Conditions for Protoplast Formation and Fusion of the Killer Yeast

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Killer 효모의 원형질체 형성 및 융합조건

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ABSTRACT: Auxotrophic mutant were isolated from wild types by the treatment with NTG as a mutagen, and the conditions of protoplast formation for them were established. The protoplasts of killer yeast *Saccharomyces cerevisiae* K 52 were formed to the level of above 70% when cells grown for 20 hr in PM medium were treated with 200 unit/ml Lyticase 50,000 at 30°C for 60 min after pretreatment of 50 mM 2-mercaptoethanol in 10 mM potassium phosphate buffer (pH 7.5) containing EDTA and 0.6 M sorbitol for 15 min. Also, the protoplast of the recipient *S. cerevisiae* S 29 were formed to the level of above 85% as it was cultured to the log phase of 24 hr in PM medium under the same conditions. The fusion frequency between the protoplast of killer yeast *S. cerevisiae* K 52 and the protoplast of recipient *S. cerevisiae* S 29 was reached to 8.2×10^{-6} when the hypertonic regeneration medium embeded with the fused protoplasts after mixing the parental protoplasts to 10^8 cells/ml in SP buffer containing 20 mM $CaCl_2$ and 30% PEG 6,000 for 15 min at 30°C were incubated.

KEY WORDS □ Killer yeast, Protoplast fusion, Regeneration.

Since Eddy and Williamson (1957) first described the formation of protoplasts from *Saccharomyces carlsbergensis* using lytic enzymes present in gut juice of the snail *Helix pomatia* and the fusogenic property of polyethylene glycol (PEG) to plant protoplasts was discovered (Kao and Michayluk, 1974), many other workers have tried to breed the new strains by the protoplast fusion (Kue and Yamamoto, 1979; Peberdy, 1979; Gunge and Sakaguchi, 1981; Ouchi *et al.*, 1983; Bortol *et al.*, 1986).

Killer factor from *Saccharomyces cerevisiae* is an extracellular protein with a toxin effect on sensitive strains (Bussey, 1981). Its synthesis is directed by a double-stranded RNA virus-like particle (Spencer and Spencer, 1983). The killer particle confers immunity from attack by other killer strains and prevents contamination by undesirable sensitive yeasts, resulting a good performance when used industrially. Killer immune character has been introduced into strains used in the production of the following products: sake (Ouchi *et*

al., 1979), white wines (Hara *et al.*, 1981), alcohol (Yamamoto *et al.*, 1984), beers (Spencer and Spencer, 1983) and bread (Bortol *et al.*, 1986).

In previous paper (Chung *et al.*, 1989), we isolated the wild killer yeasts from nature and examined the culture conditions for the production of killer toxin. The present investigations show that high yields of protoplasts can be obtained by using Lyticase 50,000 and several parameters affecing protoplast formation of the yeasts. Also we describe here the conditions of protoplast fusion and regeneration.

MATERIALS AND METHODS

Strains

The strains used to obtain protoplasts were auxotrophic mutants of *Saccharomyces cerevisiae* B 15-1 as a killer yeast and *S. cerevisiae* No. 105 as a recipient treated with N-methyl-N'-nitro-N-nitrosoguanidine (NTG), and are listed in Table 1. The sensitive strain was *S. cerevisiae* Kyokai 7 (Chung *et al.*, 1989). The procedure to induce auxotrophs were carried in accordance with the nystatin concentration method (Snow, 1966). The auxotrophic gene markers were stable over several vegetative generations, and strains were maintained on YPD medium.

Media

The compositions of YPD (CM), TTC, and YPD-MB medium were in previous paper (Chung *et al.*, 1989). The PM 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 0.2% yeast extract, was used in the process of culturing the parental strains for the protoplast formation, and the synthetic medium (MM) containing 2% dextrose, 0.2% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% KCl, 0.003% amino acids and 2% agar if needed, was used as the minimal medium. For the regeneration of protoplasts, the CM and MM containing 0.6 M sorbitol were used.

Assay of killer activity

The killer activity was assayed by well test described in previous paper (Chung *et al.*, 1989).

Protoplast formation

Protoplasts were prepared in proportion to the methods described by Yamamoto *et al.*, (1984) and Gunge *et al.* (1981). Yeast cells were separately grown in PM medium at 30°C to the log phase. The harvested cells were washed twice with sterile saline, suspended in SP buffer (0.6 M sorbitol and 50 mM potassium phosphate buffer, pH 7.5) containing 20 mM Na_2EDTA and 50 mM 2-mercaptoethanol, and incubated at 30°C for 15 min. After the cells were washed with SP buffer, they were resuspended to give a density of 5×10^8 cells per ml in SP buffer containing 0.1% 2-mercaptoethanol. To the cell suspension was added Lyticase 50,000 (200 unit/ml), and the mixture was incubated with occasional shaking at 30°C for 60 min. Protoplasts were collected by centrifuge at 3,000 rpm for 10 min, washed repeatedly with SP buffer, and resuspended in the same buffer. The Lyticase 50,000, a lytic enzyme from *Arthrobacter leuteus* (*Oerskovia xanthineolytica*), was purchased from Sigma Co., USA.

Protoplast fusion and regeneration

The parental protoplasts ($1\text{-}2 \times 10^8$ cells/ml of each auxotroph were mixed in an appropriate

Table 1. *The strains of Saccharomyces cerevisiae used.*

Strain	Killer phenotype[a]	Phenotype[b]	Remark
B15-1	K^+R^+	wild type, [KIL-k]	Isolated killer yeast
K52	K^+R^-	Met^-, [KIL-k]	NTG mutant of B 15-1
Kyokai 7	K^-R^-	wild type, [KIL-o]	Sensitive
No. 105[c]	K^-R^-	wild type, [KIL-o]	Recipient
S29	K^-R^-	Lys^-, [KIL-o]	NTG mutant of No. 105

[a] K^+ or K^- means ability or inability to secrete the active killer toxin. R^+ or R^- refers to resistance or sensitivity to the killer toxin.

[b] Killer plasmids are denoted [KIL-k]. [KIL-o] is the wild type sensitive carrying no plasmids.

[c] *Saccharomyces cerevisiae* Technical Research Institute of National Tax Administration No. 105.

combination and centrifuged. The pellet was suspended in PCSP buffer (30% polyethylene glycol 6,000, 20 mM $CaCl_2$, 0.6 M sorbitol, and 50 mM potassium phosphate buffer, pH 7.5). The suspension was incubated at 30°C for 15 min with occasional shaking. After dilution of PEG with SP buffer containing 20 mM $CaCl_2$, the pellet were centrifuged and washed twice with the same buffer. Then appropriately diluted suspension were mixed with 10 m*l* of MM or CM containing 0.6 M sorbitol and 0.85% agar which was melted and maintained at 45°C, and immediately poured onto agar plates of the same nutrient medium containing 0.6 M sorbitol. After 6 to 7 days of incubation at 30°C, the colonies which were produced were picked.

Protoplast yield and fusion frequency

The progress of protoplast formation was investigated by measuring the degree of lysis of the protoplast formed. The degree of lysis was calculated from the following equation (Yamamura *et al.*, 1975) by measuring the optical density of an appropriately diluted reaction mixture at 570 nm.

Degree of lysis (%) = $(D_o-D_t)/D_o \times 100$

where, D_o = optical density of the diluted reaction mixture at time zero.

D_t = optical density of the diluted reaction mixture at time t.

Fusion frequency was calculated by deviding the number of colonies per m*l* appearing after 7 days at 30°C on hypertonic MM by the number of colonies per m*l* on hypertonic CM.

RESULTS AND DISCUSSION

Conditions for protoplast formation

In order to investigate the optimum conditions for the preparation of yeast protoplasts in high yields, we examined some factors predicted to affect the lysis of yeast strains used. Although workers investigating the yeast protoplast have been greatly aided by the availability of suitable lytic enzymes from commercial sources including Lyticase, Zymolase, Glusulase and Novozyme 234, susceptibilities of the yeasts to the lytic enzyme differ significantly depending upon the strains and culture conditions. Generally, log phase yeasts are susceptible to protoplast formation, while stationary phase yeasts are relatively resistant. The

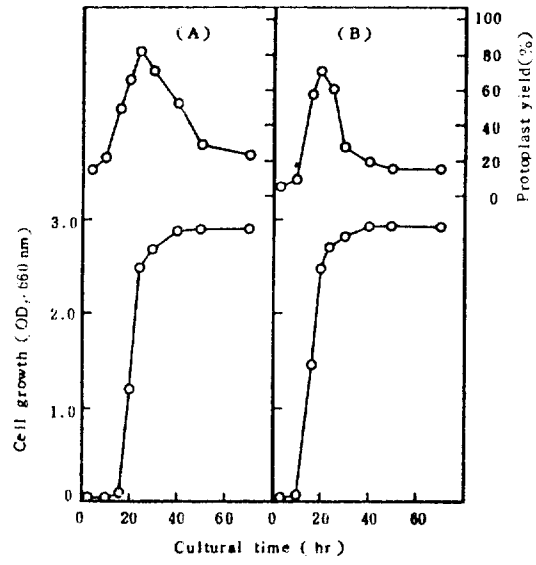

Fig. 1. *Effect of growth stage of Saccharomyces cerevisiae S29(A) and K52(B) on the protoplast formation.*

The strain S29 (NTG mutant of No. 105) and K52 (NTG mutant of B15-1) were cultured in PM medium at 30°C for the indicated times with shaking. The cells for the protoplast formation were incubated in SP buffer (0.6M sorbitol, 10mM potassium phosphate buffer (pH 6.5)) containing 200 units/m*l* of Lyticase 50,000 and 0.1% 2-mercaptoethanol at 30°C for 60 min. Detailed descriptions for the protoplast yield and cell growth are given in the 'Materials and Methods'.

protoplast formation is decreased rapidly in the process of transition from log phase to stationary phase. Such differences may be due to variations in the structure of the cell wall. Therefore the degree of lysis of yeast cell wall is influenced by those factors described above (Kue and Yamamoto, 1979; Peberdy, 1979; Koo *et al.*, 1985). As shown in Fig. 1, the cells were effectively converted into the protoplasts in the late log phase (20 and 24 hr, respectively). As appeared in Fig. 2, the optimum temperature for lysis of the cells was 30-35°C. The optimum pH was determined using 10 mM potassium phosphate buffer (pH 5.5-8.0) and 10 mM Tris-maleate buffer (pH 8.5). As shown in Fig. 3, the protoplast formation of the yeast cells was maximum at pH 7.5. These results are in accordance with the results reported by Scott and Schekman (1980). Burger *et al.* (Kue and

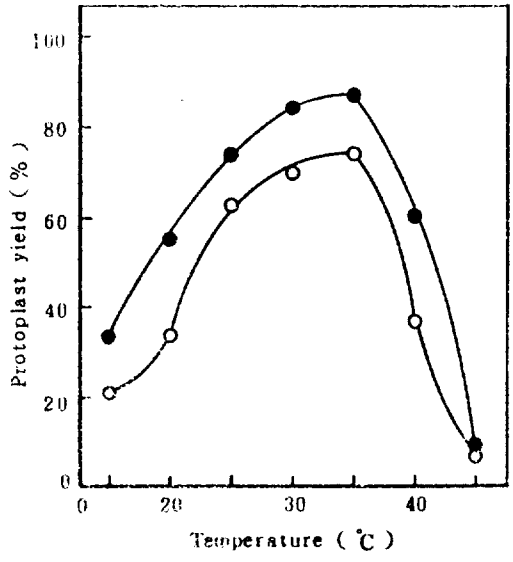

Fig. 2. *Effect of temperature on the protoplast formation of Saccharomyces cerevisiae S29 and K52.*
The cells of strain S29 (●-●) and K52 (○-○) were incubated with 200 unit/m*l* of Lyticase in SP buffer(pH 7.5) containing 0.1% 2-mercaptoethanol at the indicated temperature for 60 min.

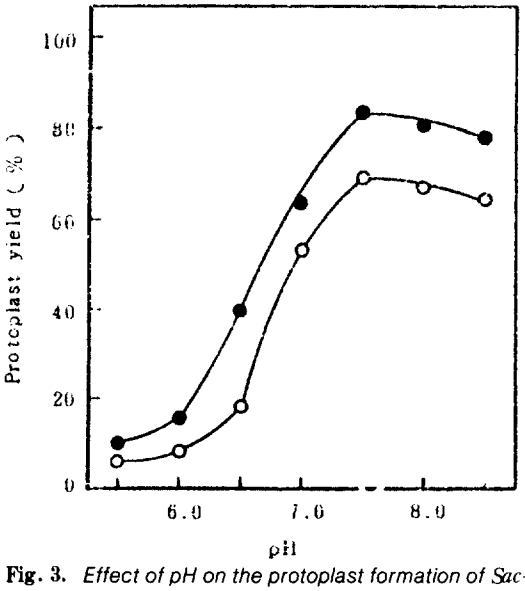

Fig. 3. *Effect of pH on the protoplast formation of Saccharomyces cerevisiae S29 (●-●) and K52 (○-○).*
The cells were incubated with 200 unit/m*l* of Lyticase in 0.6M sorbitol containing 0.1% 2-mercaptoethanol at 30 ℃ at the various pH (adjusted the pH from pH 5.5 to pH 8.0 with 10mM potassium phosphate buffer, pH8.5 with 10mM Tris-maleate buffer) for 60 min.

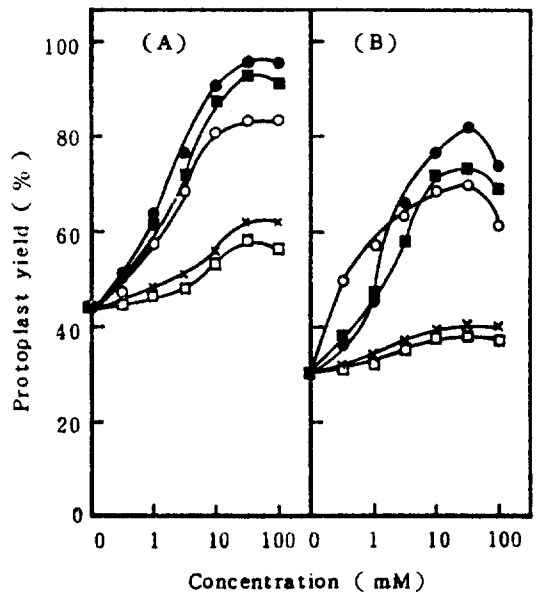

Fig. 4. *Effect of thiol compounds on the protoplast formation of Saccharomyces cerevisiae S29 (A) and K52 (B).*
The cells were pretreated with the various thiol compounds such as dithioerythritol (●-●), dithiothreitol (■-■), 2-mercaptoethanol (○-○), sodium sulfate (×-×), and thioglycollate (□-□) of the indicated concentrations in SP buffer (pH 7.5) containing 20 mM Na_2 EDTA at 30 ℃ for 15 min. Detailed descriptions for the protoplast formation are given in Fig. 1 and the 'Materials and Methods'.

Yamamoto, 1979) first reported that cysteine accelerated the preparation of protoplasts from yeasts by snail enzyme. Since then pretreatment or concurrent treatment of cells with a thiol compound such as 2-mercaptoethanol, 2-mercaptoethylamine, thioglycollate, dithiothreitol or dithioerythritol has been used to render the cells susceptible to the protoplast-forming enzyme. The effect of these compounds in enhancing protoplast release has been related to the reduction of disulfide bonds in wall proteins, thus opening up the molecules and allowing penetration of the lytic enzymes (Kue and Yamamoto, 1979). Fig. 4 shows the effect of various thiol compounds and its concentration on the cell lysis. For the cells of both strains, the effect of 2-mercaptoethanol as well as dithioerythritol for the protoplast formation was excellent, that is, the higher concentration of those

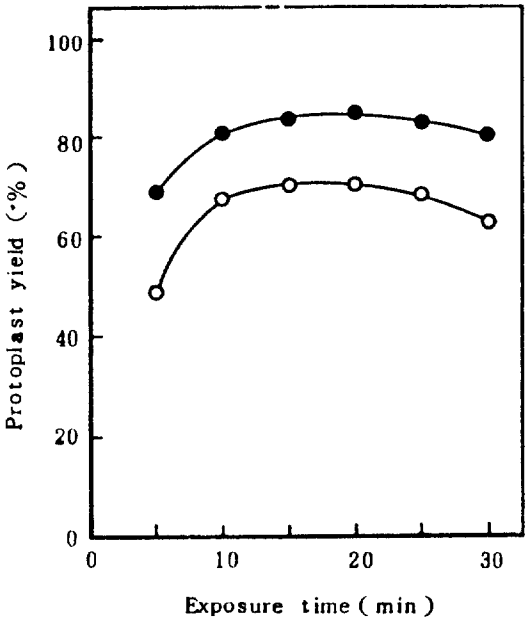

Fig. 5. *Effect of exposure time to 2-mercaptoethanol on the protoplast formation of Saccharomyces cerevisiae S29 (●—●) and K52 (○—○).*
The cells were pretreated with 50mM 2-mercaptoethanol in SP buffer (pH 7.5) containing 20 mM Na_2EDTA at 30°C for the indicated exposure times.

compounds upto 50 mM was, the higher degree of lysis was. And the exposure time against 2-mercaptoethanol on the cell lysis made little difference (Fig. 5). As the results, we used 50 mM 2-mercaptoethanol for 15 min in the reaction mixture. The effect of lytic enzyme concentration and incubation time on the protoplast formation was also investigated. In order to obtain maximum lysis, as shown in Fig. 6 and 7, the enzyme concentration should be more than about 200 unit/ml, whereas the incubation time made little difference for 60-120 min. As the incubation time was long, the protoplast yield was high, but the regeneration frequency of the protoplast is low (Kue and Yamamoto, 1979; Peberdy, 1979). We carried out the reaction for 60 min. Since protoplasts are osmotically unstable, an osmotic stabilizer is required during their formation from yeasts. So the effects of the osmotic stabilizers such as sorbitol, KCl, mannitol, $MgSO_4 \cdot 7H_2O$, and sucrose were examined for protoplast formation. But according to Villanueva *et al.* (1971), the protoplast forma-

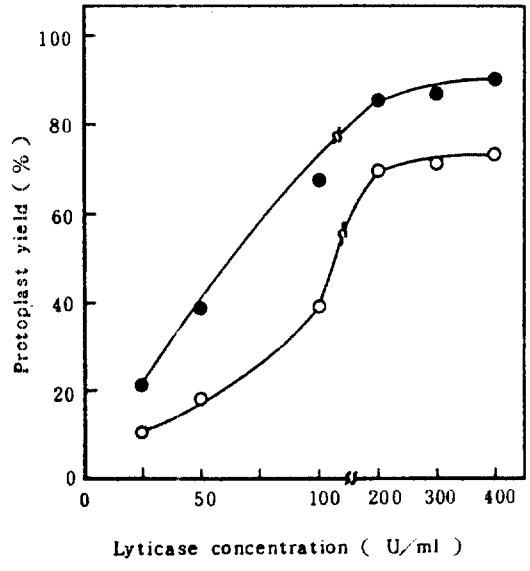

Fig. 6. *Effect of Lyticase concentration on the protoplast formation of Saccharomyces cerevisiae S29 (●—●) and K52 (○—○).*
The ce.. were incubated with different concentrations of Lyticase in SP buffer (pH 7.5) cotaining 0.1% 2-mercaptoethanol at 30°C for 60 min.

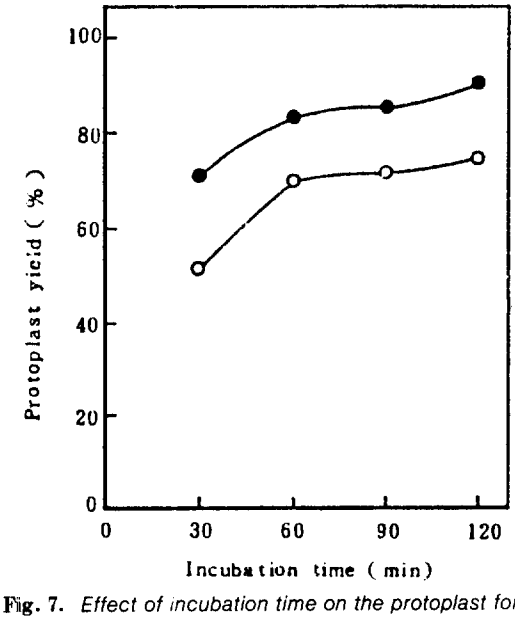

Fig. 7. *Effect of incubation time on the protoplast formation of Saccharomyces cerevisiae S29 (●—●) and K52 (○—○).*
The reaction conditions for the protoplast formation were the same as those described in the Fig. 1 except that the incubation time was varied.

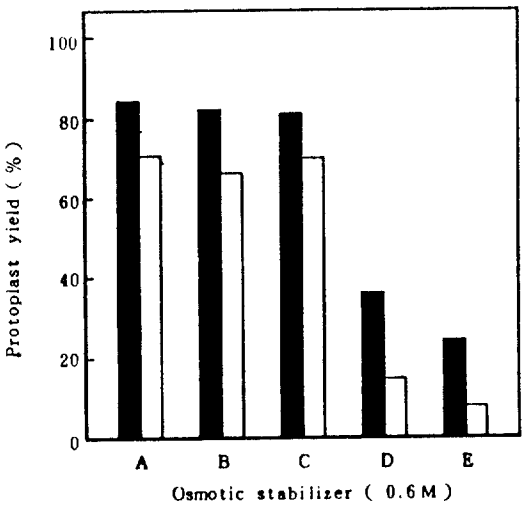

Fig. 8. *Effect of osmotic stabilizers on the protoplast formation of Saccharomyces cerevisiae S29 (■) and K52 (□).*
The cells were incubated with 200 unit/m*l* of Lyticase in the various osmotic stabilizers such as 0.6M of sorbitol (A), KCl (B), mannitol (C), $MgSO_4$ (D), and sucrose (E) containing 10mM potassium phosphate buffer (pH 7.5) and 0.1% 2-mercaptoethanol at 30 °C for 60 min.

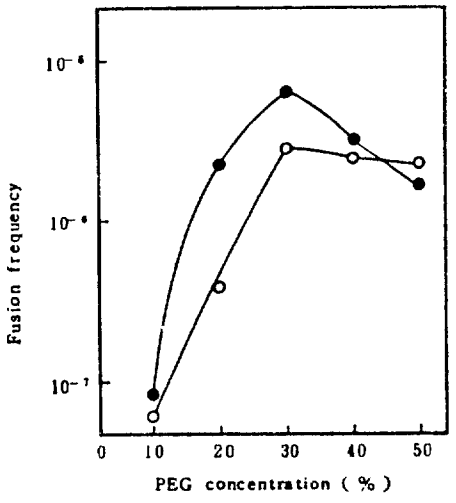

Fig. 9. *Effect of concentration and molecular weight of PEG on the fusion frequency between Saccharomyces cerevisiae S29 and K52.*
The mixture of 10^8 cells/m*l* protoplasts of each auxotroph was treated with PEG 6,000 (●–●) and PEG 4,000 (○–○) at different concentrations in SP buffer (pH 7.5) containing 20 mM $CaCl_2$ at 30 °C for 15 min. Details for the regeneration and fusion frequency of the fusants are given in the 'Materials and Methods'.

tion of some yeasts is not affected by the nature of the osmotic stabilizer. As appeared in Fig. 8, 0.6 M sorbitol showed the favorable effects on protoplast formation.

Under the optimum reaction conditions, the degree of lysis of S 29 (Lys⁻) was increased over 85%, whereas that of K 52 (Met⁻) was 70%.

Conditions for protoplast fusion and regeneration

Generally speaking, the factors affecting protoplast fusion and regeneration are molecular weight and concentration of PEG, treating time, osmotic stabilizer, addition of Ca^{2+}, regenerating method and composition of medium. Fig. 9 shows the dependence of fusion frequency on PEG concentration and molecular weight. Mixtures of the parental protoplasts were treated with various concentrations of PEG 4,000 or 6,000 in the presence of 20 mM $CaCl_2$. Although the concentration of PEG was not critical, 30% (w/v) PEG were effective regardless of molecular weight. These results showed that the maximum frequency of protoplast fusion between K 52 (Met⁻) and S 29 (Lys⁻) was 4.5×10^{-6} with PEG 4,000 and 8.1×10^{-6} with

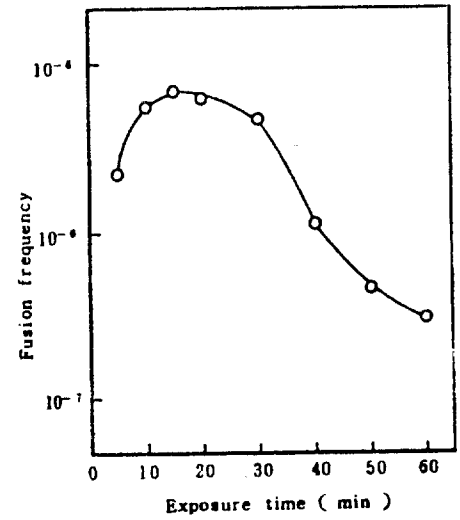

Fig. 10. *Effect of exposure time to PEG 6,000 on the fusion frequency between protoplasts of Saccharomyces cerevisiae S29 and K52.*
The reaction conditions for the protoplast fusion were the same as those described in the Fig. 9 except that the exposure time on PEG 6,000 was varied.

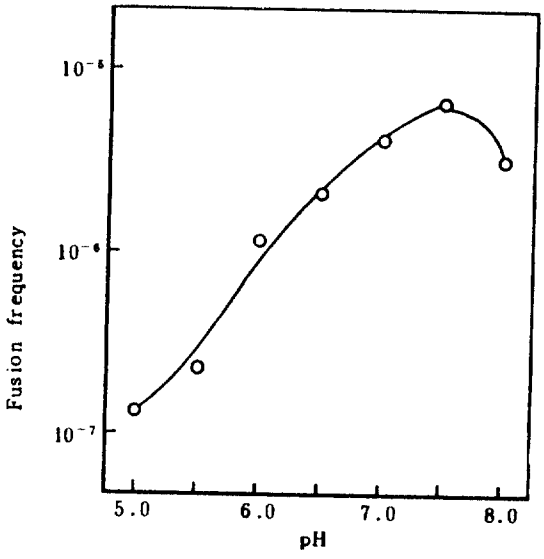

Fig. 11. *Effect of pH on the fusion frequency between protoplasts of Saccharomyces cerevisiae S29 and K52.*
The reaction conditions for the protoplast fusion were the same as those described in the Fig. 9 except that the reaction pH was adjusted from pH 5.0 to pH 8.0 with 10 mM potassium phosphate buffer.

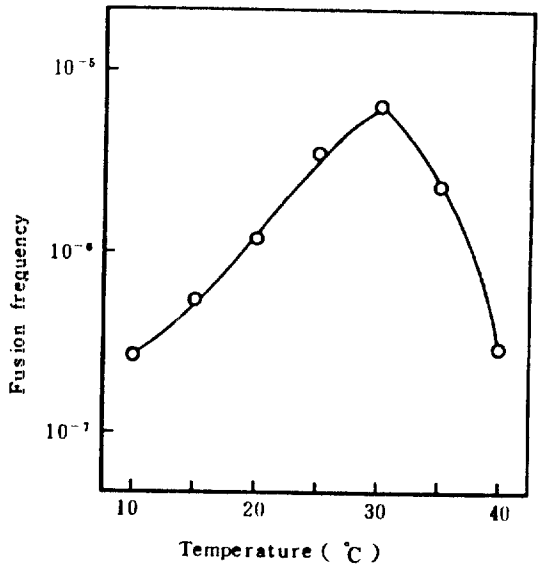

Fig. 12. *Effect of temperature on the fusion frequency between protoplasts of Saccharomyces cerevisiae S29 and K52.*
The reaction conditions for the protoplast fusion were the same as those described in the Fig. 9 except that the reaction temperature was varied.

PEG 6,000. The success of protoplast fusion for stable hybrid formation was directly concerned with the influences of various external parameters on aggregation and fusion of the parental protoplasts. All varieties of PEG were able to stablize and aggregate protoplasts and induce protoplast fusion at appropriate concentrations. Results regarding relationship between PEG exposure time and fusion frequency are shown in Fig. 10. The fusion frequency reached a maximum level after the exposure for 15 min with PEG. After 20 min exposure with PEG, no further increase in fusion frequency was observed. The optimum pH for protoplast fusion was determined using 10 mM potassium phosphate buffer at the indicated pH. Fig. 11 shows that fusion occurred at pH levels lower than pH 7.0, however, as the pH of reaction mixture increased, the fusion frequency increased and then maximized at about pH 7.5. That was similar to the result of protoplast formation with respect to optimum pH. As shown in Fig. 12, the protoplast fusion took place at 10°C. The fusion frequency was increased gradually by rising the temperature of the reaction mixture. And the optimum temperature for protoplast fusion was 30°C. This was similar to the optimum growth temperature, but lower than that of protoplast formation.

Under the optimum conditions, the fusion frequency reached to 8.2×10^{-6}. This result was similar to those of Shimoda *et al.* (1984).

적 요

원형질체 융합을 통하여 killer 효모의 유전형질을 기존의 alcohol 발효효모에 도입함으로서 야생의 killer 효모에 저항성을 가지고, 오염효모를 치사시킬 수 있는 새로운 killer 효모 균주의 개발을 시도하였다. 먼저 융합지표를 부여하기 위하여 양 친주의 영양요구 변이주를 분리하고, 원형질체 형성에 미치는 여러가지 조건을 검토한 결과, killer 효모인 *S. cerevisiae* K52는 PM 배지에서 20시간 배양한 세포를, 그리고 수용주인 *S. cerevisiae* S29는 PM 배지에서 24시간 배양한 대수 증식기의

균체를 각각 EDTA 및 삼투압 안정제로 0.6M의 sorbitol을 함유한 pH 7.5의 10mM 인산염 완충액에 현탁하여 50mM 2-mercaptoethanol로 15분간 전처리한 후 세포벽 용해효소로 Lyticase 50,000을 200unit/ml 첨가하여 30℃에서 60분간 처리했을 때 각각 70% 및 85% 이상의 수율로 원형질체가 형성되었다. 또한, 양친주의 원형질체를 동량 혼합하고, 20mM $CaCl_2$ 및 PEG 6,000을 30% 농도로 15분간 처리하여, pH 7.5의 재생배지에 매몰한 후 30℃에서 융합, 재생시킨 결과 8.2×10^{-6}의 융합 빈도수를 나타내었다.

ACKNOWLEDGEMENT

This work was partly supported by the grant from the Ministry of Education, 1988-1989.

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(Received August 23, 1989)