林試研報 Bull. For. & For. Prod. Res. Inst. No. 343, 1987 155～170

Preparation and Culture of Protoplasts of some Japanese Cultivated Mushrooms

By

Masatake OHMASA[1], Yasuhisa ABE[2], Hisahiko FURUKAWA[3]
Minoru TANIGUCHI[4] and Hitoshi NEDA[5]

Summary : Protoplasts were prepared from mycelia of 12 mushrooms, *Lentinus edodes*, *Pleurotus ostreatus*, *Flammulina velutipes*, *Pholiota nameko*, *Grifola frondosa*, *Tricholoma matsutake* and others. Mycelia of these mushrooms were cultured in liquid media, collected by filtration and treated with mixtures of commercial mycolytic enzymes. Among combinations of enzymes, enzyme systems containing a) Cellulase "Onozuka" RS and β-Glucronidase, b) Cellulase "Onozuka" RS, Driselase and β-Glucronidase and c) Cellulase "Onozuka" RS, Driselase and Zymolyase 5000 were effective for protoplast formation. Dependence of the efficiency of protoplast formation on the culture period was examined for *Lentinus edodes*, *Pholiota nameko*, *Grifola frondosa* and *Auricularia polytricha*.

The efficiency was also found to be dependent on the strain of the same mushroom. In the course of the study, the enzyme system containing Novozym 234 and chitinase was found to be highly efficient for protoplast formation from mycelia of mushrooms of the genus *Pleurotus*. Using the strain, FMC 235, of *Pleurotus ostreatus*, the authors studied conditions for protoplast formation using this enzyme system, these are, pH of the enzyme solution, effect of osmotic stabilizers, time course of protoplast formation and the effect of media used for mycelial culture. Protoplast of *Lentinus edodes* and *Pleurotus ostreatus* could be cultured to form mycelia, and culture conditions were further studied. In the course of the study, the authors found that by starting from dikaryotic mycelia of *Pleurotus ostreatus*, they could get monokaryotic mycelia as well as dikaryotic mycelia after formation of protoplast from dikaryotic mycelia and culture. The authors conclude that this is a simple method of monokaryotization of dikaryotic mycelia.

Introduction

Protoplast is a cell whose envelope lacks a cell wall and is composed only of plasma membrane. Protoplasts have been used in cell fusion of plants and fungi for their breeding[1)2)]. In addition to cell fusion, protoplasts are useful in such aspects of genetics and breeding of microorganisms as transformation[3)] and artificial mutation[4)5)]. Protoplasts are becoming more and more important also in studies of physiology of fungi as well as in genetics and breeding[6)].

In recent years protoplasts have also been studied for mushrooms[7)9)]. But for main cultivated mushrooms in Japan such as *Lentinus edodes* (Berk.) Sing., *Pholiota nameko* (T. Ito) S. Ito et Imai, *Grifola frondosa* S. F. Gray and *Pleurotus ostreatus* (Fr.) Quél., protoplasts have not been studied sufficiently. In this report we studied formation of protoplasts from mycelia of 12 edible mushrooms including main cultivated mushrooms mentioned above, and culture of *Pleurotus ostreatus* and *Lentinus edodes*. A part of this paper was presented elsewhere[10)].

Received August. 9, 1986 保 護—47 Forest Protection—47
(1) (2) (3) (4) (5) Forest Protection Division

Materials and Methods

Origanisms

Strains of 12 mushrooms, that is *Lentinus edodes, Pleurotus ostreatus, Flammulina velutipes* (Fr.) Sing., *Pholiota nameko, Grifola frondosa, Pleurotus cornucopiae* (Pers.) Rolland, *Hypsizygus marmoreus* (PK.) Bigelow, *Tricholoma matsutake* Ito et Imai, *Auricularia polytricha* (Mont.) Sacc., *Agaricus bisporus* (Lange) Sing., *Pleurotus salmoneo-stramineus* Vassilieva, *Pleurotus cystidiosus* O. K. Miller, and two *Pleurotus* spp. were used. All the strains were subcultured from stock cultures of Section of Mushroom of this institute (strains with FMC number) and were maintained on potato-dextrose-agar (PDA) medium. Monokaryotic mycelia obtained by single spore isolation from fruiting bodies of these stock cultures were also used.

Prepararion of protoplasts from mycelia

Forty ml of a liquid media in a 100 m*l* Erlenmeyer flask containing sucrose (1%), malt extracts (1%) and yeast extracts (0.4%) (SMY medium) were inoculated with the mushroom mycelia used in the experiment. After culturing at 25°C for 10—20 days depending on the kind of mushroom, mycelia were fragmented in a homogenizer (Nippon Seiki Co., Ltd.) in SMY medium at 12000 rpm for the time depending the kind of mushrooms. Except for the examination of media, 5 m*l* of the medium containing the fragmented mycelia were re-inoculated to 40 m*l* of fresh SMY medium and cultured for 2—7 days at 25°C. Mycelia were filtered by a nylonmesh and washed with a buffer solution containing 0.5 M mannitol, 0.05 M maleic acid-NaOH, pH 5.5 (buffer A).

Mycelia were resuspended in 3 m*l* of buffer A containing various combinations of enzymes listed in Table 1, and incubated in a water bath at 25°C—30°C with reciprocal shaking (60 revolutions/min.). After incubation, remaining mycelia were removed by filtration through Mylacloth (Calbiochem. Co., Ltd.). For tests of preparation of protoplasts, a number of protoplasts present in the filtered solution were counted by a haemocytometer (Thoma

Table 1. Mycolytic enzymes used in this study.

Enzyme	Source	Supplier/Manufacturer
Cellulase "Onozuka" R-10*	*Trichoderma viride*	Yakult Pharmaceutical Indust. Co. Ltd.
Cellulase "Onozuka" RS*	*Trichoderma viride*	Yakult Pharmaceutical Indust. Co. Ltd.
Chitinase	*Streptomyces griseus*	Sigma Chemical Co.
Driselase	*Irpex lacteus*	Kyowa Hakko Kogyo Co. Ltd.
β-Glucronidase	*Helix pomatia*	Sigma Chemical Co.
Macerozyme R-10	*Rhizopus* sp.	Yakult Pharmaceutical Indust. Co. Ltd.
Novozym 234	*Trichoderma harzianum*	Novo Industri A/S Enzyme Division
Zymolyase 5000	*Arthrobacter luteus*	Kirin Breweries Co. Ltd.

* Cellulase "Onozuka" R-10 and Cellulase "Onozuka" RS are abbreviated as "Cellulase R-10" and "Cellulase RS," respectively, in this paper.

counting chamber). In each test, an average of three replications were shown. For other tests, the filtered solution was centrifuged at 530×g for five minutes and precipitated protoplasts were resuspended in the buffer A.

Protoplasts were purified as follows (when necessary). Protoplasts resuspended in buffer A were layered on the 0.7 M sucrose solution containing 0.05 M maleic acid-NaOH, pH 5,5, in a centrifuge tube and centrifuged at 530×g for five minutes. Protoplasts floating on the upper surface of the sucrose layer were recovered and resuspended in 0.7 M mannitol containing 0.05 M maleic acid-NaOH, pH 5.5.

Culture of protoplasts

Protoplasts were prepared aseptically, washed once with buffer A and serially diluted with the same buffer. The protoplast solution (0.1 m*l*) was mixed with 10 m*l* of SMY or GMY medium containing the osmotic stabilizer, additives, and agar or agarose, and cultured in 9 cm plastic Petri dishes sealed with parafilm, at 25°C. For the test of the effect of osmotic stabilizers, 0.5 M sucrose was replaced with other osmotic stabilizers. For liquid cultures, 0.1 m*l* of protoplast solution was mixed with the SMY or GMY medium containing the osmotic stabilizer and additive but without agar.

Esterase isozymes

Esterase isozymes of mycelia of *Pleurotus ostreatus* cultured in the SMY medium for three weeks were separated and detected as described previously[11].

Enzymes

Enzymes used for the preparation of protoplasts in the present study are listed in Table 1.

Chemicals

Yeast extracts an nutrient broth were obtained from Oxoid Limited Co.. Malt extracts were obtained from Kyokuto Seiyaku Ind. Co., Polypeptone was obtained from Wako Pure Chemical Ind. Ltd., Potato-dextrose-agar was obtained from Nissui Seiyaku Co., Other chemicals were obtained from Wako Pure Chemical Ind. Ltd. or Nakarai Chemicals, Ltd..

Results

1. Isolation of Protoplasts from Mycelia of 12 Mushrooms

Efficiency of protoplast formation from mycelia depended firstly on the combination of enzymes used for preparation. Several combinations of commercial enzymes, except for Novozym 234 and chitinase, listed in Table 1, were tested for the preparation of protoplasts from *Lentinus edodes*, *Pleurotus ostreatus*, *Pholiota nameko* and *Auricularia polytricha*. Results are shown in Tebles 2～5. As shown in these tables, one-enzyme systems were not generally effective. In most cases, mixture of two or more commercial enzymes were effective. Enzyme systems containing a) Cellulase RS and β-Glucronidase or b) Cellulase RS, Driselase and β-Glucronidase or c) Cellulase RS, Driselase and Zymolyase 5000 showed better results. For a combination of enzymes, that is, for one enzyme system, the number of protoplasts formed were dependent on the kind of mushrooms.

The dependence of the number of protoplasts formed from cultured mycelia on different strains of the same mushroom, and on the culture period was tested using four strains each of *Lentinus edodes*, and *Pholiota nameko*, three strains of *Grifola frondosa*, and a strain of *Auricularia polytricha*. Results are shown in Figs. 1～4. The enzyme system used in this

Table 2. Number of protoplasts formed from mycelia of *Lentinus edodes* (Strain FMC 2) using several combinations of enzymes.

Combination of enzymes	Number of protoplasts per g fresh weight
Driselase 2%	0.72×10^6
Cellulase RS 2%	6.81×10^6
Macerozyme R-10 2%	0.11×10^6
Zymolyase 5000 0.2%	0.14×10^6
β-Glucronidase 0.1 ml/ml	0.17×10^6
Cellulase R-10 2%	0.00×10^6
Driselase 2%+Cellulase RS 2%+Zymolyase 5000 0.2%	12.27×10^6
Driselase 2%+Macerozyme R-10 2%+Cellulase R-10 2%	1.55×10^6
Driselase 2%+β-Glucronidase 0.1 ml/ml	0.82×10^6
Cellulase RS 2%+β-Glucronidase 0.1 ml/ml	21.93×10^6
Driselase 2%+Cellulase RS 2%+β-Glucronidase 0.1 ml/ml	25.31×10^6

Incubations were performed at 25°C for 5 hours.

Table 3. Number of protoplasts formed from mycelia of *Pleurotus ostreatus* (Strain FMC 235) using several combinations of enzymes.

Combination of enzymes	Number of protoplasts per g fresh weight
Driselase 1%	0.07×10^6
Cellulase RS 1%	17.43×10^6
Macerozyme R-10 1%	0.94×10^6
Zymolyase 5000 0.1%	0.38×10^6
β-Glucronidase 0.1 ml/2 ml	68.13×10^6
Driselase 2%+Cellulase RS 1%	73.20×10^6
Driselase 2%+Zymolyase 5000 0.1%	25.30×10^6
Driselase 2%+β-Glucronidase 0.1 ml/2 ml	20.03×10^6
Driselase 2%+Cellulase RS 1%+Zymolyase 5000 0.1%	125.53×10^6

Incubations were performed at 25°C for 5 hours.

Table 4. Number of protoplasts formed from mycelia of *Pholiota nameko* (Strain FMC 262) using several combinations of enzymes.

Combination of enzymes	Number of protoplasts per g fresh weight
Driselase 2%	0.83×10^6
Cellulase RS 2%	1.08×10^6
Macerozyme 2%	0.54×10^6
Zymolyase 5000 0.2%	0.71×10^6
Cellulase R-10 2%	0.62×10^6
Driselase 2%+Cellulase RS 2%+Zymolyase 5000 0.2%	6.97×10^6
Driselase 2%+Macellozyme 2%+Cellulase R-10 2%	3.87×10^6
Cellulase RS 2%+β-Glucronidase 0.1 ml/ml	19.30×10^6

Incubations were performed at 25°C for 5 hours.

Table 5. Number of protoplasts formed from mycelia of *Auricularia polytricha* (Strain FMC 356) using several combinations of enzymes.

Combination of enzymes	Number of protoplasts per g fresh weight
Driselase 2%	0.00×10^6
Cellulase RS 2%	2.37×10^6
Cellulase R-10 2%	0.00×10^6
Driselase 2%+Cellulase RS 2%+Zymolyase 5000 0.2%	55.73×10^6
Driselase 2%+Macerozyme 2%+Cellulase R-10 2%	1.30×10^6
Cellulase RS 2%+β-Glucronidase 0.1 ml/ml	395.47×10^6
Cellulase RS 2%+Zymolyase 5000 0.2%	15.13×10^6

Incubations were performed at 25°C for 5 hours.

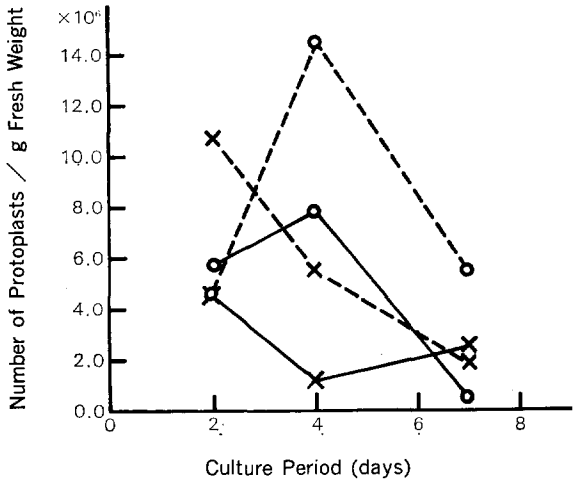

Fig. 1. Effect of culture period on the formation of protoplasts from mycelia of different strains of *Lentinus edodes*. Incubations were performed at 25°C for 5 hours.
FMC 2 : ○——○, FMC 8 : ○- - -○,
FMC 29 : ×- - -×, FMC 45 : ×——×.

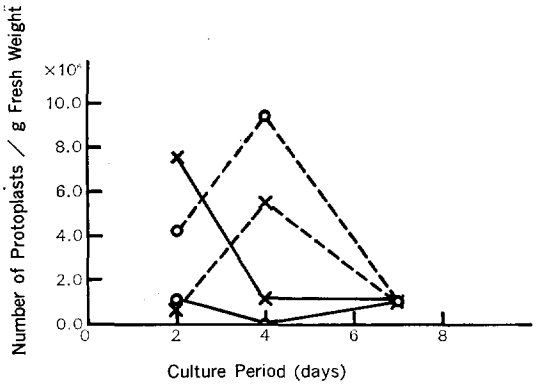

Fig. 2. Effect of culture period on the formation of protoplasts from mycelia of different strains of *Pholiota nameko*.
Incubations were performed at 25°C for 5 hours.
FMC 281:○——○, FMC 282:×- - -×,
FMC 289:○- - -○, FMC 295:×- - -×.

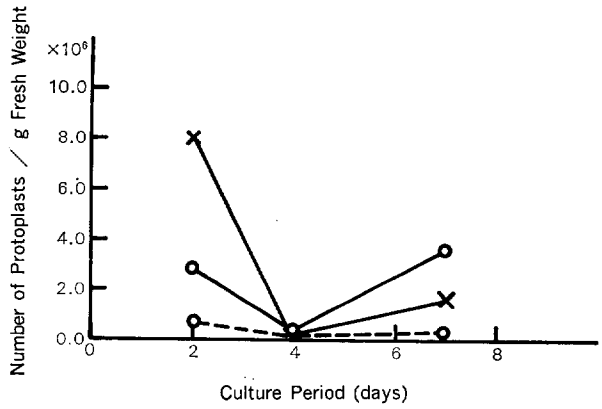

Fig. 3. Effect of culture period on the formation of protoplasts from mycelia of different strains of *Grifola frondosa*.
Incubations were performed at 25°C for 5 hours.
FMC 320:○——○, FMC 321:×---×, FMC 323:○- - -○.

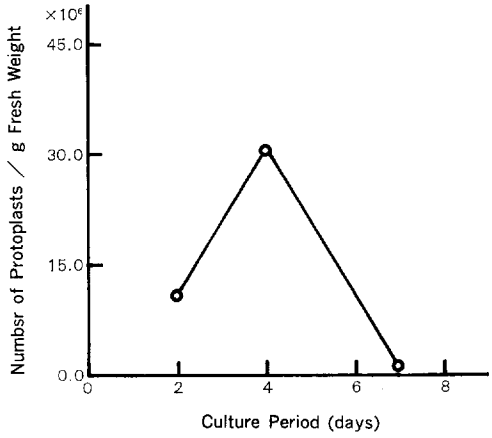

Fig. 4. Effect of culture period on the formation of protoplasts from mycelia of a strain of *Auricularia polytricha*, FMC 356.
Incubations were perfomed at 25°C for 5 hours.

experiment was Cellulase RS, 2%, Macerozyme R-10, 2%, Driselase, 2%, and Zymolyase 5000, 0.2%. As seen in these figures, number of protoplasts is dependent on the strains used for a mushroom. The effect of the culture period was also dependent on the strain. But protoplast formation at 7 days was generally lower than those at 2 days and/or 4 days.

Formation of protoplasts from mycelia was also tested for strains of seven mushrooms using enzyme system 1) Cellulase RS, 2%, Driselase, 2%, Macerozyme R-10, 2%, and Zymolyase 5000, 0.2%, and 2) Cellulase R-10, 2%, Driselase, 2%, Macesozyme, 2%, and Zymolyase 5000, 0.2%. Results are shown in Table 6. In some experiments, formation of protoplasts from mycelia of *Tricholoma matsutake* was ascertained (Fig. 5). We could not find any effective enzyme system for *Agaricus bisporus*.

2. Highly efficient formation of protoplasts from mycelia of *Pleurotus ostreatus*.

In the process of the present study, a cell wall lytic enzyme, Novozym 234, became available. So, authors tested this enzyme as well as chitinase, which was known to degrade chitin, an important component of the cell wall of fungi. Results of the test for the preparation of protoplasts from mycelia of a strain of *Pleurotus ostreatus* (FMC 235) is shown in

Table 6. Number of protoplasts formed from mycelia of several mushrooms using two enzyme systems.

Mushroom and strain	Number of protoplasts per g fresh weight	
	Enzyme system 1)	Enzyme system 2)
Pleurotus ostreatus FMC 235	131×10^6	29×10^6
FMC 236	89×10^6	70×10^6
FMC 237	89×10^6	14×10^6
FMC 238	94×10^6	22×10^6
FMC 239	61×10^6	26×10^6
FMC 240	167×10^6	26×10^6
FMC 241	23×10^6	19×10^6
FMC 242	139×10^6	34×10^6
FMC 243	59×10^6	12×10^6
FMC 244	181×10^6	129×10^6
FMC 245	178×10^6	78×10^6
FMC 246	129×10^6	36×10^6
Pleurotus cystidiosus FMC 256	75×10^6	25×10^6
Pleurotus salmoneo-stramineus FMC 252	38×10^6	23×10^6
Pleurotus sp. FMC 253	53×10^6	16×10^6
Pleurotus cornucopiae FMC 257	16×10^6	6×10^6
Hypsizygus marmoreus FMC 228	159×10^6	68×10^6
FMC 229	70×10^6	42×10^6
FMC 231	23×10^6	4×10^6
FMC 232	165×10^6	64×10^6
FMC 233	107×10^6	48×10^6
Flammulina veltipes FMC 223	78×10^6	45×10^6
FMC 224	78×10^6	56×10^6

Incubations were performed at 25°C for 5 hours.
Enzyme system 1) Cellulase RS 2%, Driselase 2%, Macerozyme R-10 2% and Zymolyase 5000 0,2%
Enzyme system 2) Cellulase R-10 2%, Driselase 2%, Macerozyme R-10 2% and Zymolyase 5000 0.2%

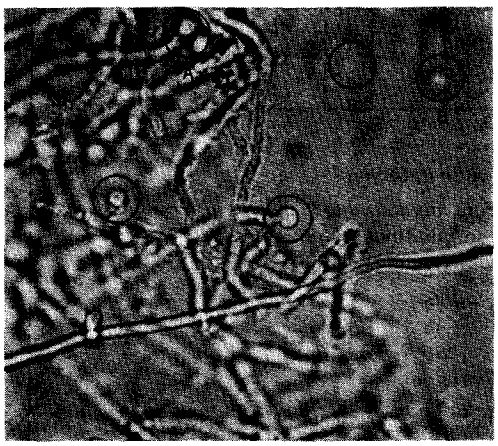

Fig. 5. Formation of protoplasts from mycelia of *Tricholoma matsutake*.
A : protruding protoplasm of mycelia formed in the process of formation of protoplasts.
B : protoplast released from mycelium.

Fig. 6. Enzyme systems containing Novozym 234 showed better results and produced a high number of protoplasts even at 2 hours incubation. Further tests were conducted on enzyme system E (Novozym 234, 1%, chitinase, 0.1%). Protoplasts formed by these enzymes from mycelia of *Pleurotus ostreatus* are shown in Fig. 7.

Because this enzyme system was very effective, authors further studied the various conditions for protoplast formation from mycelia of *Pleurotus ostreatus*. Firstly, pH dependence of protoplast formation was studied. Results are shown in Fig. 8. High yield was obtained between pH 3.5～5.5 and further experiments were performed at pH 5.5. The effect of osmotic stabilizers on the protoplast formation is shown in Fig. 9. In further experiments, authors used mannitol routinely. Fig. 10 shows the time course of protoplast formation for mycelia grown at 30°C and 15°C. Mycelia grown at 30°C produced a higher number of protoplasts in a shorter incubation time than from mycelia grown at 15°C. But mycelia grown at 15°C produced a higher number of protoplasts at 3 hours. The number of protoplasts formed from both mycelia grown at 15°C and those grown at 30°C decreased at 5 hours.

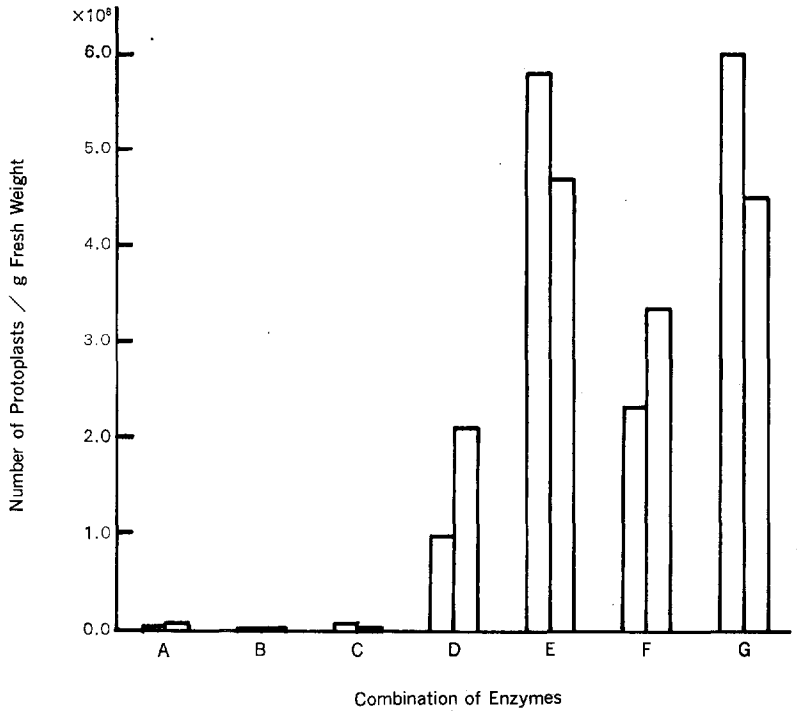

Fig. 6. Formation of protoplasts from mycelia of *Pleurotus ostreatus* (FMC 235) using several combinations of enzymes.
A : Cellulase RS 2%+Driselase 2%+Zymolyase 5000 0.1%.
B : β-Glucronidase 0.1 ml/ml.
C : β-Glucronidase 0.1 ml/ml+Cellulase RS 1%.
D : Novozym 234 1%.
E : Novozym 234 1%+Chitinase 0.1%,
F : Novozym 234 1%+β-Glucronidase 0.05 ml/ml.
G : Novozym 234 1%+Cellulase RS 1%.
For each combination of enzymes, incubations were performed at 30°C for 2 hours (shown by Ieft bar) and 4 hours (shown by right bar).

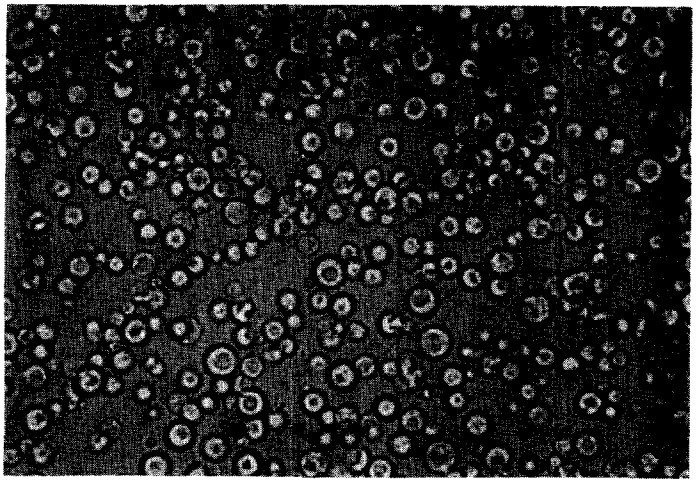

Fig. 7. Protoplasts of *Pleurotus ostreatus* prepared from mycelia using Novozym 234 and chitinase.

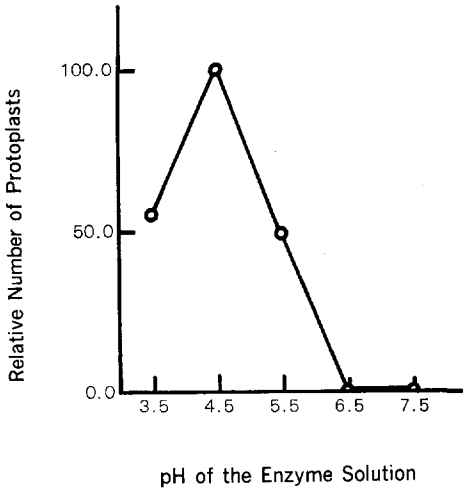

Fig. 8. PH dependance of protoplast formation from mycelia of *Pleurotus ostreatus* (FMC 235). Incubations were performed at 30°C for 2 hours.

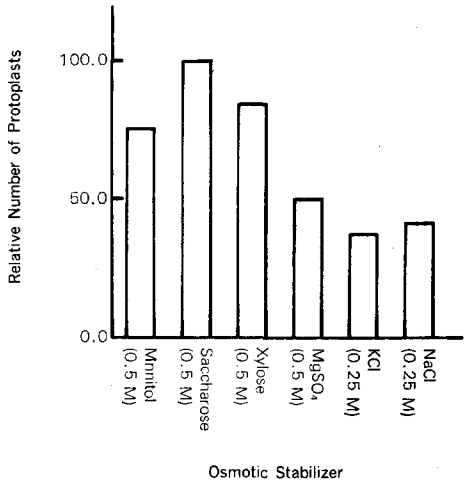

Fig. 9. Effect of osmotic stabilizers on the protoplast formation from mycelia of *Pleurotus ostreatus* (FMC 235). Incubations were performed at 30°C for 2 hours.

Efficiency of protoplast formation was found to be dependent on the medium used for culture of mycelia. Effect of media used for preparation of mycelia is shown in Table 7.

Using the method described here, we could prepare protoplasts very efficiently from mycelia of *Pleurotus ostreatus, P. salmoneo-stramineus, P. cystidiosus* and two *Pleurotus* spp.

3. **Culture of protoplasts**

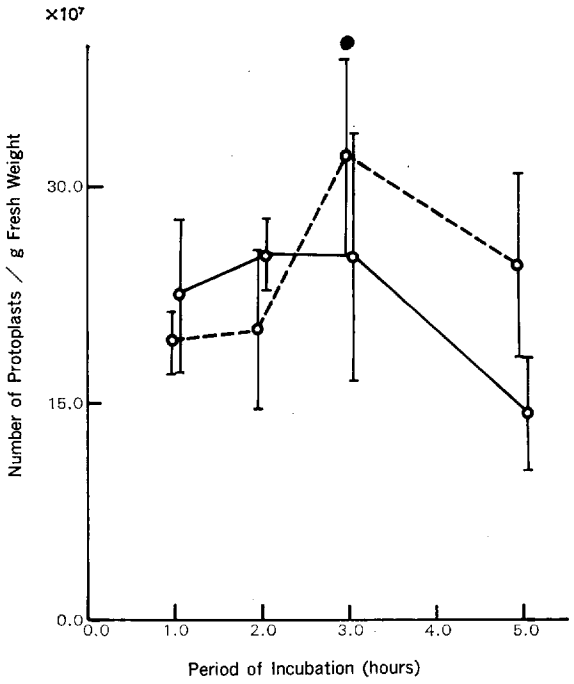

Fig. 10. Time course of protoplast formation from mycelia of *Pleurotus ostreatus* (FMC 235) grown at 30°C and 15°C.
Incubations were performed at 30°C. for 2 hours.
: Mycelia grown at 30°C.
: Mycelia grown at 15°C.

Table 7. Effect of the culture medium on the number of protoplasts formed from mycelia of *Pleurotus ostreatus* (FMC 235) grown in the medium.

Medium	No. of protoplasts per g fresh weight
MM+1% Casamino acids	0.5×10^7
MM+1% Polypeptone	14.3×10^7
MM+1% Nutrient broth	11.8×10^7
SMY	20.0×10^7
1% Sucrose+1% Polypeptone+0.4% Yeast extracts	20.1×10^7
1% Casamino acids+1% Malt extracts+0.4% Yeast extracts	26.1×10^7
1% Polypeptone+1% Malt extracts+0.4% Yeast extracts	31.9×10^7
1% Nutrient broth+1% Malt extracts+0.4% Yeast extracts	25.9×10^7
1% Malt extracts+0.4% Yeast extracts	44.6×10^7

Incubations were porformed at 30°C for 2 hours.
MM : Diammonium Hydrogenphosphate 1.5 g
Glucose 20 g
Potassium Dihydrogen Phosphate 0.45 g
di-Potassium Hydrogen Phosphate 1 g
Magnesium Sulfate 0.5 g
Thiamine Hydrochloride 120 μg
Distilled water 1l

Protoplasts could be cultured both in liquid media and in solid media. Protoplasts prepared from mycelia of *Pleurotus ostreatus* (FMC 235) and *Lentinus edodes* (FMC 2) using the enzyme system Cellulase RS. 2%, Driselase, 2%, Zymolyase 5000, 0.2%, were first cultured in the liquid media and then in solid media. Results are shown in Table 8 and Table 9. As

Table 8. Culture of protoplasts obtained from mycelia of *Pleurotus ostreatus* (FMC 235)*

Osmotic stabilizers and additives	No. of colonies for protoplasts cultured in liquid media for*				
	2 hours	1 day	2 days	3 days	4 days
Mannitol 0.5 M	7	11	6	7	5
$MgSO_4$ 0.5 M	3	23	27	62	103
KCl 0.3 M	6	3	13	15	7
Saccharose 0.5 M	3	13	29	41	41
$MgSO_4$ 0.5 M+0.1% NAG**	7	17	55	101	—
$MgSO_4$ 0.5 M+1% Polypeptone	10	10	23	141	80

* Protoplasts prepared using the enzyme system Cellulase RS 2%+Driselase 2%+Zymolyase 5000 0.2%, were first cultured at 25°C for 2 hours-4 days in liquid media containing glucose 1%, malt extracts 1%, yeast extracts 0.4% (GMY) and osmotic stabilizers and additives shown in the left column and then cultured at 25°C in solid media containing GMY, 0.6 M mannitol and 0.5% Agar. Number of colonies thus formed after 2 weeks of total culture period were counted.

** NAG : N-acetylglucosamine.

Table 9. Culture of protoplasts obtained from mycelia of *Lentinus edodes* (FMC 2)*

Osmotic stabilizers and additives	No. of colonies for protoplasts cultured in liquid media for*				
	2 hours	1 day	2 days	4 days	7 days
Mannitol 0.5 M	57	38	90	73	31
$MgSO_4$ 0.5 M	22	11	21	11	8
KCl 0.3 M	60	47	64	49	6
Saccharose 0.5 M	61	59	103	83	41
$MgSO_4$ 0.5 M+1% NAG**	29	12	11	0	4
$MgSO_4$ 0.5 M+1% Polypeptone	22	10	10	7	7

*, ** ; See legends for Table 8.

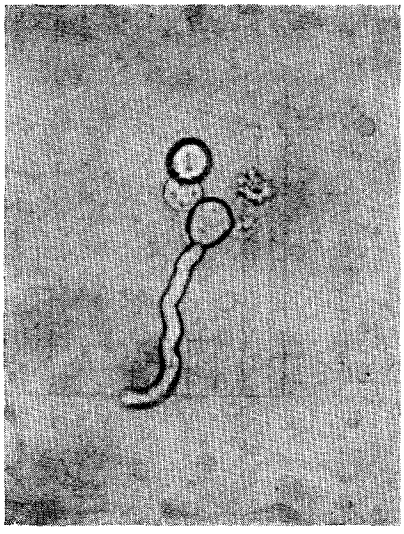

Fig. 11. Regeneration of a protoplast of *Pleurotus ostreatus.*

seen in these tables, first culture in liquid media improved the efficiency of culture of protoplasts for both mushrooms. But the effect of osmotic stabilizers and additives were different for different mushrooms. Because N-acetylglucosamine improved the culture of protoplasts of *Pleurotus ostreatus*, it was added to media in the following experiments. Examples of regenerating protoplasts and hyphae regenerated from protoplasts of *Pleurotus ostreatus* in liquid media are shown in Figs. 11 and 12.

For protoplasts of *Pleurotus ostreatus* prepared using the enzyme system, Novozym 234+ chitinase, the effect of preculture in liquid media was not so clear. The effect of osmotic stabilizers, however, was clearly observed. An example of the effect the above on the culture of protoplasts of *Pleurotus ostreatus* in solid media is shown in Fig. 13. For *Pleurotus ostreatus*, $MgSO_4$ was effective.

In the culture of protoplasts, pH of the medium had a serious effect on the culture

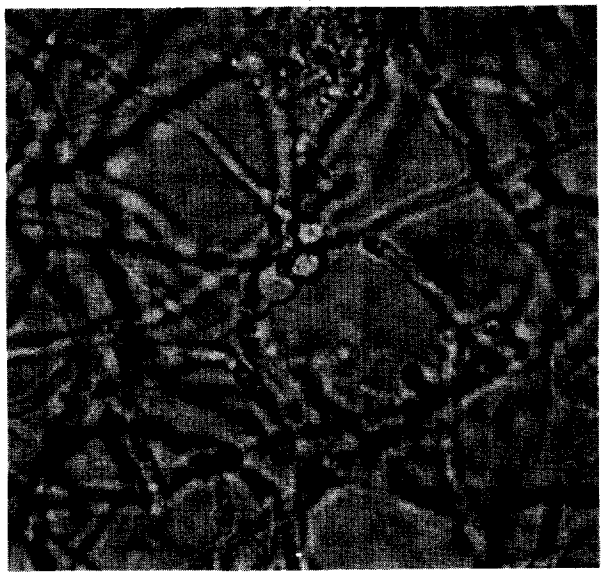

Fig. 12. Hyphae of *Pleurotus ostreatus* regenerated from a protoplast.

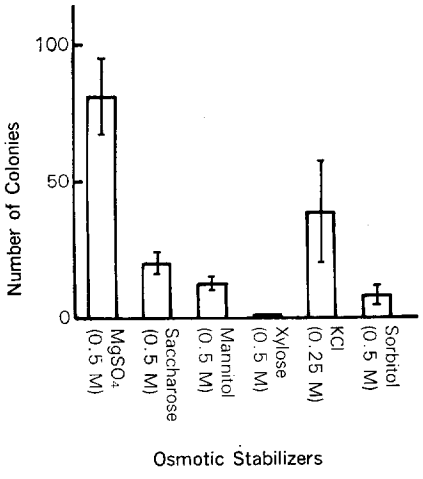

Fig. 13. Effect of osmotic stabilizers on the culture of protoplasts of *Pleurotus ostreatus* (FMC 235).

Protoplasts were prepared from mycelia of FMC 235 using Novozym 234 1%+chitinase 0.1%. They were washed and properly diluted in 0.5 M mannitol and then cultured at 25°C in solid media containing SMY, osmotic stabilizer, 0.1% N-acetylglucosamine and 0.7% agarose. After 7 days, numbers of colonies were counted.

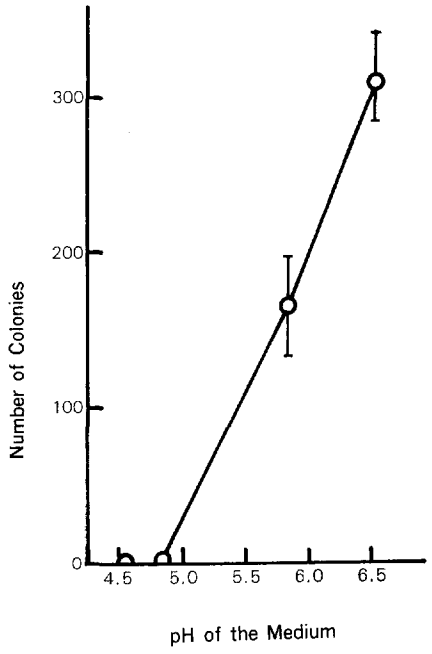

Fig. 14. Effect of pH of the medium on the culture of protoplasts of *Pleurotus ostreatus* (FMC 235). For culture of protoplasts, see the legend of Fig. 13.

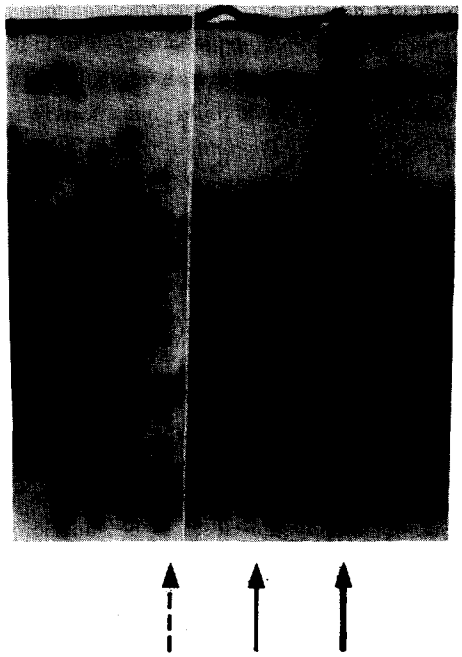

Fig. 15. Monokaryotization of dikaryotic mycelia of *Pleurotus ostreatus*, FMC 235, by formation and culture of protoplasts.
Esterase isozymes of mycelia which were obtained by culture of protoplasts prepared from dikaryotic mycelia of FMC 235, are shown here. An arrow of thick, solid line shows isozymes of the dikaryotic mycelia and arrows of narrow, solid and broken line show isozymes of two types of monokaryons.

efficiency. Protoplasts of FMC 235 were prepared and diluted as described in the legend of Fig. 13 and cultured in media containing SMY, 0.5 M $MgSO_4$, and maleic acid-NaOH buffer to adjust pH. After 9 days of culture, the number of colonies were counted. Results are shown in Fig. 14. As seen in this figure, pH near neutrality is good for the culture of protoplasts of *Pleurotus ostreatus* in the pH range tested here.

In the course of experiments of preparation and culture of protoplasts of *Pleurotus ostreatus*, we found that monokaryotic mycelia could also be obtained from the culture of protoplasts. Colonies whose hyphae have no clamps could be recovered. They could be mated with other monokaryotic mycelia of other strains of *Pleurotus ostreatus* to produce dikaryotic mycelia. Esterase isozymes were analyzed for these monokaryons, cultured from protoplasts, as well as for dikaryons, cultured from protoplasts, and original stock. The result is shown in Fig. 15. As seen from the figure, we could get two types of monokaryons.

Discussion

We were able to prepare protoplasts from mycelia of 12 species of mushrooms. Although Moore[8] prepared protoplasts from *Coprinus cinereus* using only chitinase or Helicase and de

Vries *et al*[12] prepared protoplasts from a number of mushroom using only an enzyme prepared from *Trichoderma viride*, our results indicated that generally mixed enzyme systems were more effective. This fact seems to be related to the complex nature of the cell wall of mushroom mycelia[18]. We could find effective enzyme systms for important cultivated mushrooms, *Lentinus edodes*, *Pleurotus ostreatus*, *Pholiota nameko* and *Auricularia polytricha*. Also for *Grifora frondosa* we could get a fairly high number of protoplasts.

Figs. 1-4 and Table 6 clearly shows that the efficiency of an enzyme system is highly dependent on the strain, even for the same mushroom. It is also dependent on the culture period of mycelia. As shown in Figs. 1-4, seven-day cultures were generally ineffective for protoplast formation compared with younger cultures. Table 7 shows protoplast formation from *Pleurotus ostreatus* is also affected by the media used for culture of mycelia. It was also observed that cultures containing large fractions of aerial hyphae were ineffective for protoplast formation. These facts indicate that protoplast foramation from mycelia is severely controlled by the physiological nature of mycelia.

We have established highly efficient enzyme systems for *Pleurotus ostreatus* as shown in Fig. 6. The system seem to be superior to the method described by Yamada *et el* (1983)[14] for the preparation of protoplasts from mycelia of *Pleurotus ostreatus*. One of our enzyme systems, Novozym 234+Chitinase, was effective not only for *Pleurotus ostreatus* but also for other mushrooms of the genus *Pleurotus*.

Protoplasts of *Pleurotus ostreatus* and *Lentinus edodes* could be regenerated and cultivated. Materials used for osmotic stabilizers had a serious effect on the efficiency of regeneration and cultivation of protoplasts. But as seen in Table 8, 9 and Fig. 13, an effective osmotic stabilizer was different for *P. ostreatus* and *L. edodes*. Regeneration of protoplasts of *P. ostreatus* were more effective at pH 6.5 than at more acidic pH regions.

As described in "Results", dikaryotic mycelia of *Pleurotus ostreatus* could be easily monokaryotized by protoplast formation. Two types of monokaryon could be obtained as was evidenced by isozyme analysis. This technique seems to be more effective and easier than monokaryotization by culture in the special medium[15]. Monokaryotization by protoplast formation seems to have two advantages over the conventional method of getting monokaryotic mycelia from isolated basidiospores.

1. We can get monokaryons easily and at any time.
2. We can always get monokaryons with a constant genetic nature in breeding.

Therefore monokaryotization by protoplast will be very useful in the practical breeding of mushrooms.

References

1) Chaleff, R. S.: Genetics of Higher Plants, Applications of Cell Culture, Cambridge University Press, 184 P. P., (1981)

2) Ferenczy, L. : Microbial Protoplast Fusion. In "Genetics as a Tool in Microbiology" ed. by S. W. Glover and D. A. Hopwood. Cambridge University Press., p. 1—p. 34, (1981)

3) Sandri-Goldin, R. M., A. L. Goldin, M. Levine and J. Glorioso : High-Efficiency Transfer of DNA into Eukaryotic Cells by Protoplast Fusion. Methods in Enzymology, Academic Press, 101, p. 402—p. 411, (1983)

4) Keller, U. : Highly Efficient Mutagenesis of *Claviceps purpurea* by Using Protoplasts.

Appl. Environ. Microbiol., 46, 580—584, (1983)

5) Kim, K. S., N. Y. Cho, H. S. Pai and D. D. Y. Ryu : Mutagenesis of Micromonospora rosaria by Using Protoplasts and Mycelial Fragments. Appl. Environ. Microbiol., 46, 689—693, (1983)

6) Villanueva, J. R., I. Garcia-Acha, S. Gascón and F. Uruburu : Yeast, Mould ane Plant Protoplasts, Academic Press, 381 pp., (1973)

7) de Vries, O. M. H. and J. G. H. Wessels : Release of Protoplasts from *Schizophyllum commune* by a Lytic Enzyme Preparation from *Trichoderma viriede*. J. Gen. Microbiol., 73, 13—22, (1972)

8) Moore, D. : Production of *Coprinus* Protoplasts by Use of Chitinase or Helicase. Trans. Br. mycol Soc., 65, 134—136, (1975)

9) Ushiyama, R. and Y. Nakai : Protoplasts of Shiitake, *Lentinus edodes* (Berk.) Sing Rept. Tottori Mycol. Inst. (Japan), 15, 1—5, (1977)

10) Ohmasa, M., Y. Abe, H. Furukawa, M. Taniguchi and H. Neda : Preparation, Culture and Fusion of Protoplasts of Mushrooms of the Genus *Pleurotus*. In "proceedings of the 28 th annual meeting of the Mycological Society of Japan", p. 59, (1984)

11) Ohmasa, M. and H. Furukawa : Analysis of esterase and malate dehydrogenase isozymes of *Lentinus edodes* by isoelectric focusing for the identification and discrimination of stocks. Trans. mycol. Soc. Japan, 27, 79—90, (1986)

12) de Vries, O. M. H. and J. G. H. Wessels : Effectiveness of a Lytic Enzyme Preparation from *Trichoderma viride* in Releasing Spheroplasts from Fungi, Particularly Basidiomycetes. Antonie van Leeuwenhoek, 39, 397—400, (1973)

13) Farkaš, V. : The Fungal Cell Wall. In "Fungal Protoplasts : Applications in Biochemistry and Genetics" ed. by J. F. Peberdy and L. Ferenczy, Marcel Dekker, Inc., p. 3—29, (1985)

14) Yamada, O., Y. Magai, Y. Kashiwagi, T. Shiratori and T. Sasaki : Formation and Regeneration of *Flammulina velutipes* and *Pleurotus ostreatus* Protoplasts. Nippon Shokuhin Kogyo Gakkaishi, 30, 495—500, (1983)

15) Leal-Lara, H. and G. Eger-Hummel : A Monokaryotization Method and its Use for Genetic Studies in Wood-rotting Basidiomycetes. Theor. Appl. Genet., 61, 65—68, (1982)

日本産栽培きのこのプロトプラストの調製と培養

大政正武[1]・阿部恭久[2]・古川久彦[3]
谷口　実[4]・根田　仁[5]

摘　要

プロトプラストは，細胞融合や形質転換をはじめとする，きのこではこれまで行われていなかった新しい育種法を可能にするものとして，大きな期待が寄せられている。プロトプラストの作成と菌糸の再生についてはこれまでスエヒロタケやヒトヨタケ属のきのこなどについての研究はあるがが，シイタケ，ヒラタケ，ナメコ，マイタケなど日本産の栽培きのこについては研究が少なかった。本研究は，これらの日本産の栽培きのこについて，プロトプラストの調製法と培養法を検討し，上記の新しい育種法を開拓するための基礎的知見を得ることを目的として行ったものである。

日本産栽培きのこ 12 種の菌糸からプロトプラストの調製を行った。これらにはシイタケ，ヒラタケ，エノキタケ，ナメコ，マイタケ，マツタケ他が含まれる。きのこの菌糸を液体培養し，集菌後，菌の細胞壁溶解酵素の処理でプロトプラストを得た。市販酵素の組み合せのうち，a)セルラーゼ RS，β-グルクロニダーゼ，b)セルラーゼ RS，ドリセラーゼ，β-グルクロニダーゼ，c)セルラーゼ RS，ドリセラーゼ，ザイモリアーゼ 5000 の酵素系が良い成績を示した。シイタケ，ナメコ，マイタケ，アラゲキクラゲについてプロトラプスト調製に対する菌糸の培養期間依存性を検討した。また系統の違いによってもプロトプラスト調製は異なった。ノボザイム 234 とキチナーゼの組み合せはヒラタケ属のきのこに対してきわめて良い成績を示したので，プロトプラスト調製のさらに詳しい条件，すなわち，pH 依存性，浸透圧調節剤の影響，処理時間依存性，菌糸培養の培地の影響等を検討した。シイタケとヒラタケのプロトプラストは培養を行なうことができたので，その条件をさらに検討した。その過程で，ヒラタケの 2 核菌糸からプロトプラストを得て培養すると 2 核菌糸と同時に 1 核菌糸が再生することが見出され，2 核菌糸の 1 核化の簡便法として使えることが分った。

1986年 8 月 9 日受理
(1) (2) (3) (4) (5)　保　護　部