韓 國 菌 學 會 誌 第 13 卷 第 3 號
Kor. J. Mycol. Vol. 13, No. 3, 169~177, 1985

Protoplast Formation, Regeneration and Reversion in *Pleurotus ostreatus* and *P. sajor-caju*

Seung Joo Go, Gwan Chull Shin* and Young Bok Yoo

Institute of Agricultural Sciences, Suweon 170, and Department of Agricultural Biology
*College of Agriculture, Chungnam National University**
Daejeon 300-31, Korea

느타리버섯과 여름느타리버섯의 原形質體 裸出과 再生

高 昇 柱·申 寬 澈*·劉 英 福

農村振興廳 農業技術研究所·忠南大學校 農科大學 農生物學科*

Abstract: The studies were carried out to obtain the basic data for maximizing the protoplast yields from the mycelia of *P. ostreatus* and *P. sajor-caju*. Some factors affecting the regeneration of the protoplast of both species and the productivity of their reversion were also examined. The maximum yields of protoplasts were obtained from four days cultured mycelia of both species on cellophan membrane placed on the surface of PSA or MCM media in a petri dish. The optimal concentration of lytic enzyme Novozym 234 for protoplast releasing was 5 mg per ml of 0.5 M phosphate buffer solution with 0.6 M sucrose or 0.6 M $MgSO_4$ at pH 6.0. The greatest number of protoplasts was released 3 hours after incubation of the mycelia of *P. ostreatus* and after 4 hours for the *P. sajor-caju* in the lytic enzyme solution. Among the osmotic stabilizer solutions tested 0.6 M sucrose and 0.6 M KCl showed the best regeneration rates of the protoplasts of both species. When 0.75 % agar solution was over-layed on the regeneration media immediately after inoculation of the protoplast the regeneration rates were greatly enhanced. The ampicillin added to the agar solution prevented bacteria from infection. The reverted isolates produced the sporophores and basidial spores just like their parents without any mutations when they were cultivated in a broad mouth bottle with sawdust substrates.

Keywords: Protoplast formation and regeneration, *Pleurotus ostreatus*, *Pleurotus sajor-caju*.

Protoplasts were demonstrated as a useful tool in the area of the studies on improvement of strain and genetic analysis (Hamlyn, 1982). In Streptomyces protoplast has proved to be a highly efficient procedure for obtaining genetic recombination in intraspecies crosses. Peberdy(1980) reported that *Cephalosporium* one of the recombinant isolates by protoplasts fusion showed a 40 % improvement in antibiotic production. Furthermore, in higher fungi interspecific heterokaryons were obtained between auxotrophic mutants of *Pleurotus ostreatus* and *P. florida* with polyethylen glycol+Ca^{++} induced fusion of somatic protoplast (Yoo *et al.*, 1984). This result showed a possibility of applying the protoplast even in the higher fungi for the breeding purpose.

Protoplasts were also used as a recipient cell of foreign DNA. The foreign DNA was taken up as a result of protoplast fusion when the protoplasts were exposed to in the presence of polyethylen glycol and calcium chloride (Rodri-

guez *et al.*, 1983).

Protoplasts were released from spore (Hong *et al.*, 1984), mycelia (De Vries *et al.*, 1973; Yamada *et al.*, 1983) and other somatic tissue (Bodds *et al.*, 1982) when they were treated with lytic enzymes. The lytic enzyme removed the cell wall and protoplasts were released.

One critical factor affecting protoplast yield is the physiological conditions of the organism (Santiago, 1981). The nature and concentration of the lytic enzymes, culture media, presence of osmotic stabilizers and some of environmental factors affected the protoplast yields (Peberdy, 1976).

In *P. ostreatus*, the protoplast yields were different dependent upon the nature of the lytic enzymes and authors. Chin(1984) obtained high yields of protoplast when using 1.5 % β-glucuronidase (w/v) and 0.5 % cellulase "Onozuca (w/v). However, Byun *et al.* (1984) used Novozym 234 only as a most effective lytic enzyme among commercials tested. Yoo *et al.* (1985) reported that combinations of β-D-glucanase, Novozym 234 and snail enzyme were the most effective for isolation of the protoplast.

To support osmotic stability for the protoplasts, various substances have been used in their releasing and regeneration. These substances affected the yields of protoplasts. Byun *et al.* (1984) reported that the solution of 0.8 M $MgSO_4$ or 0.8 M KCl showed good results as the osmotic stabilizer for releasing the protoplasts of *P. ostreatus*. However. Chin(1984) obtained a little different results from those of Byun. She obtained the best yield of protoplasts when used 1.0 M mannitol, 0.8 M KCl or 1.0 M NaCl as the osmotic substances.

It was neccessary that protoplasts should be as many in number as possible in order to fuse and regenerate them. In general the fusion and regeneration frequency of fungal protoplasts were significantly low. Ferenczy *et al.*(1976) reported that the best fusion frequency in *Geotrichum* was not much higher than 10^{-6} per protoplast pair when no chemical stimulating agent was added. Even though an improvement method using chemicals was applied the rate was less than 2.5×10^{-3}.

The authors, therefore, conducted experiments to obtain the basic data for maximizing the protoplast yields from mycelia of *P. ostreatus* and *P. sajor-caju*. Also some factors affecting regeneration rate of both species and the characteristics of their regeneration isolates were also examined.

Materials and Methods

Organisms

Pleurotus ostreatus ASI 2018 was used for the experiments. This organism was selected by the Institute of Agricultural Sciences as a leading cultiva in Korea among local strains in 1981 (Go *et al.*, 1981). *P. sajor-caju* ASI 2070 from India was also selected by the Institute as suitable species for cultivation during summer season in Korea (Go *et al.*, 1984). Both organisms were grown on the Potato Sugar Agar (PSA) media and preserved at 4°C refrigerator before they were used.

Media

The PSA media (Booth, 1971), Raper's (1972) Mushroom Complete Media (MCM) and Mushroom Minimal Media (MMM) were used for the experiments. The MCM consisted of dextrose 20 g, peptone 2 g, yeast extract 2 g, $MgSO_4\cdot7H_2O$ 0.5 g, KH_2PO_4 0.46 g, K_2HPO_4 1.0 g, in distilled water 1000 ml and MMM consisted of dextrose 20 g, DL-asparagin 2 g, $MgSO_4\cdot7H_2O$ 0.5 g, KH_2PO_4 0.46 g, K_2HPO_4 1 g, thiamin HCl 120 μg in distilled water 1000 ml. The regeneration media were made by adding

the osmotic stabilizer into the MCM and MMM.

Osmotic Stabilizer

The optimal concentration of magnesium sulfate for the protoplast release was tested from 0.4 mole to 1.0 mole in a pH 6.0 phosphate buffer solution. Also six kinds of osmotic substances, including the magnesium sulfate were tested for the ability to stabilize the osmotic pressures under the criterion of protoplast yields.

Lytic Enzyme

Following the good results of Byun *et al.* (1984), Novozym 234 (produced by Novo Industry in Denmark) was used as the lytic enzyme. The enzyme was solved 3 to 10 mg in 1 ml of the osmotic stabilizer solution and filtered to remove any infected organisms through the 0.2 μm membrane filter produced by Gelman Sciences Co. The enzyme solution was adjusted pH 4 to 8 by phosphate buffer to find the optimal pH for the protoplast releases. Four ml of the enzyme solution was added to the each petridish containing mycelia.

Protoplast Formation

The organisms were inoculated on the sterile cellophan membrane placed on the media in a petri dish and incubated at 25°C for 2 to 7 days. As the mycelia grew, the cellophan including the mycelia was moved from the media into new sterile petri dish and the lytic enzyme solution was added to submerge the mycelia. Then petri dish was incubated in a gentle shaking water bath at 28°C for protoplast formation. The protoplasts were counted by haemocytometer under the microscope.

Isolation and Regeneration of Protoplasts

Released protoplasts were filtered through sintered glass filter having a porosity of G-1 to isolate the protoplast from the mycelial debris. The filtered solution was centrifuged (700 rpm) two times for 10 minutes each with 0.6 M KCl osmotic stabilizer solution. The precipitation was resuspened in the KCl solution and inoculated on the regeneration media. The protoplasts were counted befor inoculation to find out the number of protoplasts to be inoculated on the regeneration media. The osmotic substances of the regeneration media were tested their effectiveness on the regeneration of the protoplasts. To enhance the regeneration rates, 0.5~2.0 % agar solutions were overlayed on the protoplast inoculated media and examined its effectiveness on the regeneration.

Reversion and Its Productivity

The regenerated colonies of both species were transferred to the MCM. The isolates were examined and their sporophore production and morphogenesis were compared with their origins. The isolates were cultivated on sawdust media as substrates (Go et *al.*, 1985) for the production of sporophores.

Results and Discussion

Production of Protoplasts

Pleurotus ostreatus and *P. sajor-caju* grew well and produced high yields of protoplast when they were cultivated on the Potato Sugar Agar (PSA) media. As shown in Table I, *P. ostreatus* and *P. sajor-caju* grew 2 and 4 mm faster on PSA media for 3 days incubation than on those of the MCM, respectively. The number

Table I. Effects of media on the mycelial growth and protoplast yields in *P. ostreatus* and *P. sajor-caju.*

Species	Media	Mycelial growth (mm/3 days)	No. of protoplast per ml
P. ostreatus	M.C.M.	5	2.4×10^6
	P.S.A.	7	1.8×10^7
P. sajor-caju	M.C.M.	6	8.0×10^6
	P.S.A.	10	3.5×10^7

* M.C.M. =Mushroom Complete Medium
P.S.A. =Potato Sucrose Agar

of protoplast obtained from mycelia grown on PSA media was 2.4×10^6 in *P. ostreatus* and 8.0×10^6 in *P. sajor-caju* and these were a little higher than those grown on the MCM. Those results indicated that the protoplast yields were related with their growth media.

P. sajor-caju showed a little higher yield of protoplasts than those in *P. ostreatus*. This indicated that the mycelium of *P. sajor-caju* was more susceptible to the lytic enzyme Novozym 234 than that of *P. ostreatus*.

In this experiment, the PSA media were made in the laboratory by the authors therefore, the media were not uniform in ingredients so that the MCM which showed uniform results of protoplast releasing were used in the subsequent experiment.

Fig. 1 shows the optimum cultivation period of mycelium for the production of protoplasts. Four days culture on cellophan membrane placed on the surface of the MCM for both species showed the best yields of protoplast. The age of the mycelium had a significant effect on the protoplast yields. The yields of protoplasts of both species increased in proportion to the cultural periods of the mycelium until the optimal day, but after the optimum day the yields decreased.

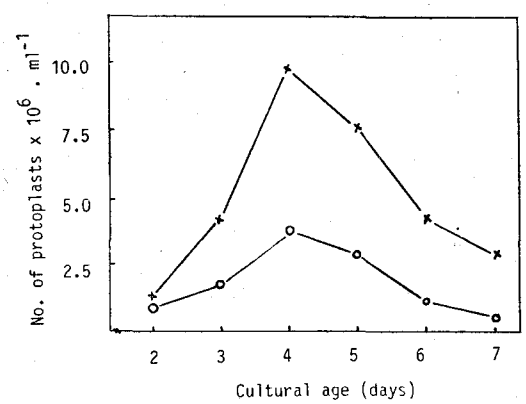

Fig. 1. Effect of growing periods on the protoplast release from the mycelium of *P. sajor-caju* (×—×) and *P. ostreatus* (○—○).

These results corresponded with those of Yamada *et al.*(1983) and Byun *et al.* (1984) who reported that the best yield of protoplast in *P. ostreatus* were obtained from three or four-day cultured mycelium. However, these results were a little different from those of Chin(1984) who reported that the protoplast yields in *P. ostreatus* were higher when the mycelia were cultured for two days rather than for four days. Generally microorganisms have usually not shown the maximum number of protoplast in the lag phase but in the late exponential growth phase (Cho, 1982). The four days incubation belonged under the exponential phase in *P. ostreatus* (Byun *et al.*, 1984).

The older mycelium was less susceptible to lysis than younger mycelium because the composition of cell wall probably changed during the aging process (Peberdy *et al.*, 1979; Santiago, 1981). De Vries *et al.* (1973) reported three wall polymers, S-glucan, R-glucan and chitin in *Schizophyllum commune* mycelia of different culture age. The amount of S-glucan remained about the same during the 6-days growth periods but the S-glucan to R-glucan and S-glucan to chitin ratios decreased 3 days after culturing and concurrently the yields of protoplasts were greatly reduced.

The concentration of the lytic enzyme Novozym 234 was affected on the release of protoplast. Although the protoplast yields were a little different in both species, the highest yield of protoplast was obtained in the same concentration of 5 mg in one ml of osmotic stabilizer solution.

As shown Fig. 2, the yields of protoplast in *P. sajor-caju* were incresed considerably in proportion to the enzyme concentration up to the optimal concentration, however beyond that the yields decreased drastically. While, in *P. ostre-*

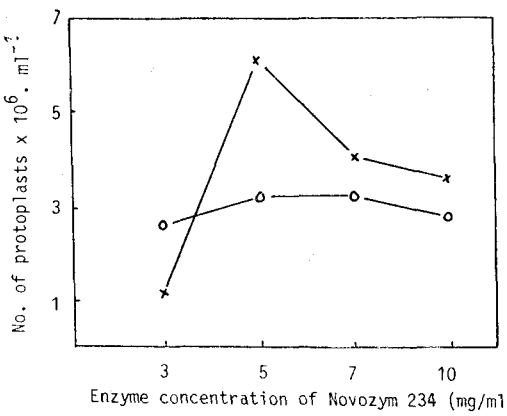

Fig. 2. Effect of enzyme concentration on the protoplast release from the mycelium of *P. sajor-caju* (×—×) and *P. ostreatus* (○—○).

atus there was only slight for the protoplast yields to be increased or decreased with changes in the enzyme concentration. This indicated that the susceptivity to enzyme solution was variable according to the species.

Novozym 234 contains a strong proteinase which is able to destroy the protein ingredient in membranal wall of protoplasts so that the protoplast were destroyed under the excessive concentration of the enzyme (Hamlyn, 1928).

Byun *et al.* (1984) reported that the optimal concentration of the lytic enzyme, Novozym 234 was 15 mg per ml of osmotic solution for protoplast releasion. Their results were however different from that of ours. The difference resulted from the different cultivation method.

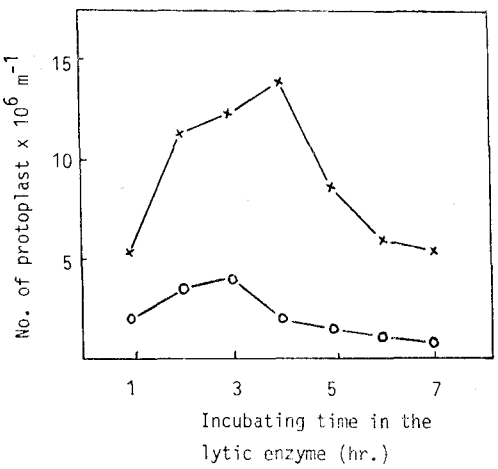

Fig. 3. Effect of incubating time of the mycelium in the lytic enzyme solution on the protoplast releasing in *P. sajor-caju* (×—×) and *P. ostreatus* (○—○).

In our experiment, the cellophan culture was employed, while Byun *et al.* (1984) used submerged cultural techniques to obtain mycelia. The wetting mycelia should need a higher concentration of lytic enzyme to release protoplast than those of the cellophan culture.

The release of protoplasts started one hour after incubating the mycelium of both species in the lytic enzyme solution at 28°C (Fig. 3). The highest yields of protoplasts were obtained 3 hours after incubation for *P. ostreatus* and 4 hour for *P. sajor-caju* in the lytic solution. The protoplast yields increased with the incubation time period until the optimal period. However, when the optimal time was exceeded yields were reduced. It was mainly due to the lysis of the protoplast. The size of the protoplast was increased with the incubation time mainly due to the increase in the volume of vacuole. During the first hour of the mycelium digestion, small or non vacuolate protoplasts were produced mainly from hyphal tips and 3 or 4 hours after much larger protoplast were released. The later protoplast contained the larger vacuole.

The influence of pH of the lytic enzyme solution on the protoplast yields was illustrated in Fig. 4. The maximum protoplast yields of both species were obtained at pH 6. Hamlyn (1982) reported that the optimum pH of Novozym 234 for the release of protoplast in *Cephalosporium acremonium* was between pH 5.5. Although the organisms used were differently about the same results were obtained.

The osmotic substances had a significant affect on the protoplast yields. As shown on Table II,

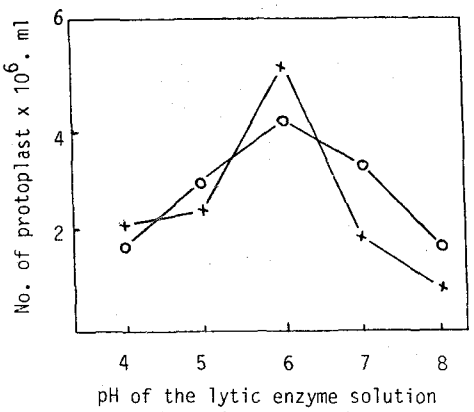

Fig. 4. Effect of pH of the lytic enzyme solution on the protoplast releasing from the mycelium of *P. sajor-caju* (×—×) and *P. ostreatus* (○—○).

Table II. Effects of somotic stabilizer on the protoplast yields in *P. ostreatus* and *P. sajor-caju.*

Osmotic stabilizer	No. of protoplast 10^6 ml^{-1}	
	P. ostreatus	*P.sajor-caju*
0.6 M KCl	4.0	8.4
0.6 M $MgSO_4$	5.6	24.0
0.6 M NaCl	3.6	23.6
0.6 M $(NH_4)_2SO_4$	5.2	19.6
0.6 M Mannitol	2.8	24.4
0.6 M Sucrose	8.8	26.4

* M: Molarity

0.6 M sucrose and 0.6 M mannitol, among various osmotic substances, showed the best results as 26.4×10^6 and 24.4×10^6 protoplasts in *P. sajor-caju*, respectively. On the other hand, in *P. ostreatus* with 0.6 M sucrose and 0.6 M $MgSO_4$ there were 8.8×10^6 and 5.6×10^6 protoplasts produced, respectively. These results corresponded with those of Yoo *et al.*(1985), but were a little different from those of Chin (1984) who failed to obtain good results when $MgSO_4$ was used as osmotic stabilizer.

$MgSO_4$ as an inorganic salt, was selected for determining the optimal molarity of the osmotic solution for the releasing of protoplasts. That

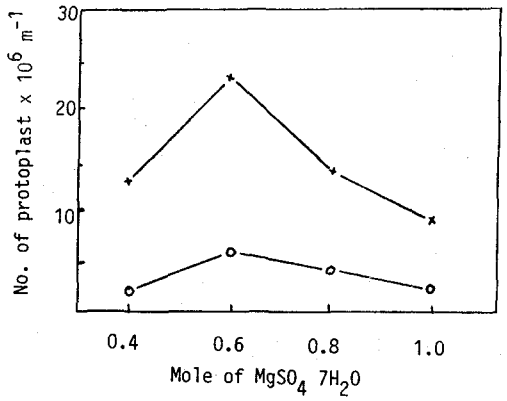

Fig. 5. Effect of osmotic stabilizer concentration on the protoplast release in *P. sajor-caju*(×—×) and *P. ostreatus* (○—○).

was because the inorganic salt was convenient to handle with and prevent contamination. The results were illustrated in Fig. 5. The maximum of the protoplast yields in both species were obtained from 0.6 mole solution, however, beyond the optimal molarity the yields were reduced drastically.

In using $MgSO_4$ Chin(1984) and Byun(1984) reported that the best yields of protoplasts were obtained from 0.8 mole solution. The molarity was little higher than that of authors. This difference was probably due to the different cultural conditions for the mycelial growth. In our experiment, the mycelia were cultivated on the cellophan membrane placed on solid media in a petri dish, but Chin(1984) and Byun *et al.*(1984) cultivated it in submerged culture media.

Regeneration and Reversion of Protoplast

The protoplast regeneration frequency in *P. ostreatus* and *P. sajor-caju* was considerably affected by various osmotic substances which were added into the regeneration media for protection against the osmotic pressure of the protoplasts during the regeneration. As shown in Table III, among the osmotic substances tested, 0.6 M sucrose and 0.6 M KCl showed 0.07 % and 0.05

Table III. Regeneration rates and incubation periods of the protoplasts of *P. sajor-caju* and *P. ostreatus* at the different osmotic stabilizers.

Csmotic stabilizer	Regeneration rates (%)		Incubation periods for regeneration (day)	
	P. ostreatus	*P. sajor-caju*	*P. ostreatus*	*P. sajor-caju*
0.6 M *KCl	0.03	0.05	14	12
0.6 M $MgSO_4$	0.01	0.03	12	10
0.6 M NaCl	—*	0.01	16	13
0.6 M Mannitol	0.02	0.02	10	9
0.6 M Sucrose	0.03	0.07	10	9

M=Molarity —=below 0.01 %

% regeneration frequencies, respectively, in *P. sajor-caju*. In 0.6 M sucrose and 0.6 M KCl *P. ostreatus* showed a high regeneration frequncy. However, in 0.6 M NaCl *P. ostreatus* produced no regeneration colonies. The periods required to regenerate the protoplasts of both species were shorter in 0.6 M sucrose and 0.6 M mannitol as organic substances than in 0.6 M KCl, 0.6 M $MgSO_4$ and 0.6 M NaCl as inorganic salts. The regeneration media containing 0.6 M $MgSO_4$ did not harden well, mainly due to its acidity. So, 0.6 M KCl was used instead of 0.6 M $MgSO_4$ for the subsequent experiments as osmotic stabilizer.

The regeneration frequencies differed due to kinds of organisms in higher fungi. The regeneration rates of *Schizophillum commune* (De Vries *et al.*, 1975) *Coprinus cinereus* (Akamatsu *et al.*, 1983) and *Tricholoma matsutake* (Abe *et al.*, 1982) were 50 %, 35~55 % and about 10 %, respectively. Those kinds of mushroom had high-regeneration rates. However, *Volvariella volvacea* showed a lower regeneration rate, only about 1~3 %, mainly due to the abscence of nuclei in the protoplast (Santiago, 1981).

In the authors experiments, the rates were much lower, only 0.07 % in *P. sajor-caju* and 0.03 % in *P. ostreatus* than those of other fungi. The reason for this differences was not found. The regeneration colonies from the protoplast of both species on MCM are shown in Figure 6.

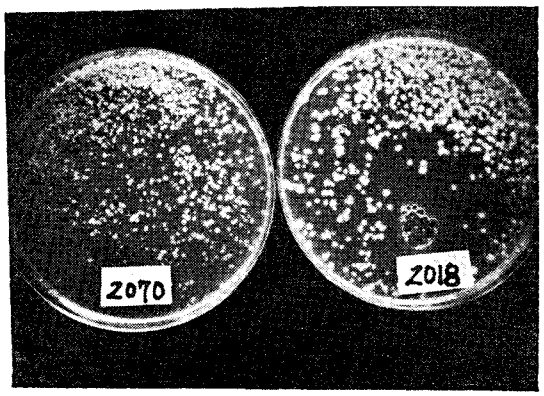

Fig. 6. The regeneration colonies from the protoplast of *P. ostreatus* (2018) and *P. sajor-caju* (2070) on MCM for 14 day incubation at 25°C.

Table IV. Influences of agar contents in the overlayed solution on the media on the rates of regeneration in *P. ostreatus*.

Agar contents(%) in the overlayed solution	Regeneration rate(%)
no overlaying	0.022
0.50	0.025
0.75	0.044
1.0	0.034
2.0	0.026

* Media: Mushroom Complete Media+0.06M KCl

To enhance the regeneration frequency, various concentrations of agar solutions were overlayed on the regeneration media immediately after inoculation of the protoplasts. The influe-

Table V. Influence of ampicillin on regeneration rate in *P. ostreatus* and *P. sajor-caju*.

Species	Regeneration rate(%)	
	No treatment	Ampicillin treatment
P. ostreatus	0.032	0.035*
P. sajor-caju	0.034	0.037

Table VI. The sporophores yields of reversion isolates in *P. ostreatus* and *P. sajor-caju*.

Isolates	Yield[1] (g/bottle)	Individual weight(g)	Spore production
P. ostreatus(R)[2]	63	10.3	abundant
〃 (W)	74	11.0	〃
P. sajor-caju(R)	72	13.7	〃
〃 (W)	67	13.4	〃

1) bottle: 800 cc PP bottle.
2) R: Reversion isolates, W: Wild strain.

nce of agar solution on the regeneration rate showed in Table IV. The maximum rate in *P. ostreatus* obtained when 0.75% agar solution was overlayed. In comparison with no overlaying that result was enhanced two times as the rates.

Even though 0.6 M KCl was used as an osmotic stabilizer during the regeneration of the protoplasts, in some instances the media were contaminated by bacteria. To prevent contamination, 300 ppm ampicillin as antibotic was added to the 0.75% agar solution. The influence of ampicillin on the regeneration frequency was shown on Table V. The antibiotic substance did not show any influences on the regeneration rate in both species, however it did prevent contamination.

The reversion isolates produced the same sporophores as wild types in both species without any mutations when they were cultivated in the broad mouth bottle with sawdust substrates (Table VI).

The morphology of sporophore and the basidial spore in both species were quite normal.

摘 要

느타리버섯*Pleurotus ostreatus* 및 여름느타리버섯 *Pleurotus sajor-caju*의 原形質體는 감자培地(PSA) 및 버섯 完全培地 (MCM)에 cellophan 半透析膜을 被覆하여 培養한 菌絲體에서 2.4×10^6~3.5×10^7 으로 많이 裸出되었다. 버섯 完全培地에 4日間 培養한 菌絲體를 pH가 6.0인 燐酸 緩衝溶液에 細胞壁 分解酵素인 Novozym 234를 5 mg/ml 濃度로 溶解한 酵素溶液에 4時間 處理하였을 때 原形質體의 裸出數가 가장 많았다. 이들 菌株의 原形質體 裸出 時 滲透壓 調節劑로는 0.6 M sucrose와 0.6 M $MgSO_4$가 가장 適合하였다. 한편 原形質體의 再生 時에는 滲透壓 調節劑로 0.6 M sucrose와 0.6M KCl를 使用하였을 때 높은 再生率을 보였다. 原形質體는 再生用 培地에 接種한 후 0.75% agar 培地를 被覆하였을 때 再生率이 向上되었으며, 이때 300 ppm ampicillin 을 0.75% agar 培地에 混合하여 處理한 結果 細菌의 汚染을 防止할 수 있었다. 이들 菌株의 原形質體는 正常的인 菌絲體로 還元되었으며 菌絲體는 正常的으로 生長하여 子實體와 擔子胞子를 形成하였다.

References

Abe, M., Umetsu, H., Nakai T. and Sasage, D. (1982): Regeneration and fusion of mycelial protoplast of *Tricholoma matsutake*. *Agric. Chem.* **46**:1955~1957.

Akamatsu, K., Kamada, T., and Takemaru, T. (1983): Release and regeneration of protoplast from the oidia of *Corprinus cinereus*. *Trans. Mycol. Soc. Japan* **24**:173~184.

Bodds, J. H. and Roberts, W. (1982): *Experiments in Plant Tissue Culture*. Cambridge University Press, London.

Booth, C. (1971): Fungal cultural media. ed. C. Booth. Methods in microbiology. Vol. 4, Academic Press, London.

Byun, M.O., Go, S.J., Park, Y.H. and Shin, G.C. (1984): Some factors affecting the protoplast releasing from *Pleurotus ostreatus*. *Kor. J. Mycol.* **12**: 9~14.

Chin, K.H. (1984): Studies on the protoplast formation and reversion in *Pleurotus ostreatus*. Thesis for

M.S. Degree, Sug Myung University.

Cho, N.J. (1982): Production of protoplast and protoplast fusion in *Trichoderma koningii*. Thesis for M.S. Degree, Seoul National University.

De Vries, O.M.H. and Wessels, J.G.H. (1973): Release of protoplast from *Schizophyllum commune* by combined action of purified α-1, 3-glucanase and chitinase derived from *Trichoderma viridae*. *J. Gener. Microbiol.* **76**:319~330.

De Vries, O.M.H. and Wessels, J.G.H. (1975): Chemical analysis of cell wall regeneration and reversion of protoplasts from *Schizophyllum commune*. *Arch. Microbiol.* **102**:209~219.

Ferenczy, L., Kevei, F. and Szegedi, M. (1976): Fusion of fungal protoplasts induced by polyethylene glycol. 177~187 ed. J.F. Peberdy. A.H. Rose, H.J. Regers and E.C. Cocking, *Microbial and plant protoplast*, Academic Press, London.

Go, S.J., Cha, D.Y. and Park, Y.H. (1981): Intra-and intermatings among strains of *Pleurotus ostreatus* and *P. florida*. *Kor. J. Mycol.* **9**:13~18.

Go, S.J., Byun, M.O., You, C.H. and Park, Y.H. (1984): Selection of *Pleurotus sajor-caju* as suitable species for cultivation under summer climatic conditions in Korea. *Kor. J. Mycol.* **12**:53~58.

Hamlyn, P.F. (1982): Protoplast fusion and genetic analysis in *Cephalosporium acremonium*. Thesis of Ph. D., the Univ. of Nottingham.

Hong, S.W., Han, Y.C. and Park, H.M. (1984): The conidial protoplast fusion of cellulolytic fungus, *Trichoderma koningii*. *Kor. J. Microbiol.* **22**: 207~214.

Peberdy, J.F. (1976): Factors affecting protoplast release in some filamentous fungi. *Trans. Br. Mycol. Soc.* **67**:23~26.

Peberdy, J.F. (1980): Protoplast fusion-a tool for genetic manipulation and breeding in industrial microorganism. *Enzymes Microb. Technol.* **2**:923~929.

Raper, C.A., Raper, J.R. and Miller, R.E. (1972): Genetic analysis of the life cycle of *Agaricus bisporus*. *Mycologia* **64**:1088~1117.

Rodrinez, R.L. and Tait, R.C. (1983): *Recombinant DNA techniques*. Addison Wesley Publishing Co.

Santiago, C.M. (1981): Studies on the physiology and genetics of *Volvariella volvacea* (Bull ex Fr.) Singer. Thesis for Ph. D. in University of Nottingham.

Yamada, O., Magae, Kashiwagi, Y. Y., Shiratori, T. and Sasaki, T. (1983): Formation and regeneration of *Flammulia velutipes* and *Pleurotus ostreatus* protoplast. *Nippon Shokuhin Kogyo Gakkaishi* **30**:495~500.

Yoo, Y.B., Byun, M.O., Go, S.J. and You, C.H. (1984): Characteristics of fusion products between *Pleurotus ostreatus* and *Pleurotus florida* following inter specific protoplast fusion. *Kor. J. Mycol.* **12**: 164~169.

Yoo, Y.B., Peberdy, J.F. and You, C.H. (1985): Studies on protoplast isolation from edible fungi. *Kor. J. Mycol.* **13**:1~10.

〈Received August 12, 1985;
Accepted September 17, 1985〉