The fusant contains 11.1% more unsaturated fatty acid than *K. fragilis*. Particularly, the content of linoleic aicd ($C_{18:2}$) was 2.6 times higher as compared with *K. fragilis*. The intracellular ethanol concentrations of the fusant and *K. fragilis* during the fermentation were shown in Fig. 5. Similar profile was obtained, for which the intracellulr ethanol was rapidly accumulated due to the higher

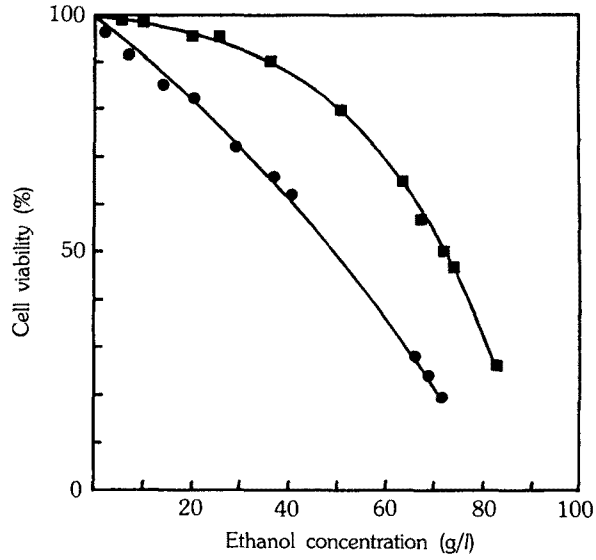

Fig. 3. The cell viability of *K. fragilis* (●) and fusant (■) as a function of ethanol concentration in culture broth.

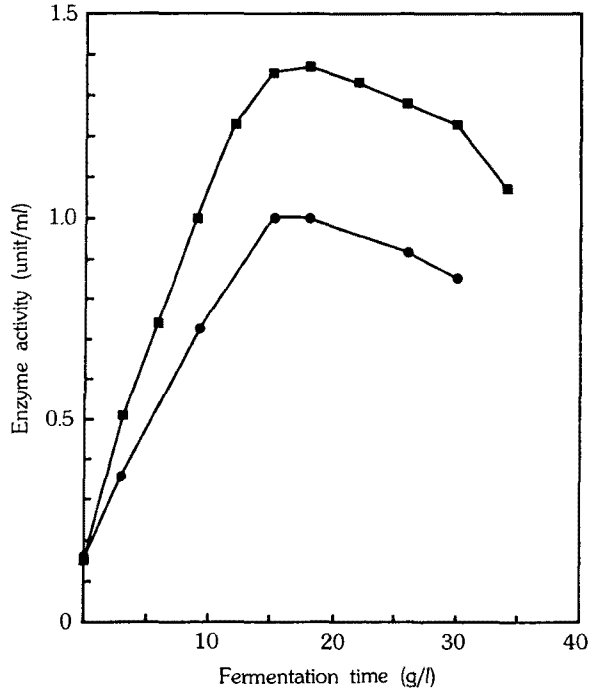

Fig. 4. The extracellular β-galactosidase activity of *K. fragilis* (●) and fusant (■) during the lactose fermentation.

ethanol production rate than ethanol diffusion into medium during an early fermentation stage and, after reaching the maximum level of intracellular ethanol concentration, the accumulation of ethanol in cells was rapidly decreased to the extracellular level in the late fermentation stage because the ethanol diffusion rate was higher

Table 2. The relative fatty acid composition of *K. fragilis* and fusant (F-3-19) at 30°C under aerobic condition

Starins	Culture time (h)	Fatty acid composition (%)									Sum (%)	
		$C_{12:0}$	$C_{14:0}$	$C_{14:1}$	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	Saturated	Unsaturated
K. fragilis	9	2.3	2.1	4.2	16.2	15.9	15.2	20.5	20.7	2.9	35.8	64.2
	18	0.1	2.3	2.2	12.4	13.8	15.3	45.1	8.5	0.3	30.1	69.9
	30	1.6	0.6	0.8	15.7	22.2	10.2	42.3	6.6	0.0	28.1	71.9
	Average	1.3	1.7	2.4	14.8	17.3	13.6	35.9	11.9	1.1	31.3	68.7
F-3-19	9	0.5	0.6	–	16.2	18.4	5.1	33.6	25.6	–	22.4	77.6
	18	–	0.8	–	11.9	14.1	6.0	34.5	32.7	–	18.7	81.3
	30	–	0.8	0.8	7.8	10.1	10.7	33.5	36.3	–	19.3	80.7
	Average	0.2	0.7	0.3	12.0	14.2	7.3	33.9	31.5	–	20.1	79.9

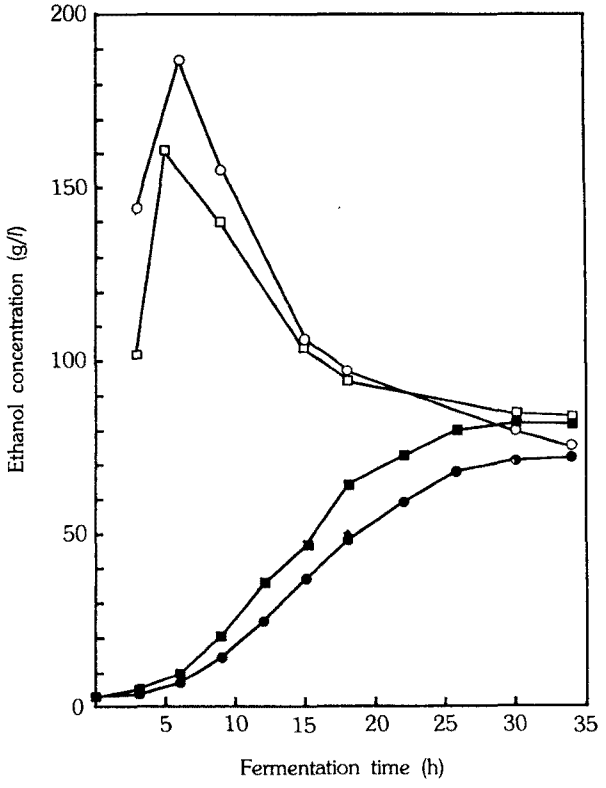

Fig. 5. The accumulation of extracellular (●, ■) and intracellular (○, □) ethanol during the fermentation time of *K. fragilis* (●, ○) and fusant (■, □).

than the ethanol production rate. However, the maximum intracellular ethanol concentration of the fusant was lower than that of *K. fragilis*.

From these results, it was suggested that ethanol tolerance of the fusant was increased with unsaturated fatty acids content, especially linoleic acid, showing moderate ethanol toxicity due to enhanced ethanol diffusion rate (17, 20, 26) and the cell growth and ethanol production rates were also increased by the higher hydrolysis of lactose due to enhanced secretion of β-galactosidase into medium. Thus, it was concluded that unsaturated fatty acids act a decisive role in the increase of ethanol tolerance of yeast strains.

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