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Protoplast Isolation and Reversion from *Lyophyllum ulmarium*

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만가닥버섯의 原形質体 分離 및 還元

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ABSTRACT: This experiment was undertaken to investigate proper conditions for protoplast formation from *Lyophyllum ulmarium*. Combination of Novozym 234, β-Glucuronidase and β-D-Glucanase with 0.6 M Sucrose was the most effective for isolation of protoplasts. The optimal reaction time of mycelium with the lytic mixture was 3 hrs in shaking condition at 120 strokes min^{-1}. When the mycelium of *L. ulmarium* was cultured for 6 days on yeast glucose agar medium at 25°C, the formation of protoplasts was effective.

The yeast glucose agar medium stabilized with 0.6 M sucrose was the most effective for reversion of protoplasts.

KEYWORDS: Protoplast formation and reversion. *Lyophyllum ulmarium*, Basidiomycetes.

Protoplasts have proved a valuable tool in the study of genetics and breeding in fungi(Hamlyn and Ball, 1979; Yoo *et al.*, 1984; Ferenczy, 1985). Two decades have been passed since the first report on protoplast isolation from *Polystictus versicolor*, basidiomycetes, after treatment with the gut-juice of *Helix pomatia*(Strunk, 1965).

In relation to the protoplast formation many investigations have been studied on basidiomycetes including *Agaricus brunnescens*(Anderson, 1984), *Collybia velutipes*(Yamada *et al.*, 1983), *Coprinus lagopus*(Moore, 1975), *Lentinus edodes*(Ushiyama and Nakai, 1977), *Phanerochaets chrysosporium*(Gold *et al.*, 1983), *Pleurotus cornucopiae*(Lee *et al.*, 1986a), *Pleurotus ostreatus*(Byun, 1984; Yoo, *et al.*, 1985), *Pleurotus sajor-caju*(Go *et al.*, 1985), *Tricholoma matsutake*(Abe *et al.*, 1982) and *Volvariella volvacea*(Santiago, 1981). De Vries and Wessels (1973) obtained spheroplasts from 33 species.

This experiment was undertaken to find out the effectiveness of some lytic enzymes and the optimum conditions for the production of protoplasts from the hyphae of *Lyophyllum ulmarium*. Proper osmotic stabilizer for reversion of protoplasts was also tested.

Materials and Methods

Strains and Growth Conditions

Lyophyllum ulmarium ASI 8007 was obtained from the Agricultural Sciences Institute. It was maintained on the yeast glucose agar(YGA; Conney and Emerson, 1964), containing(g/*l*) yeast extract 5.0, glucose 10.0, agar 20.0. For the reversion of protoplasts, osmotically stabilized agar medium was prepared. It consists of(g/*l*) yeast extreact 5.0, glucose 10.0 and Bacto-agar 20.0 and is supplimented with 0.6 M KCl, mannitol, sorbitol or sucrose. Bottom agar was 2.0%

while overlaying soft agar was of 0.75% Bacto -agar(Difco).

Lytic Enzymes

Novozym 234 was provided from Novo Industri, Denmark. β-D-Glucanase was purchased from BDH Chemicals Ltd, U.K. β-Glucuronidase was purchased from the Sigma Chemical Company Ltd. U.S.A. Each enzyme was dissolved 5 mg ml^{-1} in 0.6 M osmotic stabilizer solution and sterilized by Gelman filtration system(pore size 0.2 um).

Protoplast Formation

Disks of sterile cellophan membrane(Spectrum Medical Industries Inc., USA) were placed on the surface of YGA in petri dishes. The mycelial disks were ready for protoplast production when mycelia had grown over the disks. Mycelial disks of *Lyophyllum ulmarium* from 3-7 days culture at 25℃ were removed to clean sterile petri dishes and the lytic enzyme stabilizer solution was added immediately. The petri dishes incubated on reciprocal shaker(120 strokes min^{-1}) at 28℃ for 1-24 hours. The process of protoplast release was examined under microscope.

Protoplast Reversion

Protoplasts from lytic mixture were separated from mycelial debris by filtration through sintered glass filters(porosity 1), and sedimented by centrifugation at 1,000 rpm. The pellet of protoplasts was washed twice with 0.6 M sucrose to remove lytic enzyme. Protoplast yields were based on haemocytometer counts on the filtrates following lytic digestion. Appropriate dilutions were made of the protoplast suspensions and inoculated into hypertonic YGA at 25℃ for 7-14 days. A colony count was made to assess the reversion frequency of the protoplasts.

Results and Discussion

Table I. Comparison of commercial enzymes for their ability to induce protoplast release from *Lyophyllum ulmarium*

Enzyme	Protoplast yield ($\times 10^6$/ml)
Novozym 234	4.65
Novozym 234+β-Glucuronidase	5.20
Novozym 234+β-Glucuronidase+ β-D-Glucanase	7.20

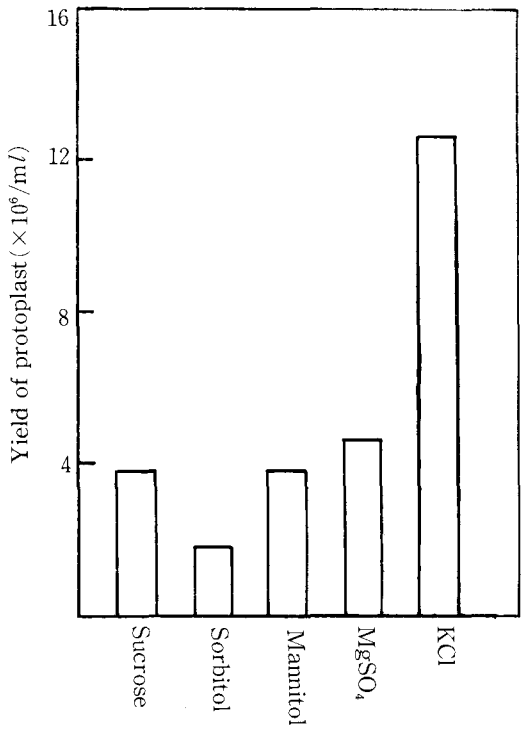

Fig.1. Protoplast formation in *Lyophyllum ulmarium* using different osmotic stabilizers. The digestions were carried out for 3h at 28℃.

Factors Affecting Protoplast Release

Lytic Enzymes: Some commercial lytic enzyme preparations were tested as to their ability to induce protoplast release(Table I). A combination of the enzymes Novozym 234, β-D-Glucanase and β-Glucuronidase gave more results than those obtained using Novozym 234 alone.

Novozym 234 was the most effective enzymes with *Pleurotus ostreatus*(Byun *et al.*, 1984; Yoo *et al.*, 1985), *Pleurotus cornucopiae*(Lee *et al.*, 1986) and *Volvariella volacea*(Hamlyn *et al.*, 1981). In general, combination of enzymes was required to obtain high yields of protoplasts in filamentous fungi(Hamlyn *et al.*, 1981; Yoo *et al.*, 1985; Lee *et al.*, 1986a).

Osmotic stabilizers: Inorganic salts, sugar and sugar alcohols were tested for their effect on protoplast isolation(Fig.1). The osmotic stabilizers varied in their ability to support the release and maintain the stability of protoplasts. Sucrose gave the best yield of protoplasts. In the higher fungi, some reporters examined a variety of stabilizer solutions for protoplast isolation from *Pleurotus*

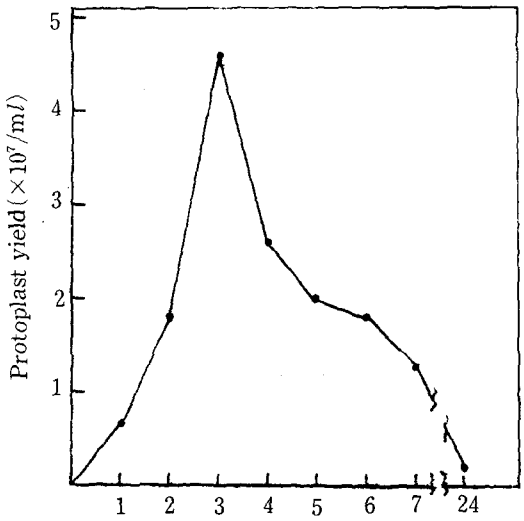

Fig.2. Effect of incubation time on protoplasts release. Lytic mixture stabilized with 0.6 M Sucrose stabilizer.

ostreatus(Flint, 1982), *Pleurotus sajor-caju*(Go *et al.*, 1985) and *Pleurotus cornucopiae*(Lee *et al.*, 1986a), and found that sucrose gave the best result.

Incubation time: The number or protoplasts increased to a maximum after 3 to 4h and prolonged incubation had no effect(Fig.2). Over 15% of the total protoplast yield was obtained within the first hour of incubation with the lytic enzymes and after 24 hrs. only 4% had survived due to lysis.

It has been shown in some fungi that incubation after 3h to 4h give the best protoplast yields(Santiago, 1981; Yamada *et al.*, 1983).

Age of Mycelium: Mycelium of different ages

Table II. Production of protoplasts from mycelial colonies of different ages in *Lyophyllum ulmarium*

Culture age (day)	Colony area (cm^2)	Protoplast yield ($\times 10^6$/ml)	Protoplast yield per unit area ($\times 10^5$/ml)
3	8.49	3.70	4.35
4	10.20	5.65	5.53
5	17.61	13.86	7.87
6	20.91	26.15	12.50
7	33.95	12.25	3.60

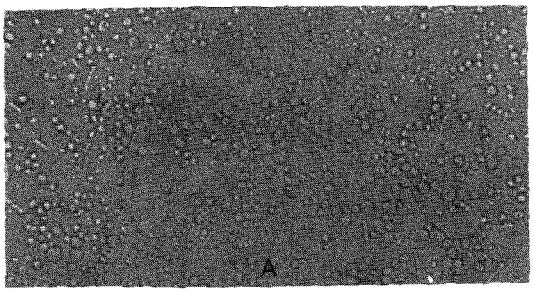

Fig.3. Suspension of harvested protoplasts from mycelium of *L. ulmarium* after treatment with lytic enzyme(A).

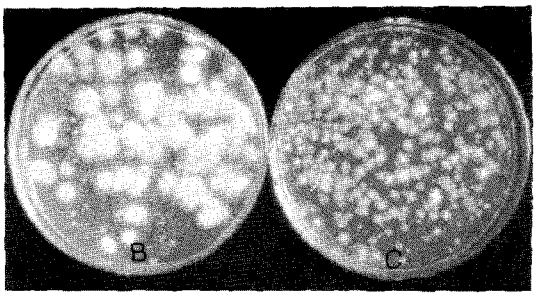

Fig.4. Reversion of protoplasts of *L. ulmarium*. Protoplasts were incubated at 25℃ for 10 days on 0.6 M Mannitol(B) and Sucrose(C) stabilized YG.

was tested for protoplast isolation(Table II). The yield of protoplasts increased considerably with increasing age of mycelium up to 6 days. The crude protoplast preparation obtained from the mycelia of *L. ulmarium* by filtration through sintered glass filters(Fig.3).

Maximum yields of protoplast were obtained from culture in the exponential phase(Peberdy, 1976; Peberdy *et al.*, 1976).

Protoplast Reversion

The effect of various osmotic stabilizers was

Table III. Effect of different osmotic stabilizers on the reversion of protoplasts of *Lyophyllum ulmarium*

Osmotic stabilizer (0.6M)	Percentage of protoplast reverted on YG agar
KCl	0.0002
Mannitol	0.17
Sorbitol	0.11
Sucrose	0.23

tested for their ability on protoplast reversion to the hyphal state. Protoplasts of *L. ulmarium* were able to form colonies at highest frequency on YGA supplemented with 0.6 M sucrose as the osmotic stabilizer(Table III). After 7-14 days of incubation period, bunches of aerial mycelia formed were tough and strong texture(Fig.4).

Hamlym(1982) and Lee *et al.*, (1986b) found that sucrose proved to be effective when incorporated in the regeneration medium and *Pythium* protoplast was inhibited by inorganic salts(Sietsma and De Boer, 1973).

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