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Factors Affecting Protoplast Formation of Yeast

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酵母의 原形質体 形成條件

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As an essential previous step towards the development of cell fusion to breed a new brewing yearst strain, several factors predicted to affect the protoplast formation of *S. cerevisiae, C. tropicalis* and *E. fibuligera* were investigated in order to obtain the protoplasts in high yields. The optimum pH and temperature for the protoplas formation were 7.5 and 35°C, respectively. Pretreatment of the yeast cells with 2-mercaptoethanol stimulated the protoplast formation and 50 mM of the reagent was found as effective. Among several osmotic stabilizers tested for their effect on protoplas formation, 0.6M KCl was comparatively favorable.

The availability of procedure for the protoplast formation is a primary essential step towards the development of cell fusion which can be applied to the breeding of new strains. Since Eddy and Williamson[1] first demonstrated the routine preparation of large numbers of yeast protoplasts using lytic enzymes present in the gut-juice of the snail Helix pomatia, many other workers have tried to replace this source of lytic enzymes with more appropriate ones obtained from microorganisms.[2,3,4,5] Although the general picture of yeast cell walls is a complex structure composed of a highly branched glucan, part of which is associated with chitin and mannans which are firmly bound to protein,[6] most yeasts are easily converted to protoplasts by treatment of the commercially available lytic enzymes. The present investigations show that high yields of protoplasts from *S. cerevisiae* HAKKOKEN 1 GO, *C. tropicalis* IFO 0589 and *E. fibuligera* IAM 4247 can be obtained by using zymolyase 5,000 and several parameters affecting protoplast formation of the yeasts.

Materials and Methods

Strains

The strains used to obtain protoplasts were *Saccharomyces cerevisiae* HAKKOKEN 1 GO, *Candida tropicalis* IFO 0589 and *Endomycopsis fibuligera* IAM 4247.

Media and Culture Conditions

The susceptibility of yeast cells to lytic enzymes was found to be affected by the culture conditions.[7] With respect to the effect of growth phase on subsequent protoplast formation, Riggsby et al,[6] reported that the exponential phase cells were more susceptible to attack by lytic enzyme. For this purpose, the yeast cells for the protoplast formation were grown at 30°C for 18 hours with shaking in production medium containg 20g dextrose, 3g $(NH_4)_2SO_4$, 1g yeast extract, 1g KH_2PO_4, 0.5g $MgSO_4 \cdot 7H_2O$, 0.5g KCl and 10 mg $FeSO_4 \cdot 7H_2O$ in a liter of distilled water.

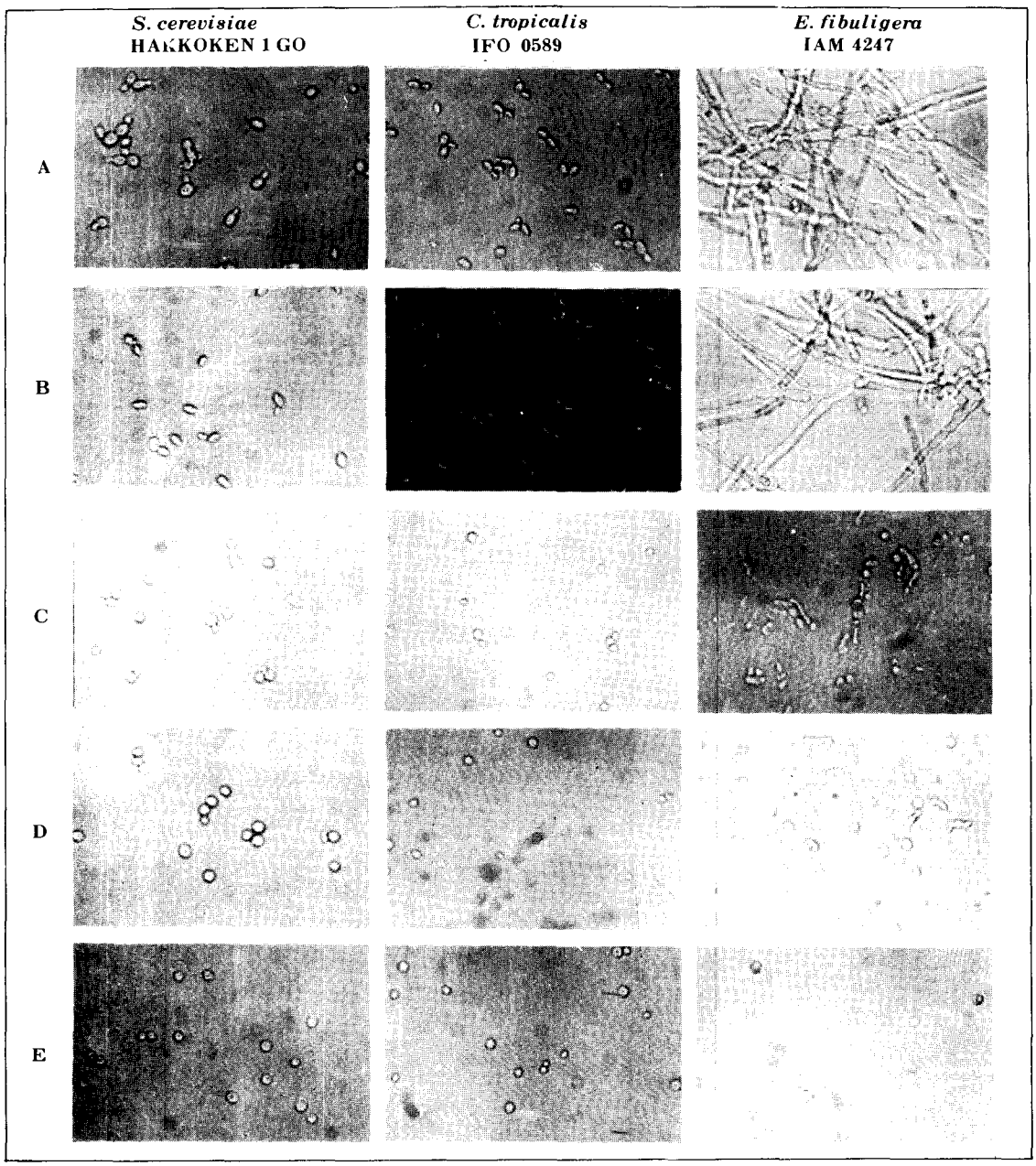

Fig. 1. Micrographs of protoplast formation of yeast cells (x400).

The intact cells (A) were treated with PTP buffer and then incubated with zymolyase for 0 min (B), 15 min (C), 30 min (D) and 45 min (E).

Preparation of Protoplasts

The method of Fournier[8] was modified and applied to protoplast formation of the yeast cells. The yeasts (*S. cerevisiae* and *C. tropicalis*, 5×10^8 cells; *E. fibuligera*, 400 mg wet cell weight) harvested in exponential growth phase were washed twice with sterile distilled water and resuspended in PTP buffer (99 mM Tris, 860 μM EDTA and 50 mM 2-mercaptoethanol, pH 8.0) and incubated at 30°C for 10 minutes. The cells were washed twice with 1.2 M KCl and resuspended in 4ml of the enzyme solution: 2ml of 1.2M KCl, 1ml of 50 mM 2-mercaptoethanol, 1ml of zymolyase 5,000 (4 mg) and the mixture was incubated at 30°C for an

appropriate period. The zymolyase 5,000, a lytic enzyme of *Arthrobacter luteus*, was purchased from Kirin Brewery Co. Ltd., Japan.

Measurement of protoplast formation

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[9] by measuring the optical density of the ten fold diluted reaction mixture at 620 nm.

$$\text{Degree of lysis (\%)} = \frac{Do - Dt}{(Do)} \times 100$$

Do = Optical density of the diluted reaction mixture at time zero.

Dt = Optical density of the diluted reaction mixture at time t.

Results

Microscopical observation on protoplast formation

The formation of yeast protoplasts as related to time is shown in Fig. 1. The digestion of the yeast cell wall was easily recognized by microscopy because the protoplast was spherical shape. After pretreatment in PTP buffer for 10 minutes, the morphological changes of the cells were not observed. When each cells of *S. cerevisiae* and *C. tropicalis* was incubated with zymolyase in 0.6M KCl for 15min, the protoplasts began to appear, and 30 min later the cells were almost completely transformed into protoplasts. Meanwhile, the filamentous-form cells of *E. fibuligera* were divided into small fragments at the points of septa as the lysis proceeded and finally transformed into round-shaped protoplasts after 45 minute incubation.

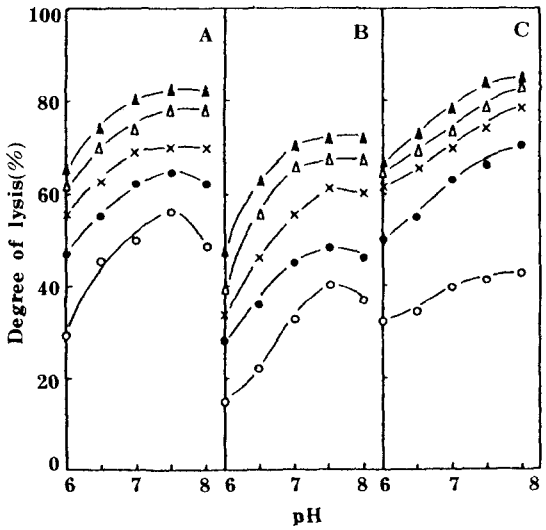

Fig. 2. Effect of pH on protoplast formation of *C. tropicalis*(A), *E. fibuligera*(B) and *S. cerevisiae*(C).

The cells were incubated with 1.0mg/m*l* of zymolyase in 0.6M KCl solution containing 50 mM of 2-mercaptoethanol at various pH for 15 min(○), 30 min(●), 60 min(×), 90 min(△) and 120 min(▲).

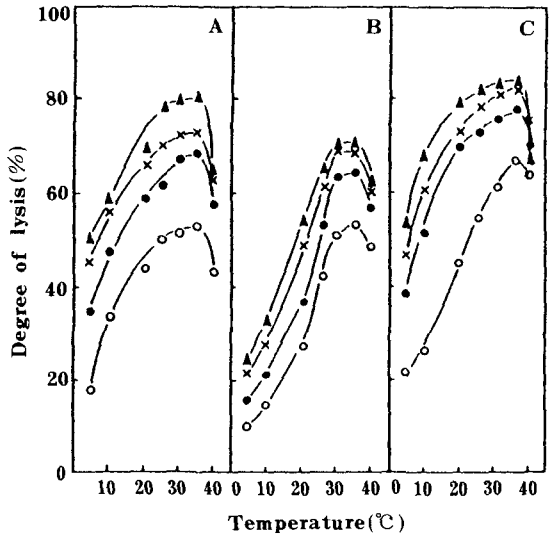

Fig. 3. Effect of temperature on protoplast formation of *C. tropicalis*(A), *E. fibuligera*(B) and *S. cerevisiae*(C).

The cells were incubated with 1.0mg/m*l* of zymolyase in 0.6M KCl solution containing 50mM of 2-mercaptoethanol at different temperature for 30 min(○), 60 min(●), 90 min(×) and 120 min(▲).

Conditions for protoplast formation

In order to investigate the optimum conditions for the preparation of yeast protoplasts in high yields, we examined several factors predicted to affect the lysis of *S. cerevisiae*, *C. tropicalis* and *E. fibuligera*. The optimum pH was determined using 0.01 M phosphate buffer containing 0.6 M KCl. As shown in Fig. 2, although the initial velosities of lysis were maximum at pH 7.5, the degree of lysis of the yeast cells were nearly similar in the pH region from 7 to 8 with increasing time. As appeared in Fig. 3, the optimum temperature for lysis of the cells was 35°C. Fig. 4 shows the effect of 2-mercaptoethanol concentration on the cell lysis. For the cells of *S. cerevisiae* and *E. fibuligera* pretreated with PTP buffer, the effect of 2-mercaptoethanol was not so significant and increasing the concentration over 100 mM caused a marked decrease in the cell lysis. But for the *C. tropicalis*,

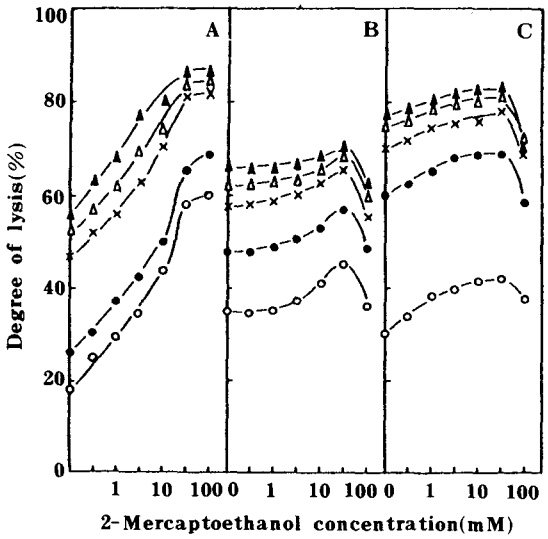

Fig. 4. Effect of 2-mercaptoethnol concentration on protoplast formation of *C. tropicalis*(A), *E. fibuligera*(B), *S. cerevisiae*(C).

The cells were incubated with 1.0mg/m*l* of zymolyase in 0.6 M KCl solution containing various concentration of 2-mercaptoethanol at 30℃ for 15 min(○), 30 min(●), 60 min(×), 90min(△) and 120 min(▲).

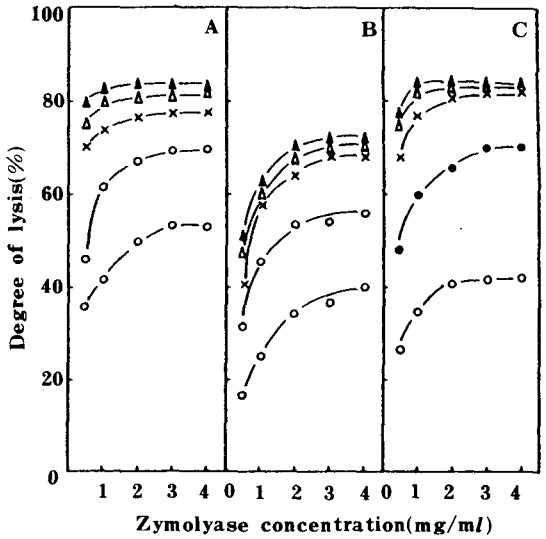

Fig. 5. Effect of zymolyase concentration on protoplast formation of *C. tropicalis*(A). and *E. fibuligera*(B) and *S. cerevisiae*(C).

The cells were incubated with different concentration of zymolyase in 0.6 M KCl solution containing 50 mM of 2-mercaptoethanol at 30℃ for 15 min(○), 30 min(●), 60 min(×), 90 min(△) and 120 min(▲).

this compound exhibited a significantly important effect, that is, the higher concentration of 2-mercaptoethanol tested was, the higher degree of lysis was observed. As the results, we decided to use 50 mM of the reagent in the reaction mixture. The ratio of the lytic enzyme concentration to the cell concentration was also investigated. The results shown in Fig. 5, where the cell concentration was fixed at 10^8 cells/ml (*S. cerevisiae* and *C. tropicalis)* and 400mg wet cell weight/ml (*E. fibuligera)*, revealed the similar relationship between the cells and the enzyme concentrations. To obtain maximum lysis after 120 minute incubation, the enzyme concentration should be more than about 1 mg/ml in the case of *S. cerevisiae* and *C. tropicalis*, and 2mg/ml in the case of *E. fibuligera*. The Effects of sorbitol, sucrose, KCl and $MgSO_4$ were examined as osmotic stabilizers for protoplast formation. As appeared in Fig. 6, 0.6 M KCl showed a favorable effect on protoplast formation common to *S. cerveisiae*, *C. tropicalis* and *E. fibuligera*.

Effect of incubation time on cell lysis under optimum condition

The results shown in Fig. 7 were obtained when *S. cerevisiae*, *C. tropicalis* and *E. fibuligera* were subjected to

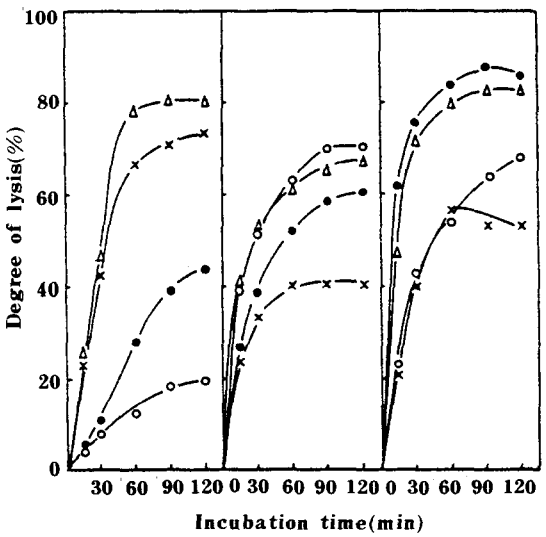

Fig. 6. Effect of osmotic stabilizer on protoplast formation of *C. tropicalis*(A), *E. fibuligera* (B) and *S. cerevisiae*(C).

The cells were incubated with 1.0mg/m*l* of zymolyase and 50 mM of 2-mercaptoethanol in 0.6 M sucrose(○), sorbitol(●), KCl(△) or $MgSO_4$(×) at 30℃ for 120 min.

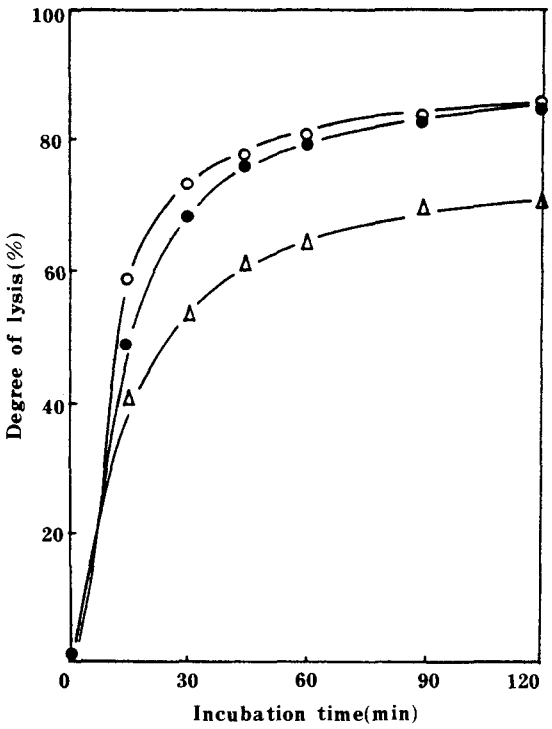

Fig. 7. Effect of incubation time on protoplast formation under optimum condition.

C. tropicalis (●), *S. cerevisiae* (○) and *E. fibuligera* (△) were suspended in 0.6M KCl solution containing 2.0 mg/ml of zymolyase and 50 mM of 2-mercaptoethanol, and incubated at 35℃ for 120 min.

lysis under the optimum reaction condition. The cells were effectively converted to the protoplasts after the incubation for 60 to 120 min, although the initial velocities of the lysis were slightly different each other. In this case, the degree of lysis of both *S. cerevisiae* and *C. tropicalis* were increased up more than 80%, whereas that of *E. fibuligera* was 70%.

Discussion

Although workers investigating the yeast protoplast have been greatly aided by the availability of suitable lytic enzymes from commercial sources including zymolyase, β-gluculonidase and novozym 234, susceptibilities of the yeasts to the lytic enzyme differ significantly depending upon the strains, and culture conditions. Since cell wall composition varies with the growth phase,[10,11] exponential phase cells are generally more susceptible than stationary phase cells to attack by lytic enzymes. Kitamura et al.[12] reported that the cells cultured in a medium containing organic nitrogen as the sole nitrogen source showed low susceptibility, whereas those of cultured in the same medium containing inorganic nitrogen as the sole nitrogen source showed high susceptibilty. Therefore, the cells grown in production medium to exponential phase were used for protoplast formations. As shown in Fig. 1, intact cells could hardly be found microscopically after 45 minutes incubation in the reaction mixture. Because the protoplasts are osmotically sensitive, the progress of protoplast formation can be monitored by measuring the degree of cell lysis.[9] Namely, zymolyase lysed viable yeast cells resulting in decrease of the turbidity of the reaction mixture which was diluted ten fold in distilled water. The turbidity decreased rapidly at the initial stage but became approximately constant after 60 minute incubation. The optimum pH and temperature for lysis of viable yeast cells were 7.5 and 35°C, repectively. These results are in accordance with the results reported by Kitamura et al.[5] Pretreatment with mercapto compounds such as 2-mercaptoethanol and dithiothreitol is used to render cells more susceptible to lytic enzyme digestion,[13] as the results of weakening or dissolving of the mannan-protein complex.[14] Athough the effect of 2-mercaptoethanol on protoplast formation varies in strains, 50 mM of the reagent was found as more effective. According to Villanueva et al.[15] and Dooijewaard-Kloosterziel et al.,[16] protoplast formation of yeasts in some species is not affected by the nature of the osmotic stabilizer. However, in our observation 0.6M KCl was comparatively favorable. Although *S. cerevisiae, C. tropicalis, E. fibuligera* showed a similar degree of lysis and reached maximum after 60 minute incubation, the time course study on lysis suggested that there should exist a certain difference among the susceptibilites of the cells.

요 약

이속 효모간 원형질체 융합에 의해 새로운 알콜발효 균주를 개발하기 위한 전단계로서, ***Arthrobacter luteus***에서 얻은 zymolyase 5,000을 세포벽 분해효소로 사용하여 전분분해 효모인 ***C. tropicalis, E. fibuligera*** 및 알콜발효 효모인 ***S. cerevisiae*** 등의 원형질체 형성조건에 대하여 검토하였다. 원형질체 형성의 최적 pH와 온도는 각각 pH 7.5와 35℃였다. 또한 원형질체 형성에 대한 2-mercaptoethanol의 효과는 50 mM 농도에서 가장 좋았으며 삼투압 안정제로는 0.6M KCl이 적당하였다.

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