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Formation and Regeneration of *Penicillium verruculosum* Protoplasts

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*Penicillium verruculosum*의 원형질체 형성과 재생

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Optimal conditions for the formation and regeneration of protoplasts of the cellulolytic fungus *Penicillium verruculosum* were investigated. Among the various commercial cell wall lytic enzymes tested, 0.5%(w/v) Novozym 234 was the most effective for protoplast formation. The highest yield of protoplasts exceeding 4.5×10^6/ml obtained when 400mg of 20 hr-old mycelia was incubated with 0.5%(w/v) Novozym 234 at 30°C for 1 hr. The best osmotic stabilizer for the isolation and regeneration of protoplasts was 0.7M sorbital (pH 5.6) and 0.6M $MgSO_4$ (pH 5.6), respectively. When 0.6M $MgSO_4$ was added as osmotic stabilizer to the complete medium, the maximum regeneration frequency obtained was 4.6-27.8%. Micromorphological change of giant protoplasts into hyphae was observed during incubation in the regeneration liquid medium.

Microbial conversion of cellulosic biomass into useful products, such as liquid fuel, chemical feedstock and food materials, can be one of the most promising to solve the resource shortage problems (1). However, enzymatic conversion of cellulose into low molecular materials has not been achieved yet commercially due to the high costs for producing cellulases by available microbial strains. Thus, a key to the success of the process of enzymatic conversion of cellulose is the availability of high activity-cellulases. Maximization of cellulase productivity at a reduced cost could be achieved by improvement of the strain and optimization of fermentation conditions(2,3).

Even though many microorganisms are actively cellulolytic, only a few of them have received special attention(4). *Trichoderma* species, particulary *T. reesei* and its mutants had been considered as the best source of extracellular cellulase suitable for practical saccharification of cellulose. A cellulolytic fungus *Penicillium verruculosum* has received recent attention due to its enzyme complexes, which degrade very efficiently crystalline cellulose like cotton and Avicel(5,6).

In connection with the strain improvement of useful industrial microorganisms, protoplast fusion technique has been proved to be valuable for intra- and interspecific hybridization(7-10). Unfortunately, neither the genetic background of various aspects of cellulase production nor genetic manipulation of *P. verruculosum* using protoplasts has been reported.

In this paper we report the optimal conditions for the protoplast formation and regeneration, which are essential steps for the genetic manipulation through protoplast fusion.

Materials and Methods

Organisms

P. verruculosum F-3 (IFO 31136) was used. The fungus was isolated by Chung *et al.*(6) as a new po-

Key words: *Penicillium verruculosum*, Protoplast formation and regeneration
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tent producer of cellulase complex and its properties was described in detail in the previous papers (11-13).

Media and culture condition

P. verruculosum F-3 was grown on potato dextrose agar(PDA) slants at 30 °C for 7 days and stored at 4 °C. Modified Mandels' medium(14) containing 1% glucose was used as the minimal medium (MM). The regeneration complete medium (RCM) and regeneration minimal medium (RMM) were prepared by adding 0.6M magnesium sulfate to PDA and MM, respectively. To obtain mycelia for preparing protoplasts, 5m*l* of the suspended spores(2×10^6 spores/m*l*) developed on the slants of PDA were inoculated in 250m*l* flasks containing 50m*l* of PDY medium (200g of peeled and diced potato, 20g of dextrose and 5g of yeast extract in 1 liter of deionized water) and incubated on a shaker (spm 110, 2.5cm) at 30 °C.

Lytic enzymes

The enzymes used are Chitinase (Sigma Chemical Co., St. Louis, Missouri, U.S.A.), Laminarinase (Sigma), β-Glucuronidase (Sigma), Novozym 234 (Novo Biolabs, Novo Alle' DK-2880 Bagsvaerd, Denmark), Driselase (Kyowa Hakko Kogyo, Tokyo, Japan), Zymolase-20T (Seikagaku Kogyo, Nihonbashi, Tokyo, Japan), Macerozyme R-10 (Yakult Honsha, Nishinomiya, Japan), Cellulase Onozuka RS (Yakult Honsha) and Meicelase (Meijiseika Kaisha Ltd. Tokyo, Japan). Lytic enzyme preparations were sterilized by filtration.

Formation of protoplasts

About 400mg of damp mycelium grown in liquid PDY medium for 18 hr were harvested and then washed twice with the osmotic stabilizer. The osmotic stabilizer used were 0.05M citrate buffer, pH 5.6, containing 0.6M each of KCl, $(NH_4)_2SO_4$, NaCl, $MgSO_4$, mannitol and sorbitol at final concentration. The mycelia were then treated with 1m*l* of the lytic system in the stabilizer and reacted at 30 °C on a reciprocal shaker(spm 108, 2.0cm) for up to 120 min. The number of protoplasts formed were counted periodically under phase contrast microscope, using haemacytometer. Protoplasts were concentrated by centrifugation (1,000x*g* for 10 min), after removing mycelial debris by filtration through a sintered glass filter (porosity; 40-60 μm) and washed twice with the same buffered osmotic stabilizer.

Regeneration of protoplasts

The protoplasts prepared were resuspended in the stabilizer to adjust the population density and plated on the RCM or RMM for regeneration. After incubation at 30 °C for 4-7 days, regeneration frequency was estimated according to Lee *et al.*(15) by the following equation.

$$\text{Regeneration frequency} = \frac{(B\text{-}C)}{A} \times 100(\%)$$

, where A is the total number of protoplasts observed under microscope, B the number of regenerated cells, and C the number of osmotic resistant cells in deionized water.

Results and Discussion

Effect of cell wall lytic enzymes on protoplast formation

Various kinds of lytic preparations are now

Table 1. Comparison of commercial enzymes for their ability to induce protoplast formation from *P. verruculosum**

Enzyme solution	Number of protoplasts per m*l* $\times 10^6$
Chitinase (Sigma 21 U/m*l*)	1.0
Laminarinase (0.5%)	0
β-Glucuronidase (0.5%)	13.0
Chitinase (Sigma 21 U/m*l*) + Laminarinase (0.5%) + β-Glucuronidase (0.5%)	1.5
Novozym 234 (0.5%)	32.4
Driselase (0.5%)	11.0
Zymolyase-20T (0.5%)	2.3
Macerozyme R-10 (0.5%)	0
Cellulase Onozuka RS (0.5%)	0
Meicelase (0.5%)	0

*Mycelia of 400mg as wet weight were treated at 30 °C for 60 minutes with 1 m*l* of enzyme solution in 0.7M sorbitol buffered at pH 5.6 with 0.05M citrate buffer.

commercially available, but they vary in their enzyme contents and activities. In order to select a suitable lytic system, various commercial enzyme preparations were tested as to their ability to release mycelical protoplasts. As shown in Table 1, the enzyme preparations such as Laminarinase, Macerozyme R-10, Cellulose Onozuka RS and Meicelase were not effective to form protoplast, while Chitinase and Zymolase-20T were slightly effective. Novozym 234 was the most effective, yielding 32.4 × 10^6 protoplasts per m*l* (after incubation for 60 min at 30 °C). This high efficiency of Novozym 234 for protoplasts formation seems to be due to the fact that the cell wall of *Penicillium* contain chitin and β-1, 3-glucan as main cell wall components(16), and Novozym 234 contains a number of enzymes, i.e., chitinase, β-1, 3-glucanase, α-1,3-glucanase, and protease(17,18).

Effect of enzyme concentration on protoplast formation

To find an optimal enzyme concentration, Novozym 234 was added at four different concentrations (i.e., 3,5,8 and 10mg per m*l*) and reacted at 30 °C for different periods from zero to 120 min. As shown Fig. 1, the protoplast yield appeared to increase linearly with the enzyme concentration and incubation time up to the optimal concentration (5 mg/m*l*) and the optimal incubation time (60 min). However, the yield decreased drastically beyond the optimal concentration and time. As reported by Hamlyn *et al.*(18), Novozym 234 contains strong proteinase, therefore, the protoplasts seemed to be easily destroyed at high concentration of the enzyme or extended incubation. The addition of 5 mg/m*l* of Novozym 234 gave the highest yield of protoplasts which exceeded 4.5 × 10^6/m*l*.

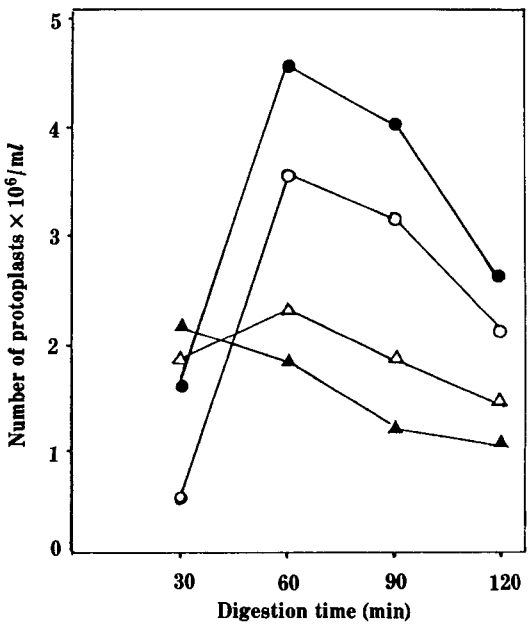

Fig. 1. Effect of enzyme concentration on the formation of protoplasts from *P. verruculosum*.
○ - ○ ; 3 mg/m*l*, ● - ● ; 5 mg/m*l*, △ - △ ; 8 mg/m*l*, ▲ - ▲ ; 10 mg/m*l*.

Effect of mycelial age on protoplast formation

The physiological age of the culture markedly influence the protoplast yield. When 400mg as wet weight of mycelia at different ages were incubated on 1m*l* of 0.5% Novozym 234 at 30 °C for 1hr, the maximum yield of protoplasts was obtained from 20hr-old mycelia (Fig. 2). Use of mycelia older than 20hr resulted in a significant decrease in protoplast yield. This result corresponds with those of Peberdy *et al.* (19) and Picataggio *et al.* (20) who reported that the protoplast yield was the highest when the cultures at exponential growth phase were used.

Effects of osmotic stabilizers and pH on protoplast formation

As osmotic stabilizers for protoplast formation each of 0.6M sorbitol, mannitol, KCl, $(NH_4)_2SO_4$,

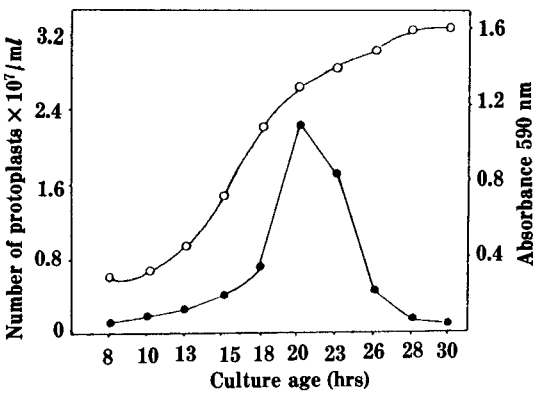

Fig. 2. Effect of mycelial ages on the formation of protoplasts from *P. verruculosum*.
● - ● ; Protoplasts yield, ○ - ○ ; Mycelial growth.

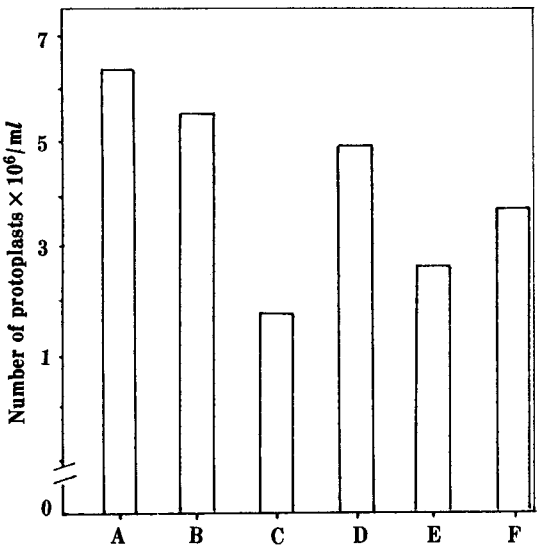

Fig. 3. Effect of osmotic stabilizers on the formation of protoplasts from *P. verruculosum*.
A: Sorbitol, B: KCl, C: $(NH_4)_2SO_4$, D: Mannitol, E: $MgSO_4$, F: NaCl.

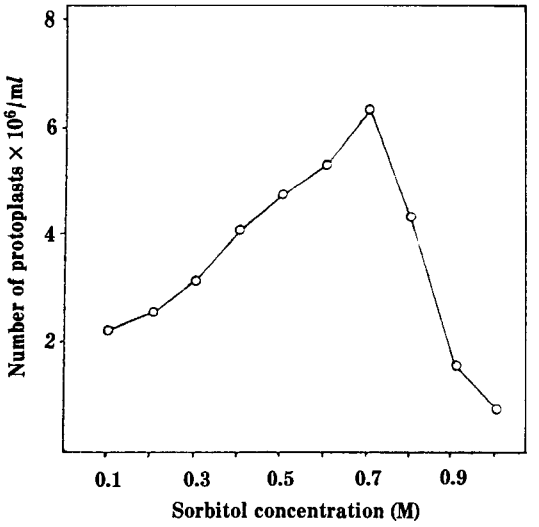

Fig. 4. Effect of sorbitol concentration on the formation of protoplasts from *P. verruculosum*.

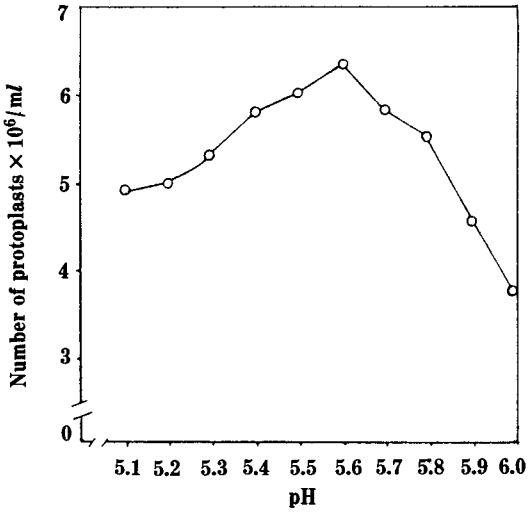

Fig. 5. Effect of pH on the formation of protoplasts from *P. verruculosum*.
The digestion was carried out at 30 °C for 60 minutes with 1 ml of 0.5% Novozym 234 solution in 0.7 M sorbitol buffered at pH 5.1-6.0 with 0.05 M citrate buffer.

$MgSO_4$ or NaCl added to 0.05M citrate buffer (pH 5.6) were examined. As shown in Fig. 3, sorbitol and KCl showed better protective effect than other stabilizers at 0.6M. Optimum concentration of sorbitol was observed to be 0.7M, and the protoplast yield sharply dropped when the sorbitol concentration was higher than 0.7M (Fig. 4). Optimum pH for protoplast formation was determined using 0.05M citrate buffer containing 0.7M sorbitol, and it was observed that the maximum yield of protoplast was obtained at pH 5.6 (Fig. 5). Hamlyn (21) reported that the optimum pH of Novozym 234 for the release of protoplast in *Cephalosporium acremonium* as pH 5.5. The results were similar even though the organisms tested were different.

Effects of temperature and incubation time on protoplast formation

The enzyme reaction was carried out at 25 °C-35 °C for 30-120 min on a slow shaker (108 spm, 2cm). The highest yield of protoplasts was obtained by incubating at 30 °C for 60 min (Fig. 6). The incubations at temperatures higher or lower than 30 °C resulted in significantly low yield of protoplast. The duration of incubation affected not only the number of protoplasts formed but also their shape and size(Fig. 7). The protoplasts obtained from the incubation for 90 min were as large as 2 times than those from 60 min incubation. The disrupted protoplasts were frequently observed from the incubation for 120min. Regeneration of protoplasts was dependent upon the length of exposure time of the mycelia to the lytic enzyme; higher rege-

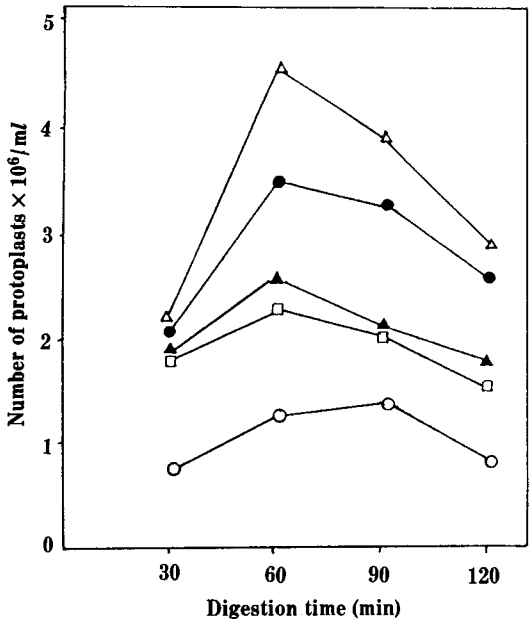

Fig. 6. Effect of digestion temperature on the formation of protoplasts from *P. verruculosum*.
○-○; 25 °C, ●-●; 28 °C, △-△; 30 °C, ▲-▲; 33 °C, □-□; 35 °C.

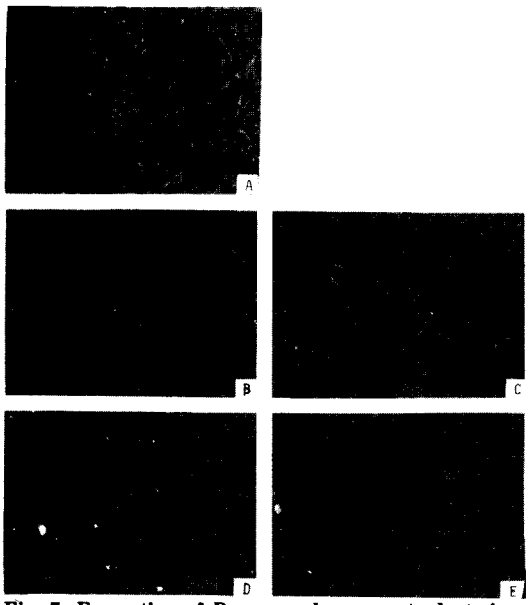

Fig. 7. Formation of *P. verruculosum* protoplasts in a 0.5% (w/v) solution of Novozym 234.
A; 0 time treatment, B: 30 min treatment, C: 60 min treatment, D: 90 min treatment, E: 120 min treatment. All photographs were taken at a magnification × 400 through a light microscope.

neration frequency was obtained from the protoplasts formed by shorter time of hydrolytic treatment(20). Hong *et al.*(22) and Go *et al.*(23) also reported that when the protoplasts were prepared by prolonged period of incubation, the regeneration frequency was very low due to the damage of protein or lipoprotein components of the plasma membrane by proteolytic enzymes contaminated in the lytic enzyme preparation. Consequently, based on the results described above, it was expected that the regeneration frequency would be very high because of the optimal enzyme reaction time for this species was much shorter than those for *P. chrysogenum* (24,25), *P. baarnense* (25), and *Tricholoma matsutake* (26).

Regeneration of protoplasts

In order to examine the effect of osmotic stabilizers on protoplast regeneration, various osmotic stabizers were added to the RMM and RCM. The protoplast suspension was diluted with the same stabilizer solution and an aliquot was placed on the RMM and RCM containing osmotic stabilizers. As shown in Table 2, among the osmotic stabilizers tested, 0.6M KCl and 0.6M $MgSO_4$ showed 2.3-6.8% and 4.6-27.8% regeneration frequencies, respectively. These results differed from those in protoplast formation where 0.7M sorbitol was the most effective as osmotic stabilizer. Thus 0.6M $MgSO_4$ was chosen as the osmotic stabilizer for protoplast regeneration. Difference in regeneration frequency on the RCM and RMM was not noticed. Regeneration of protoplast also occurred in the liquid media(RLM) containing 0.7M sorbitol in MM. As

Table 2. Regeneration frequency and incubation periods of the protoplasts of *P. verruculosum* at the different osmotic stabilizers

Osmotic stabilizer	Regeneration frequency (%)	Incubation periods for regeneration (day)
0.7M Sorbitol	0.2-0.4	4
0.6M KCl	2.3-6.8	6
0.6M $(NH_4)_2SO_4$	1.7-2.8	7
0.6M Mannitol	1.8-2.6	4
0.6M $MgSO_4$	4.6-27.8	4

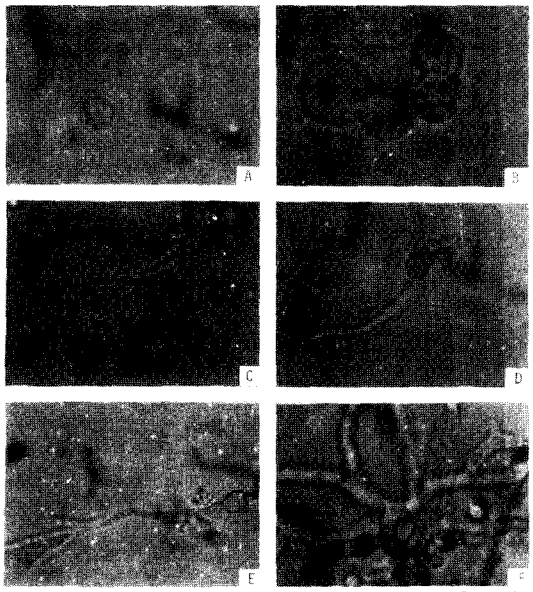

Fig. 8. Regeneration of *P. verruculosum* protoplasts in RLM medium.
A: Pyriformed protoplast after 8h.
B: Formation of a short chain of yeast-like cells after 15h.
C: Aberrant tube developed after 18h.
D: Formation of the first hypha after 20h.
E: Lengthened and branched hypha after 22h.
F: Well-developed hypha after 24h.
All photographs taken at a magnification × 400 through a light microscope.

shown in Fig. 8, the protoplasts transferred to RLM initiated regeneration in 8 hr after incubation. The process started with the formation of a prototrusion from the original expanded protoplast(A), and a short chain of "yeast-like cells" were formed in 15 hr after incubation(B), and then from the distal end of this chain a large aberrant tube emerged(C). As the aberrant tube disappeared, normal mycelium developed from the terminal bud of the yeast-like chain in 20hr after incubation(D). This pattern of regeneration was similar to the result of Cho *et al.*(27). When further incubated typical branched mycelia of *Penicillium* were observed to develop (E,F).

요 약

섬유소 분해효소를 생산하는 *P. verruculosum* F-3의 세포융합에 관한 연구의 일환으로 균사체로부터의 원형질체 형성과 재생 최적조건을 검토하였다. 세포벽 분해효소로 유효한 각종시판효소제제중 0.5%(w/v) Novozym 234가 가장 효과적이었고 PDY 배지로 30℃ 20시간 배양한 균사체 400 mg을 30℃ 1시간 반응시 가장 많은 원형질체가 형성되었다. 원형질체 형성을 위한 최적 삼투압 조절제는 0.7 M sorbitol(pH 5.6)이었고 원형질체 재생을 위한 최적 삼투압 조절제는 0.6 M $MgSO_4$(pH 5.6)이었으며 RCM에서의 재생율은 4.6~27.8%였다. 원형질체를 RLM에 배양시 균사체로 환원되는 형태적 변화가 관찰되었다.

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