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Studies on Protoplast Formation and Regeneration of *Lyophyllum decastes*

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*Lyophyllum decastes*의 원형질체 분리와 재생에 관한 연구

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ABSTRACT: This experiment was carried out to investigate proper conditions for protoplast isolation and regeneration from mycelia of *Lyophyllum decastes*. Novozym 234(10 mg/ml) with 0.6 M $MgSO_4$ in phosphate buffer(pH 4.0) was proper for protoplast isolation. The optimal reaction time of the mycelium with the lytic enzyme was four hours in shaking condition at 120 strokes per min. When the mycelium of *L. decastes* was cultured at 24℃ for 5 days, the formation of protoplasts was effective. The liquid medium was more effective for protoplast isolation than the solid medium. In the liquid medium, high yields of protoplasts were obtained from 0.6 M $MgSO_4$ osmotic stabilizer. Protoplasts of *L. decastes* were regenerated to normal hyphal growth and the regeneration frequency of the protoplasts in the complete agar medium containing Triton X-100(0.0025%) was 5.94~8.32%. The regeneration medium stabilized with 0.6 M sucrose was the best for regeneration of the protoplasts. In contrast to protoplast formation, regeneration was inhibited by the inorganic salts used as osmotic stabilizer.

KEYWORDS: Basidiomycetes, *Lyophyllum decastes*, protoplast isolation, protoplast regeneration, osmotic stabilizers

Introduction

The antitumor activities of polysaccharides of higher fungi have been demonstrated. For example, lentinan from *Lentinula edodes* and PS-K from *Coriolus versicolor* have shown strong antitumor activities against sarcoma 180 and other tumors (Kim *et al.*, 1979; Cho *et al.*, 1988; Hyun *et al.*, 1990; Chihara *et al.*, 1969; Ohno *et al.*, 1975).

On the other hand, several reports on protoplast isolation from yeasts(Eddy *et al.*, 1957), filamentous fungi(Bachmann *et al.*, 1959; Ferrer *et al.*, 1985) and plants(Negrutiu *et al.*, 1984) began to appear. The naked protoplasts were characterized by their osmotic fragility and loss of the rigidity characteristic of the mycelial cells. These protoplast bodies appeared to have the same synthetic abilities as whole cells from which they were derived, including the ability to make a new wall. The regeneration of fungal protopalst is of current interest because of the importance of cell wall formation in morphogenesis. Removing the wall and exposing the protoplast membrane allow for manipulation involving fusion or uptake of nucleic acids, processes that are less achievable or impossible with intact cells.

Since the first protoplast isolation was carried out in *Polyticus versicolor*, many reports have been

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published: *Schizophyllum commune*(De Vries & Wassels, 1972), *Tricholoma matsutake*(Abe *et al.*, 1982), *Phanerochate chrysosporium*(Gold *et al.*, 19 83), *Coprinus macrophizus*(Yanagi & Takebe, 1983), *Pleurotus ostreatus*(Byun, 1984), *P. sajar-caju*(Go, 1985), *Coriolus versicolor*(Bok, *et al.*, 1990). Protoplast reversion or regeneration has been attempted to elucidate the details of wall polymer biogenesis and deposition(Peberdy & Gibson, 1971; Kreger & Kopecka, 1978).

Up to now, interest has been focused on the use of protoplasts as genetic tools. Fusion and transformation systems depend on the avalibility of protoplasts in large numbers and in most cases protoplasts seem to provide the means of isolating DNA from fungal cells for transformation. Protoplast fusion holds great potential as a tool for improvement of industrially important microorganisms(Hamlyn & Ball, 1979). Fusion of fungal protoplasts after treatment with polyethylene glycol (PEG) has been described as a new technique for intraspecific heterokaryon formation at high frequency(Anne & Peberdy, 1975, 1976). In fungi, interspecies protoplast fusions have been studied such as *Aspergillus nidulans* and *A. rugurosus* (Bradshaw *et al.*, 1983), *A. nidulans* and *A. fumigatus*(Ferenczy, *et al.*, 1977), *Mucor pusilus* and *M. miehei*(Onuki *et al.*, 1982), *Penicillum roquefirtii* and *P. chrysogenum*(Anne *et al.*, 1976), *P. chrysigenum* and *P. cyneo-fulvum*(Peberdy *et al.*, 1977), *Ganoderma lucidum* and *G. applanatum*(Park *et al.*, 1988), *P. citrium* and *P. cyano-fulvum*(Anne & Eyssen, 1978).

Lyophyllum decastes belongs to Eumycota(Kingdom), Basidiomycotina(Division), Eubasidiomycetes(Class), Agaricales(Order), Tricholomataceae (Family), *Lyophyllum*(Genus)(Rinaldi & Tyndalo, 1974). Studies on the antitumor activities of protein-bound polysaccharide of *L. decastes* has been already conducted in our laboratory(Kim *et al.*, 1984). The mycelia of *L. decastes* were cultured in artificial media and a new antitumor component which showed potent antitumor activity against sarcoma 180 implanted in mice, was isolated and named "Lyophyllan A"(Lee *et al.*, 1986, 1987, 19 87).

In this work, an attempt was made to examine some of the factors which affect the isolation and regeneration of protoplasts.

Materials and Methods

Fungal Strain

The organism used in this study was a dikaryo-

Table 1. Media Composition

Medium ingredient (g/l)	Medium									
	MMM	MCM	CCM	MCM'	GCM	A	B	LCM	D	PDA
$MgSO_4 \cdot 7H_2O$	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
KH_2PO_4	0.46	0.46	0.46	0.46	0.46	0.46	0.46	0.46	0.46	0.46
K_2HPO_4	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Peptone		2.0	5.0	2.0	4.0	5.0	5.0	2.0	2.0	
Yeast Extract		2.0	5.0	2.0	10	5.0	2.0	5.0	2.0	
Glucose	20	20	20	20	30	50	20	20	50	
PDA										39
Sucrose					20					
Casamino acid					5.0					
Mineral sol'n* (ml/l)				10		10	10	10	10	
Agar	20	20	20	20	20	20	20	20	20	5

*Mineral sol'n (mg/l), $ZnSo_4 \cdot 7H_2O$: 4, $CuSO_4 \cdot 5H_2O$: 1, $MnCl_2 \cdot 4H_2O$: 7, $FeSO_4 \cdot 7H_2O$: 10

tic strain of *Lyophyllum decastes* (Fr.) Singer obtained from Department of Microbial Chemistry, College of Pharmacy, Seoul National University, Seoul, Korea.

Media

The cultural conditions used to produce mycelia for protoplast isolation were described in Table 1. All media were autoclaved at 121℃, 1.2 kg/cm^2 for 15 min. Regeneration media were prepared by adding an osmotic stabilizer to medium which contains $MgSO_4 \cdot 7H_2O$ 0.5 g, KH_2PO_4 0.46 g, K_2HPO_4 1.0 g, peptone 2.0 g, yeast extract 5.0 g, glucose 20 g, mineral soln 10 m*l* and agar 20 g in distilled water of 1000 m*l*(LCM).

Protoplast Formation and Regeneration

1) Enzyme Preparations

Novozym 234(Novo Industry, Denmark), Cellulase-Onozuka R-10(Yakult Honsha, Japan) and β-Glucuronidase(Sigma Chem. Co., U.S.A.) were tested. Each enzyme of 5 to 15 mg was dissolved in 1 m*l* of the osmotic stabilizer solution. The enzyme complex solution containing the osmotic stabilizer was filtered through membrane filter(Gelman Sciences Co. 0.2 μm).

2) Pretreatment of Organism

The mycelia of *Lyophyllum decastes* were incubated with 2-mercaptoethanol for 30 min. They were harvested and washed in distilled water.

3) Osmotic Stabilizers

For the optimal conditions of the protoplast release, 0.4 M to 0.6 M of KCl, $MgSO_4$, NaCl, mannitol and sucrose were tested.

4) Formation of Protoplast

Mycelia to be used for the preparation of protoplast were grown for 6~8 days on LCM plate. The 8-day cultured mycelia were incubated on the cellophane membrane surface for 5 days at 24℃. Also, 20 m*l* of the liquid medium in an Erlenmeyer flask(100 m*l*) were inoculated with the mycelia, and incubated for 5 days without shaking. They were harvested and added with the lytic enzyme solution. The lytic mixture was then incubated at 28.5℃ with gentle shaking(120 strokes/min) for 4 hours. Protoplasts were readily detected microscopically as osmotically sensitive spherical bodies and were separated from the rest of the mycelia debris by filtration with sintered-glass filter(porosity 1).

Protoplast Regeneration

The damp mycelia cultured for 9 days were incubated in 10 m*l* of 0.6 M $MgSO_4$ containing 10 mg/m*l* Novozym 234. After the released protoplasts were filtered, their filtrates were centrifugated at 1,000 rpm for 15 min. Thirteen milliliters of 0.6 M sucrose solution were added to the filtrate containing the protoplasts, and the filtrate was centrifugated. This procedure was repeated once more to obtain the protoplast precipitate. The prepared protoplast suspension was diluted serially to 10^3, 10^4, and 10^5 /m*l* by adding the osmotic stabilizer(0.6 M sucrose). The regeneration medium containing a suitable osmotic stabilizer, Triton X-100 and 2% agar was prepared. Protoplasts of 0.5 m*l* suspended in 0.6 M sucrose solution were plated on the regeneration medium and covered with 5 m*l* of the regeneration medium containing 0.75% agar with a pasteur pipette. The covering agar solution was previously kept at 45 ℃. In order to calculate regeneration frequency, the number of regenerated colonies were counted and the regeneration ratio was calculated.

$$\text{Regeneration ratio}(\%) = \frac{\text{No. of colonies}}{\text{No. of protoplasts}} \times 100$$

Results and Discussion

Mycelial Growth Conditions

The mycelia of *L. decastes* were incubated for 12 days at 24℃. Among 10 media of Table 1, LCM which made fast mycelial growth and formed compact aerial mycelia was optimal(Table 2). The mycelia of *L. decastes* with clamp connections were shown in Fig. 1.

Factors Influencing Protoplasts Formation

1) Effects of Lytic Enzymes

The purified protoplasts of *L. decastes* were osmotically hypersensitive: spherical bodies were

Table 2. Influence of different media on the mycelial growth (24℃)

Medium	Colony radius (cm)			Degree of aerial mycelium
	4 days	8 days	12 days	
MMM	2.0	4.6	6.5**	+*
MCM	1.9	4.5	6.9	++
CCM	1.9	3.8	6.5	+++
MCM	1.7	4.3	6.8	+++
SCM	1.5	2.9	4.4	+++
GCM	1.4	2.9	4.3	+++
A	1.4	2.7	4.0	++
B	2.0	4.4	6.7	++
LCM	2.1	4.8	7.6	+++
D	1.5	2.9	4.1	++
PDA	2.9	6.0	8.0	++

*+++: indicates degree of aerial mycelium
**mean values of three petri-dish

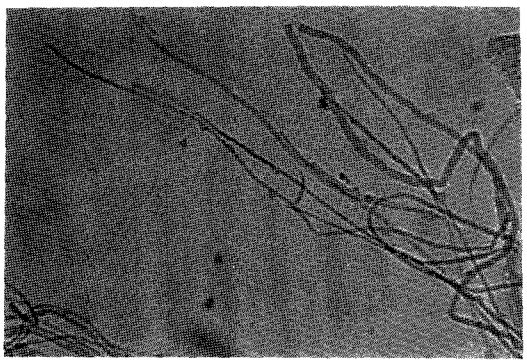

Fig. 1. The mycelia of *L. decastes* with clamp connection (×800).

observed under a microscope(Fig. 2). Several enzymes were tested alone or in various combinations (Fig. 3, Table 3). Novozym 234 was the most effective enzyme with *L. decastes* and combinations of enzymes did not improve on protoplast formation. Commercial preparations of Novozym 234 have been used to produce high yields of protoplasts from several filamentous fungi, and its effectiveness has been compared with that of other commercially available enzymes(Hamlyn *et al.*, 1981). The subsequent work was done only with

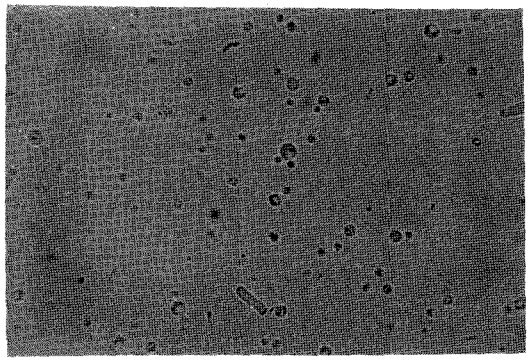

Fig. 2. Protoplasts of varying size released from the hypha of *L. decastes* (×800).

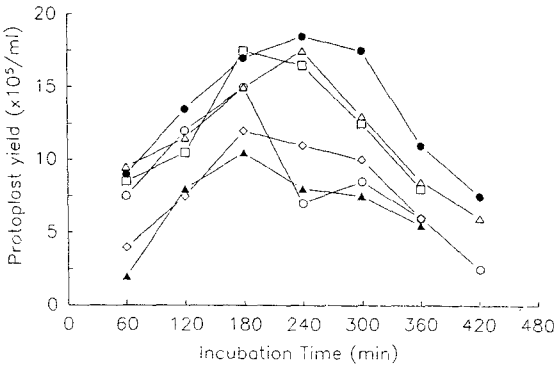

Fig. 3. Effect of enzyme concentration on the protoplast release.

Novozym+Cellulase−Onozuka		
	(mg/ml)	(mg/ml)
○	5	0
●	10	0
△	15	0
▲	10	5
□	10	10
◇	10	15

Table 3. Comparison of different enzyme preparations for the release of protoplasts

Enzyme	Protoplast yield(×10⁵/ml)
Novozym[a]	15.0
Novozym[b]	18.5
Cellulase[b]	0.5
Novozym[b]+Cellulase[b]	17.5
Novozym[b]+β-glucuronidase[a]	7.5
Novozym[b]+β-glucuronidase[b]	8.5

Table 4. Effect of mycelial pretreatment with 2-mercaptoethanol on protoplast release

Concentration(mM)	Protoplast yield($\times 10^5$ /ml)
100	17.5
10	9.0
0	18.5

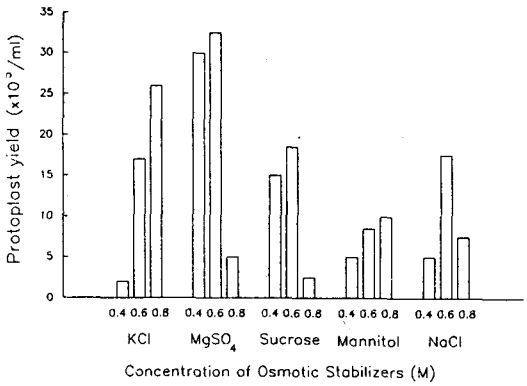

Fig. 4. Effect of different osmotic stabilizers on the formation of protoplasts.

Novozym 234.

Although mechanical and other non-enzymatic methods for protoplast isolation have been reported(Berliner, 1971; Berliner & Reca, 1970), they have not been used extensively. Eddy & Williamson(1957) were the first to introduce the use of *Helix pomatis* digestive juice as lytic enzyme. The isolation of protoplasts from microbial cells involves the total digestion or localized punching of the cell wall by enzymes, allowing the cell contents enclosed by the plasma membrane to escape. The criteria for defining protoplasts were the absence of cell wall and osmotic sensitivity of the spherical cells.

2) Pretreatment of Organism

As shown in Table 4, pre-incubation of the mycelia of *L. decastes* with 2-mercaptoethanol did not affect protoplast formation. Pre-incubation of some filamentous fungi with thiol compounds stimulated protoplast formation(Dookjewaard-Kloosterziel *et al.*, 1973).

3) Effects of Osmotic Stabilizers

An osmotic stabilizer is clearly essential to provide osmotic support to the protoplasts following the removal of the cell wall. Sucrose, mannitol, NaCl, KCl and $MgSO_4$ were examined as osmotic stabilizers for protoplast formation. As shown in Fig. 4, inorganic salts(0.6 M $MgSO_4$) showed a better protective effect than organic stabilizers(sucrose, mannitol). In general, sugars are more suitable for the filamentous fungi and sugars and sugar alcohols for the yeasts.

Table 5. Effect of pH on protoplast release

pH	Protoplast yield($\times 10^5$ /ml)
4.0	165
5.0	30
6.0	7.5

Enzymes were dissolved in 50 mM Na-phosphate buffer. 0.6 M $MgSO_4$ was used for the osmotic stabilizer.

Magnesium sulfate has promoted the release of highly vacuolated protoplasts from *Sch. commune*(De Vries & Wessels, 1972). These protoplasts can be easily isolated from mycelial debris as they float on the supernatant after centrifugation. This magnesium sulfate-induced buoyancy was also found in the protoplasts of *Aspergillus nidulans*.

4) Influence of Incubation Time

Progress of protoplast formation was followed by repeated hemacytometer counts made during incubation. The yield of the released protoplasts reached the maximum after 4 hours of incubation (Fig. 3). The length of incubation time is an important factor that may cause the early-formed protoplasts to degenerate.

5) pH and Buffer Effects

The pH of the incubation medium may affect protoplast yield. The maximum yield of the protoplast was observed at 50 mM Na-phosphated buffer, pH 4.0(Table 5).

6) Influence of the Age of Mycelia

The cells from the stationary or late stationary phase of growth gave a reduced protoplast yield. Indeed, the best protoplast yields were obtained from the 5-day old mycelia of *L. decastes* grown on LCM medium although the older cultures were

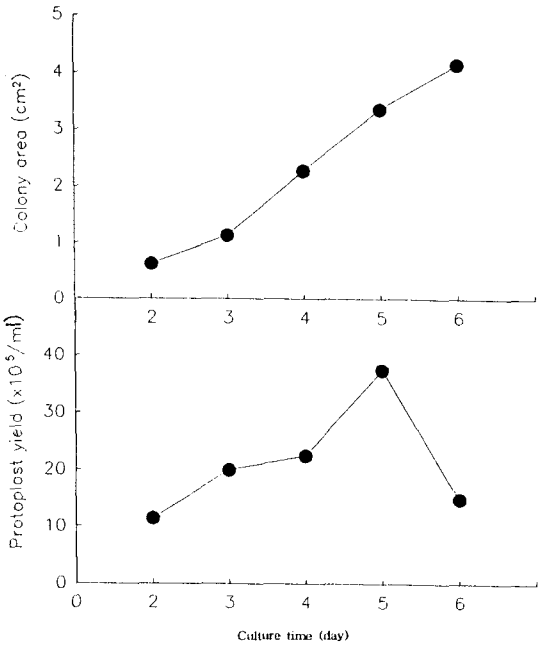

Fig. 5. The yield of protoplasts per unit area of mycelial colony.

still susceptible to lytic enzymes(Fig. 5). The culture age of the mycelia strongly affected the yield of protoplasts. The protoplast yield was much greater from the mycelium in the exponential phase of growth(Peberdy *et al.*, 1976; Anne, 1977). It is not clear why mycelia yield fewer protoplasts in the late exponential growth phase and in the stationary phase. However, it may be due to its composition and ratio change in the cells according to the culture age(Hamlyn *et al.*, 1981).

7) Culture Conditions

As shown in Fig. 6, the liquid medium was more effective for protoplast isolation than the solid medium. In the liquid medium, protoplasts were made by Novozym 234 and 0.6 M $MgSO_4$ without buffer and adjusting pH.

Influence of Osmotic Stabilizers on Regeneration

A protoplast was considered to regenerate when it could grow out to a visible colony on an agar plate. The phenomenon of regeneration serves as a model system for the study of general and special cell processes. The regeneration of protoplasts

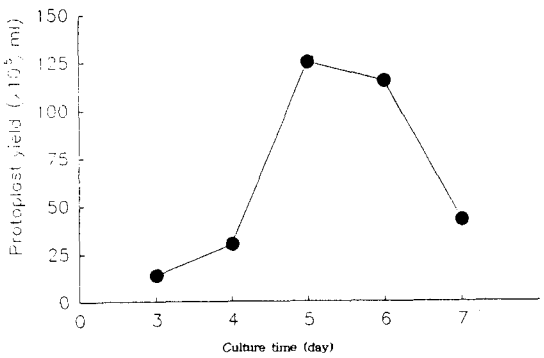

Fig. 6. Production of protoplasts in a liquid complete medium.

Table 6. Effects of osmotic stabilizers on the regeneration frequency of the protoplasts of *L. decastes*

Osmotic stabilizer (0.6 M)*	Regeneration frequency (%)		
	0.0025%	0.005%	0.007%**
Sucrose	8.32	0.20	0
Mannitol	5.94	0.14	0
KCl	0	0	0
NaCl	0	0	0
$MgSO_4$	0	0	0

*Each stabilizer was added into the complete agar medium containing Triton X-100 to give a final concentration of 0.6 M

**Percentage of Triton X-100 used

Table 7. Radial growth rate of *L. decastes* on complete medium containing Triton X-100

Concentration of Triton X-100(%)	Radial growth rate (μm/h)	Relative value of Kr (%)
0	239± 5	100.0
0.005	160± 4	67.3
0.01	113± 4	47.7
0.05	58± 2	25.1

$$Kr = \frac{R_1 - R_0}{t_1 - t_0} \ (\mu m/h)$$

R_1: diameter of colony at time t_1
R_0: diameter of colony at time t_0

is also a prerequisite for all experiments employing somatic hybridization and other genome mani-

pulations in fungal cells.

In the case of the protoplast regeneration of *L. decastes* in the complete medium without Triton X-100 it did not make a visible colony. Therefore, Triton X-100 was used as growth inhibitor. As shown in Table 6, the regeneration frequency of *L. decastes* in the complete agar medium containing Triton X-100(0.0025%) as growth inhibitor was 5.94~8.32%(Table 7). Inorganic salts such as NaCl, $MgSO_4$ and KCl completely inhibited regeneration, whereas organic compounds such as sucrose and mannitol did not inhibit this process. The percentage of protoplasts able to regenerate is very low. The reason for this observation may be due to the absence of nuclei, or the defection of organelles(Garicia *et al.*, 1966).

摘 要

담자균류인 잿빛 만가닥버섯 *Lyophyllum decastes*은 이미 항암작용이 있다고 보고된 바, 이 버섯의 유전연구와 균주개발을 위해 기초적 연구인 원형질체 분리와 재생에 관하여 실험하였다. 원형질체 분리의 최적 조건으로는 여러 효소의 혼용보다 Novozym 234(10 mg/ml)를 단용하였을 때 더 우수하였고, 삼투압 안정제로 0.6 M $MgSO_4$에서 효과적이었으며, 24℃에서 5일 배양한 균사와 효소액을 4시간 반응시켰을 때 수득율이 높았다. 완충용액과 pH는 Na-phosphate buffer(pH 4)에서 원형질체 분리가 양호하였으며, 고체배지보다 액체배지에서 자란 균사를 완충용액과 pH를 조절하지 않은 상태에서 효소와 반응시켰을 때 12.5×10^6 cell/ml로 높았다. 원형질체 재생시 균사가 배지 전면에 확산되어 성장하여 성장억제제로 Triton X-100(0.0025%)을 사용하였다. 원형질체 분리시와는 대조적으로 0.6 M sucrose와 mannitol에서 재생이 효율적이었고, 각각 8.32%와 5.94%의 재생 빈도를 나타내었다.

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