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Studies on Protoplast Formation and Regeneration of *Coriolus versicolor*

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구름버섯의 原形質體 形成과 再生에 관한 研究

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ABSTRACT: To establish basic techniques for protoplast fusion of *Coriolus versicolor* several factors affecting protoplast formation and regeneration were investigated. Protoplast isolation was at maximum with 2.5-day cultured mycelia of *C. versicolor* treated with the combination of two enzymes, Novozym 234 (10 mg/m*l*) and cellulase Onozuka R-10 (15 mg/m*l*), for 3-4.5 hours at 30°C. As an osmotic stabilizer for stabilizing the protoplast, 0.6 M sucrose was the best for formation and regeneration of the protoplast from the mycelia of the fungus and the regeneration frequency was 3.48%. Protoplast fusion was made by a modified method of Peberdy using PEG (M.W. 4,000). The fusion frequency between two mutants of *C. versicolor* was 1.86% and the fusion products showed differences in growth rate and colony morphology.

KEYWORDS: Protoplast formation, Regeneration and fusion, *Coriolus versicolor*, Osmotic stabilizer, Regeneration and Fusion frequency

It has been reported that polysaccharides prepared from several species of wild and cultured basidiomycetes markedly inhibited the growth of transplanted solid tumor of mice (Ito *et al.*, 1974; Kim *et al.*, 1979; Park *et al.*, 1979; Cho *et al.*, 1988; Radner *et al.*, 1958; Maeda *et al.*, 1973; Yoshioko *et al.*, 1972). Of these polysaccharides, the mycelial protein-bound polysaccharide from *Coriolus versicolor*, PS-K (Tsukagoshi *et al.*, 1974), is used clinically in combination with other anticancer drugs. This polysaccharide was most effective with much less toxicity. PS-K is a biological response modifier in that PS-K can induce interferon and augment natural killer (NK) cell activity. Antitumor effects of other polysaccharides have been demonstrated mainly by intraperitoneal injection, and modes of the action have been considered to be host mediated (Nakashima *et al.*, 1979; Zakany *et al.*, 1980) or directly cytocidal.

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 (Cove, 1979; Negrutiu *et al.*, 1984) began to appear. The first report of DNA-mediated transformation of a fungal species was published in 1973 by Tatum (Mishra *et al.*, 1973). The species was *Neurospora crassa,* and the strain transformed was an inositol-requiring mutant (in*l*). These early results were received with some scepticism. However, it was not until fungal transformation was extended to other gene and other species that it became widely accepted as a working technique.

The first breakthrough was accomplished with *Saccharomyces cerevisiae.* Hutchinson and Hartwell (1982) had devised a way of preparing *S. cerevisiae* protoplast (or spheroplast-the names are interchangeable) by dissolving the cell walls with a commercial glucanase preparation and stabilizing the resulting protoplasts

with 1 M sorbitol. More recently, interest in protoplasts has again moved to their usefulness as tools to certain new approaches to the genetic modification of bacteria, fungi and plants. Removing cell wall and exposing protoplast membrane allow for mainpulation involving fusion or uptake of nucleic acid (Ferenczy *et al.*, 1982), whereas these processes are less achievable or impossible with intact cell.

Numerous studies on the isolation of fungal protoplasts were reported; *Polystictus vericolor* (Strunk, 1965), *Trichoderma viride* (Nutsubidze *et al.*, 1988), *Podospora anserina* (Ferrer *et al.*, 1985), *Schizophyllum commune* (De veries *et al.*, 1972), *Coprinus cinereus* (Moore, 1975), *Lentinus edodes* (Ushiyama *et al.*, 1977), *Trichoderma matsutake* (Abe *et al.*, 1982), *Flammulina velutipes* (Yamada *et al.*, 1983), *Pleurotus cornucopiae* (Lee *et al.*, 1986), *P. flolida* (Yoo *et al.*, 1985), *Lyophyllum ulmanium* (Yoo *et al.*, 1987) and *Ganoderma lucidum* (Choi *et al.*, 1987).

Since the first protoplast fusion was carried out in *Geotricum candidium* (Frenczy *et al.*, 1974), many attempts at controlled fusion have been made. The most important development followed the introduction of polyethylene glycol (PEG) as a fusogenic agent (Anne *et al.*, 1976). Current research into electrofusion method was able to avoid the most of disadvantages of the chemically induced fusion procedures (Zimmermann *et al.*, 1981; Tsoneva *et al.*, 1989). In fungi, interspecies protoplast fusion 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), *Penicillium roquefortii* and *P. chrysogenum* (Anne *et al.*, 1979), *P. chrysogenum* and *P. cyaneo-fulvum* (Peberdy *et al.*, 1977) and *Ganoderma lucidum* and *G. applanatum* (Park *et al.*, 1988).

Furthermore, the original protocols have been varied and improved in detail, but have not been fundamentally changed, except for the adoption by some groups of the use of high concentration of lithium ions as a means of rendering cell walls permeable to DNA without forming protoplast (Ito *et al.*, 1983). Costanzo and Fox (Costanzo *et al.*, 1988) have reported sucessful transformation of *Saccharomyces cerevisiae* cells by suspending them in growth medium supplemented with 1 M sorbitol and DNA, adding glass beads, and agitating the mixture at the highest speed of a vortex mixer for 30 seconds.

Recently, PS-K almost completely blocked the cytophathic effect such as giant cell formation and human immunodeficiency virus-specific antigen expression both in MT-4 cells and MOLT-4 cells at a concentration of 0.4 and 0.6 mg/m*l*, respectively (Tochikura *et al.*, 1987). And it inhibited reverse transcriptase *in vitro* (Hirose *et al.*, 1987).

Therefore, protoplast fusion between the different strains may produce a new strain which will show several activities. As the basis to investigate those, attempts were made to determine optimal factors for protoplast formation and regeneration of *Coriolus versicolor*. Furthermore, protoplast fusion between *C. versicolor* mutants (CE-6 and CE-17) was carried out.

Materials and Methods

Fungal strain

Coriolus versicolor (Fr.) Qu'el. (the family Polyporaceae) was provided by Department of Microbial Chemistry, College of Pharmacy, Seoul National University, Seoul Korea.

Media

Among nine media of Table I, SCM which made fast mycelial growth and formed compact aerial mycelia and MMM were used. The pH of this medium was adjusted to 5.5 and autoclaved at 121°C, 1.1 kg/cm^2 for 15 min. Regeneration media were prepared by adding an osmotic stabilizer to SCM and MMM.

Protoplast formation and regeneration

1) Enzyme preparations

Novozym 234 (Novo Industry, Denmark) and cellulase-Onozuka R-10 (Yakult Honsha, Japan) 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 solution. The enzyme complex solution containing the osmotic stabilizer was filtered through 0.2 μm membrane filter (Gelman Sciences Co.). To make the most effective

Table I. Media composition

Medium ingredient (g/l)	Medium						
	MCM	CCM	SCM	GCM	PDA	YG	PDY
$MgSO_4 \cdot 7H_2O$	0.5	0.5	0.5	0.5			0.5
KH_2PO_4	0.46	0.46	0.46	0.46			0.46
K_2HPO_4	1.0	1.0	1.0	1.0			1.0
Peptone	2.0	5.0	2.0	4.0			5.0
Yeast extract	2.0	5.0	15	10		5.0	5.0
Glucose	20	20	50	30		10	30
Sucrose				20			
PDA (Bacto)					39		
Casamino acid				5.0			
Agar	20	20	20	20		20	5.0

release conditions of protoplasts, these commercial enzymes were examined and their appropriate mixed concentrations were tested.

2) Osmotic stabilizers

For the optimal conditions for the protoplast release, 0.6 M of $MgSO_4 \cdot 7H_2O$, KCl, NaCl, mannitol and sucrose were tested.

3) Protoplast isolation

The mycelia of the fungus were grown for 3-4 days in SCM plate prior to inoculation on cellophane membrane in petri-dish (Plate 1). The four-day cultured mycelia were incubated on the cellophane membrane surface for 2.5 days at 30°C. They were harvested and added with the lytic enzyme solution. The lytic mixture was then incubated at 30°C with gentle shaking (120 strokes/min) for five hours. Protoplasts were readily released as osmotically sensitive spherical bodies and collected by filtration with sintered-glass filter (porosity 1) to remove the mycelial debris.

Protoplast regeneration

After the released protoplasts were filtered, their filtrates were centrifugated for 15 min (1,000 rpm) and then washed with 0.6 M sucrose (2 times). The prepared protoplast suspension was diluted serially to 10^3, 10^4, and 10^5/ml by adding the osmotic stabilizer, 0.6 M sucrose. Regeneration medium containing a suitable osmotic substance was prepared prior to plating serially diluted protoplast 0.5 ml and spread it uniformly. Five milliliters of top agar (0.75%, 42°C) was immediately overlayed and mixed with protoplasts. In order to calculate regeneration frequency, the number of regenerated colonies were counted and the regeneration ratio was calculated.

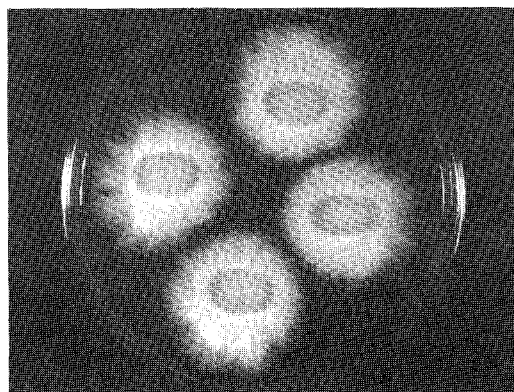

Plate 1. The mycelial morphology of four colonies growing on cellophane containing SCM.

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

Survival ratio after UV irradiation

To prepare mycelial fragments, mycelia were harvested at logarithmic phase and homogenized in distilled water (1,000 rpm for 10 min) to produce a homogeneous suspension of mycelial fragments. An aliquot (15 ml) of the

suspension in a petridish was exposed to UV irradiation for an appropriate period, with constant agitation, to give a survival of 0.3-15%. UV lamp (emission = 39 erg/sec cm^2) was activated for 30 min before irradiation at the distance of 10 cm. After the treatment, several dilution of mutagenized suspension were plated onto SCM agar media colonies were counted.

$$\text{Survival ratio (\%)} = \frac{\text{No. of irradiated colonies}}{\text{No. of unirradiated colonies}} \times 100$$

Fusion of protoplasts and selection of fusion products

Protoplasts isolated from mutant strains were fused by using polyethylene glycol (PEG), mol. wt. 4,000 (Sigma), in the similar method to that described by Anne and Peberdy (1976) and Ferenczy *et al.* (1974). The protoplasts were separated from the mycelial debris by filtration with sintered-glass filter (porosity 1) and washed twice in 0.6 M sucrose. An equal number of protoplasts of each strain (about 10^7/m*l* of each) was mixed and centrifugated (2,000 rpm for 10 min). The pellet was then resuspended in 1 m*l* of 30% (w/v) PEG containing 0.01 M $CaCl_2$ and 0.05 M glycine (adjusted to pH 8.0 with NaOH) and incubated at 30°C for 10 min. Finally the suspension was diluted with 5 m*l* of 0.6 M sucrose and then resuspended in 5 m*l* of 0.6 M sucrose.

The PEG-treated protoplast suspension was plated by 0.5 m*l* onto the hypertonic medium with 0.75% top agar medium. The plates were incubated for 2-3 weeks at 30°C. Those plates with well separated colonies were selected. The colonies were picked off with a coocktail stick, transferred to a fresh medium lacking the osmotic stabilizer and incubated further for 6-25 days. Finally, a representative single colony isolated from each plate was studied independently for further analysis.

To examine backmutation of the auxotrophic markers, the control plates were inoculated with protoplasts of the parental strains. The standard scheme for carrying out protoplast fusion was shown in Fig. 1.

The fusion frequency is defined as ratio of colonies growth on minimal medium and complete medium.

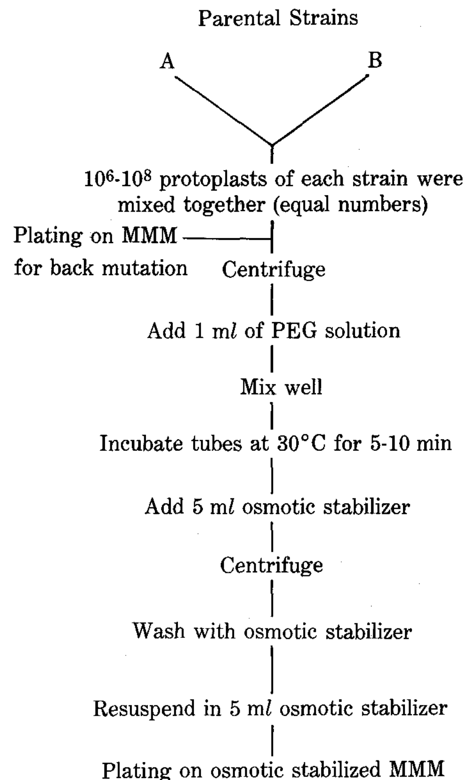

Fig. 1. Standard scheme for carrying out protoplast fusion.

Electrophoretic patterns of esterase isozymes

1) Culture

Wild types, parental mutants and fusants, grown on SCM liquid media for two weeks, were harvested and freeze-dried at −70°C. They were finely ground with mortar and the same volume of the 0.1 M Tris-HCl buffer (pH 7.5) as those of the ground mycelia was added to them. The mixtures were centrifugated at 12,000g for 30 min and then the supernatants were as samples.

2) Electrophoretic system

A high pH discontinuous system using non-denaturing polyacrylamide slab gel (T = 10%, C = 5%) was employed. Fifty to two-hundred microliters of the samples were applied and electrophoresised for 7-9 hours.

3) Staining

The treated gel was stained as described in Sigma diagenostics on α-naphthyl acetate esterase (Procedure No. 90). After fixed with

Table II. The mycelial growth of *C. versicolor* in the different media

Medium	Colony diameter (cm)		Degree of aerial mycelium
	3 days	6 days	
MCM	2.05	3.90	+ +
CCM	1.90	3.95	+ + +
SCM	2.25	4.55	+ + + + +
GCM	2.05	4.05	+ + + + +
PDA	2.65	5.35	+ +
YG	2.15	4.95	+
PDY	2.15	4.30	+ + +

+ + + +: indicates degree of aerial mycelium

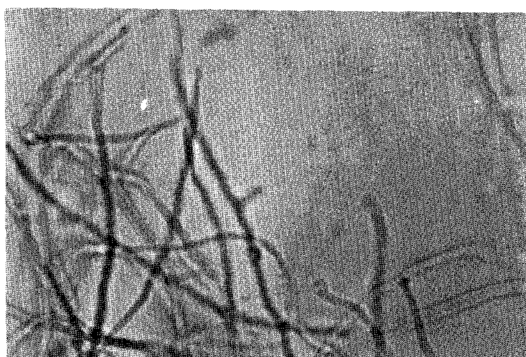

Fig. 2. The mycelia of *C. versicolor* with clamp connection (×800).

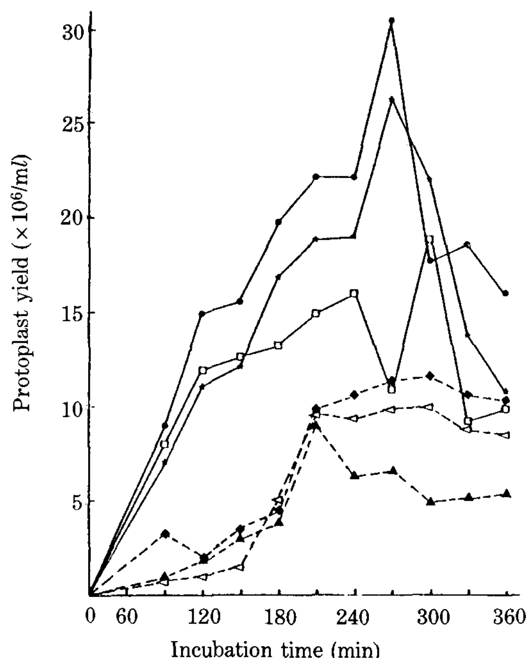

Fig. 3. Influence of enzyme concentrations on the formation of protoplasts.

	cellulase-Onozuka (mg/ml)	+ Novozym (mg/ml)
▲ :	0	5
◆ :	0	10
◁ :	0	15
□ :	5	10
★ :	10	10
● :	15	10

citrate-acetone-methanol fixative containing 0.0383 M citrate buffer (pH 5.4) 18 ml, ACS grade acetone 27 ml and absolute methanol 5 ml, the slide was placed in the staining solution containing α-naphthyl acetate 20 mg, ethylene glycol monoethyl ether 2 ml, fast blue RR salt (Sigma) and 20 mM trizmal buffer (pH 7.6) 50 ml for 30 min at 37°C until the bands appeared evidently.

Results and Discussion

Conditions of mycelial culture

The mycelia of *C. versicolor* were aseptically transferred into eight media including SCM and cultured for 6 days at 30°C±1. Compared with other media in the colony diameter and the degree of aerial mycelia, SCM was the best. GCM also produced compact mycelial growth was compact as much as that of SCM (Table II). And the morphology of the mycelia was shown in Fig. 2.

Various factors on protoplasts foramtion

1) Effect of lytic enzymes

The lytic enzyme was one of the major factors for protoplast formation. The effectiveness of two different commercial enzymes in releasing protoplasts from *C. versicolor* was shown in Fig. 3. The combination Novozym 234 (10 mg/ml) and cellulase Onozuka R-10 (15 mg/ml) was the most effective. Protoplasts of *C. versicolor* was shown in Fig. 4. A combination of the enzymes, Novozym 234 and Cellulase CP gave the best results with *Aspergillus nidulans* (Peberdy, 1976). Novozym 234 was the most effective enzyme with *Penicillium*

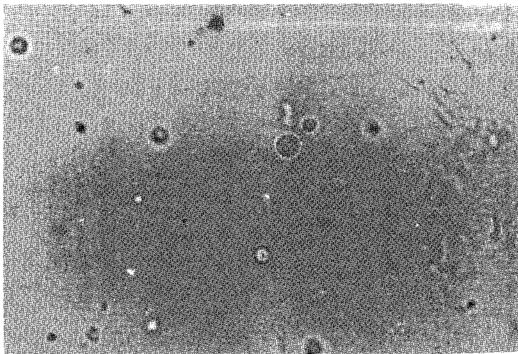

Fig. 4. Protoplasts of varying sizes released from the hypha of *C. versicolor* (×800).

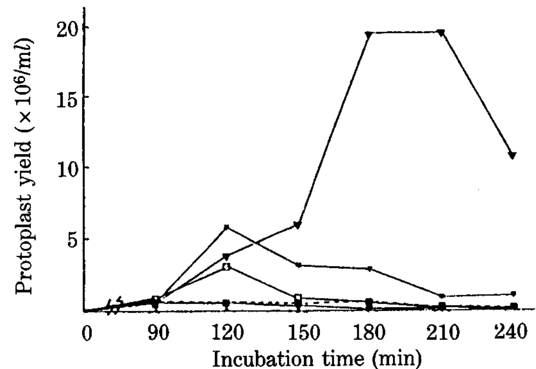

Fig. 5. Comparison of protoplast yields when using different osmotic stabilizers.
□: $MgSO_4 \cdot 7H_2O$, ▼: Sucrose, ★: Mannitol, ●: KCl, ■: NaCl

chrysogenum and *Volvariella volvaceae*. Generally, Novozym 234 was effective in most of fungi because it may be combination of different enzymes (Hamlyn *et al.*, 1981). Some enzymes have been used by many other workers with several strains. Various Actinomycetes produce enzymes that are lytic against yeasts but these are not commercially available (Gascon *et al.*, 1965).

2) Influence of osmotic stabilizers

An osmotic stabilizer is clearly essential to provide osmotic support for maintaining the integrity of protoplasts during their release and for subsequent studies. The large ranges of inorganic salts, sugars and sugars alcohols have been successfully used and have proved more effective for filamentous fungi, and sugars or sugar alcohols were more effective for yeasts. However, the virtues of particular stabilizers were understood only in a empirical sense and differences in effectiveness must relate to as yet unknown factors in the uptake and utilization of the particular compounds.

Five 0.6 M osmotic stabilizers (sucrose, mannitol, KCl, NaCl and $MgSO_4$) were tested for protoplast formation from the mycelia of *C. versicolor* (Fig. 5). Among them, 0.6 M sucrose solution was found to be the best osmotic stabilizer and used for subsequent experiments. The results agreed with those of Rodriguez-Aquirre *et al.* (1964) and Buckley (1976). But they were different from the results that inorganic salts proved to be more effective stabilizers than sugars (Buckley *et al.*, 1976). For 0.6 M $MgSO_4$ and NaCl, it was observed that the mycelia became extensively fragmented in the early period of lytic digestion. Exclusively, a large protoplast released had large vacuoles in 0.6 M $MgSO_4$ and large vacuoles offered buoyancy when they were centrifugated.

3) Influence of incubation time

After the treatment of the enzyme solutions, the protoplasts began to appear. The number of protoplasts was checked every 30 min. The yield of the released protoplasts reached the maximum after 4.5 hours in case of 2.5 day-cultured mycelia and 6.5 hours in time of 3.5 day-cultured mycelia and then decreased gradually. In case of 2.5 day-cultured mycelia, more than 70% of the total protoplast yields were obtained within 3.5 hours of incubation with the lytic enzyme (Fig. 3).

4) Influence of the age of mycelium

The age of the mycelium used for the preparation of protoplasts also influenced protoplast release. Experiments with many other filamentous fungi showed that maximum number of protoplasts was obtained from the cultures in the exponential phase of growth (Buckley *et al.*, 1976; Issac *et al.*, 1978) and protoplasts were greatly reduced as mycelia were old. As it was, the best protoplast yields were obtained from 2.5- and 3-day old mycelia of *C. versicolor* grown on SCM medium although the older cultures were still susceptible to lytic enzymes (Fig. 6). The significance of mycelial age for induction

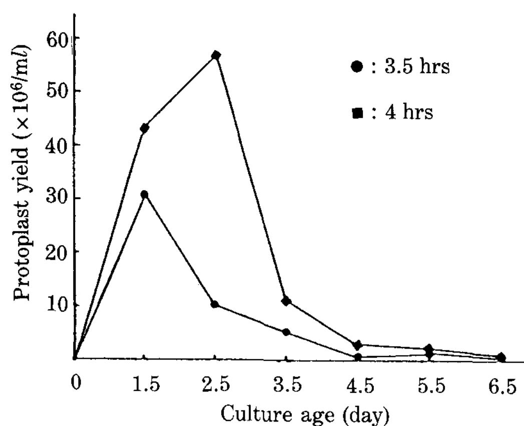

Fig. 6. Effects of the culture age on the yield of the protoplast per unit area of the mycelial colony of *C. versicolor*.

Table III. Effects of osmotic stabilizer on the regeneration of the protoplasts of *C. versicolor*

Stabilizer*	Regeneration frequency
0.6 M Sucrose	3.48 ± 0.34**
0.6 M KCl	0.41 ± 0.09
0.6 M NaCl	0.53 ± 0.01
0.6 M Mannitol	2.86 ± 0.74

*: Each stabilizer was added into the minimal agar medium to give a final concentration of 0.6 M.
**: Mean ± standard deviation

of protoplasts was not known clearly; however, it may be due to its composition and ratio change in the cell wall depending on the culture age (Hamlyn *et al.*, 1981).

Influence of osmotic stabilizers on regeneration

The process of wall regeneration and subsequent reversion has been the aspect of fungal protoplasts most extensively studied. It has been investigated with respect to the mechanism of wall polymer biogenesis and deposition in an attempt to gain an understanding of the processes in the intact cell. The developmental aspects of protoplast reversion are also of interest, providing a model system for investigation into the basis for changes in shape and the possible role of the cell wall.

The protoplast regeneration frequency was affected by various osmotic substances which were added into the regeneration media for protection against the osmotic pressure of the protoplast during regeneration. As shown in Table III, among the osmotic stabilizers tested, 0.6 M sucrose and 0.6 M mannitol showed 3.5% and 2.9% regeneration frequencies, respectively. However, the regeneration media containing 0.6 M $MgSO_4$ did not harden easily because of its acidity and precipitation with agar.

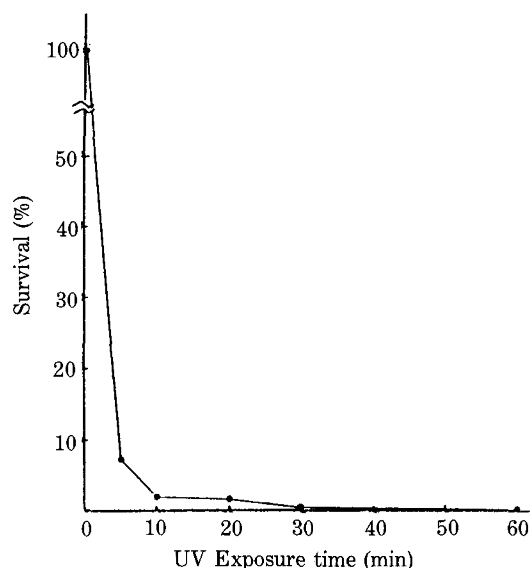

Fig. 7. Survival curves of the mycelia of *C. versicolor* after UV irradiation.

Survival ratio after UV irradiation

Repair mechanism plays such a crucial role in UV mutagenesis of prokaryotes that much effort has been spent on finding UV- sensitive strains of eucaryotic microoragnisms and testing their mutational behavior. The results are usually interpreted in analogy with Witkin's models of error-prone and error-proof repair, but so far there has been no direct evidence for these mechanisms in eukaryotes. But UV irradiation was the mutagenic agent of choice for fungi. After UV irradiation, 278 colonies survived and survival ratio of 5 min UV irradiation was only 7.4% (Fig. 7). And a list of mutants of *C. versicolor* is shown in Table IV.

Protoplast fusion

The overall process involved the agregation of protoplasts in the presence of PEG. It was assumed that fusion occurs after the PEG had

Table IV. List of auxotrophic mutants of *C. versicolor*

Mutant number	Genetic marker	Mutagen	Exposure time (min)	Morphology
10	PABA	UV	10	Dark brown circle
22	PABA, Glu	UV	10	Dark brown circle
CE-3	PABA, Asc	UV-EtBr	60	Release brown pigment
CE-6	Glu	UV-EtBr	20	Release brown pigment
CE-17	Arg	UV-EtBr	30	Very thin mycelia
CE-30	Glu	UV-EtBr	10	Very thin mycelia
CE-33	Glu, Folic acid	UV-EtBr	10	Irregular growth

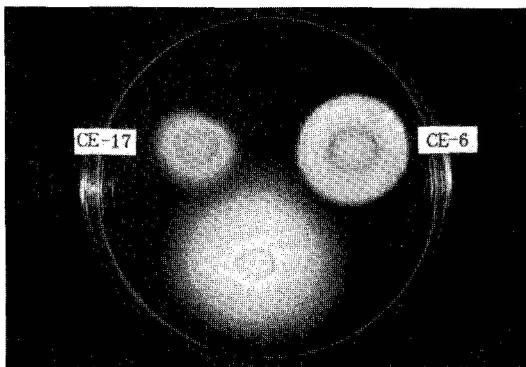

Plate 2. The mycelial morphology of the wild strain, and auxotrophs CE-6 and CE-17.

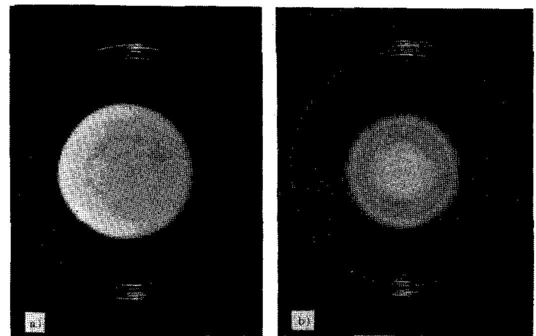

Plate 3. The mycelial morphology of protoplast fusants on SCM.
a) Fusant No. 5 of CE-6+CE-17
b) Fusant No. 14 of CE-6+CE-17

been diluted or washed away. The mechanism of fusion has been also studied in detail (Ankong *et al.*, 1975; Cullies *et al.*, 1978). Protoplast fusion of auxotrophs CE-6 and CE-17 was carried out at 30°C for 10 min with PEG 4,000. The fusion frequency between CE-6 and CE-17 was 1.86% and reversion frequency of CE-6 and CE-17 was 6×10^{-3}% and 9.78×10^{-4}%, respectively. The reversion frequency was calculated as follows:

$$\text{Reversion frequency} = \frac{\text{No. of colonies formed in MMM}}{\text{No. of protoplasts of mutants}} \times 100$$

Characterization of fusants

After 7-20 day culture on hypertonic MMM and SCM, the fusion products showed sectors of densely growing mycelia. When transferred to the MMM plates, the sectors gave normal mycelia. The dense colony might be attributed to the presence of the osmotic stabilizer in the mycelia. The fusion products were different from the parental colony forms (Plate 3). Differences were detected in the cultural morphology (Plate 2) and growth rate (Fig. 8 and Table V). For the morphology, the fusants showed more compact of fast growing mycelia than the parents and grew uniformly during the successive transfers on SCM. However, a limited number of fusion products gave rise to parental segregation when transferred to SCM medium from the fusion medium. The growth rate of CE-6 and CE-17 was 1.64 and 2.3 mm/hr when compared with that of the wild type. They appear to be petite mutants. Further investigation on the genetic properties of the fusion products is required.

The fusants displayed mixed profiles or to-

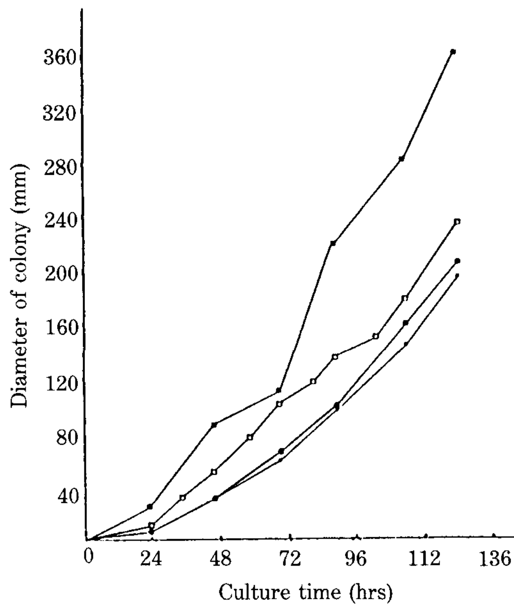

Fig. 8. Growth curves of the parents and fusant of *C. versicolor*.
■: Wild type, □: CE-6, ★: CE-17, ●: Fusant No. 2 (CE-6+CE-17)

Table V. Radial growth rate of the parents and fusant of *C. versicolor*

Strain	Radial growth rate* (mm/hr)	Relative value of Kr1**	Relative value of Kr2
CE-6	1.89	1	–
CE-17	1.35	–	1
Fusant No.2 (CE-6+CE-17)	1.63	0.86	1.20

*Radial growth rate $=\frac{dl}{dt}$ { t: log phase time, l: colony diameter (mm) }
**Kr1: Relative growth ratio of fusants against parent strain CE-6
Kr2: Relative growth ratio of fusants against parent strain CE-17

tally novel ones resulting from altered assemblies of the protein subunits involved in the esterase structure (Fig. 9). The isozyme components of the mycelial extracts were a potentially useful tool for assessing the interaction of different genomes in fusant. This was particularly so where the known genotypic differences between the species were few.

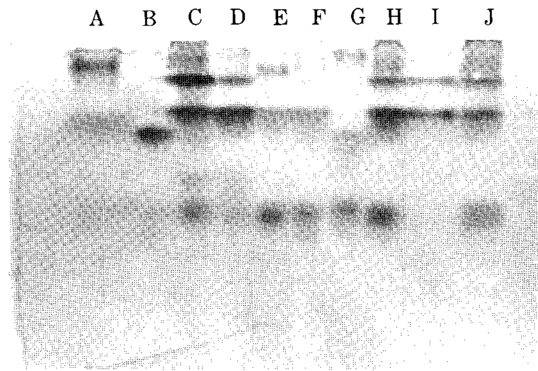

Fig. 9. The electrophoretic patterns of esterase isozymes of protoplast fusants on non-denaturing polyacrylamide gel (T=10%, C=5%).
Lane A: *C. versicolor* Wild
B: CE-17
C: CE-6
D: Fusant No. 7 of CE-6+CE-17
E: Fusant No. 30 of CE-6+CE-17
F: Fusant No. 2 of CE-6+CE-17
G: Fusant No. 3 of CE-6+CE-17
H: Fusant No. 14 of CE-6+CE-17
I: Fusant No. 25 of CE-6+CE-17
J: Fusant No. 16 of CE-6+CE-17

Conclusion

Several factors affecting protoplast formation, regeneration and fusion of *Coriolus versicolor* were examined and characteristics of the fusants were determined. Among the several media for the mycelial growth, SCM showed the best growth. When several enzymes and their combinations for removing the cell wall of the mycelia were compared, a combination of Novozym 234 and cellulase Onozuka R-10 yielded the highest numbers (3.14×10^{7} cells/m*l*) of the protoplasts. As osmotic stabilizer, 0.6 M sucrose was the best choice for the formation and regeneration of the protoplast from the mycelia. The regeneration frequency of the protoplast on SCM containing 0.6 M sucrose and 0.6 M mannitol was 3.5% and 2.9%, respectively. Survival ratio of the mycelia after UV-irradiation was 0.02-7.4%. Reversion frequencies of auxotrophs CE-6 and CE-17 were 6×10^{-3}% and 9.78×10^{-4}%, respectively. Protoplast fusion was made by a modified method of Peberdy and fusion frequency of CE-6 and CE-17 was 1.86%.

The fusants were examined for cultural morphology and growth rate. For the morphology, the fusants showed more compact mycelia than the parents and grew uniformly during the successive transfers on SCM. However, the fusant No. 15 of CE-6 and CE-17 gave rise to segregation. The growth rates of CE-6 and CE-17 were 1.64 and 2.3 mm/hr when compared with the wild type. It appears that the auxotrophs CE-6 and CE-17 were petite mutants.

摘 要

구름버섯은 항종양작용이 인정되었으며 근자에는 AIDS virus에 대한 억제효과가 보고되고 있다. 이와 같은 약효를 인정받고 있는 구름버섯 균주간의 세포융합이나 구름버섯과 타 유효한 버섯과 세포융합을 시행함으로써 더욱 효능이 우수하거나 다양한 균주의 약효를 한 균주내에서 기대할 수 있는 새로운 균주의 개발이 가능하다. 이러한 목적으로 구름버섯의 원형질체융합을 시행하기 위하여, 원형질체분리, 재생 및 두 영양요구주의 융합에 관하여 실험하였다. 균사체를 2.5일간 셀로판지위에서 배양하여 Novozym 234와 cellulase Onozuka R-10이 각각 15 mg/ml와 10 mg/ml 포함되어 있는 0.6 M sucrose 용액에 넣어 30℃에서 3-4.5시간 반응시킬 때 원형질체 수득률이 가장 높았다. 삼투압 안정제로 0.6 M sucrose는 원형질체 형성과 재생에서 최적조건이었다. 0.6 M sucrose를 포함하는 고체배지에서의 재생빈도는 3.48%이었으며 UV 조사시 생존률은 0.02-7.4%이었다. 원형질체융합은 30℃에서 10분 동안 polyethylene glycol(M.W. 4,000)을 이용하여 시행하였는데 그 융합빈도는 1.86%이었다.

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