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Liquid Culture Enhances Protoplast Formation from the Auxotroph (Ser^-) of *Lentinula edodes*

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The optimal conditions for the production and regeneration of the protoplasts from *Lentinula edodes* were studied. Protoplast formation from the mycelia of *L. edodes* which were cultured in liquid medium showed a significantly high yield compared with that of the mycelia which were cultured on cellophane covered agar media. A mixture of Novozyme 234 (15 mg/ml) and Cellulase Onozuka R10 (10 mg/ml) in 0.6 M mannitol (pH 4) was optimal lytic enzyme for the protoplast release. The optimal incubation time and mycelia age were 3.5-4 hours at 30°C and 6-8 days, respectively. Regeneration frequency was 0.18% plated onto a medium containing 0.6 M sucrose, and 0.08% plated onto a medium containing mannitol. But hardly any regeneration was observed in the media containing NaCl, KCl, or $MgSO_4$. More than 90% of the protoplasts contained nuclei and the nucleus number per protoplast was 1.1. The DNA content per nucleus was 5.1 pg. The diameter of the protoplast was 3-5 μm and it had a well defined cell structure.

Key words : Basidiomycete, *Lentinula edodes,* Liquid culture, Osmotic stabilizer, Protoplast, Regeneration, Ultrastructure

INTRODUCTION

Protoplasts are one of the most useful tools for the study in biochemistry and genetics of mushrooms, including protoplast fusion, transformation and transfection. Conditions for the formation, regeneration and reversion of protoplasts from fungi have been reported since 1950s (Eddy *et al.*, 1957; Abe *et al.*, 1984; Anne, 1983; Benitez *et al.*, 1975; Bok *et al.*, 1990 and 1994; De Vries *et al.*, 1972; Eyssen, 1977; Lee *et al.*, 1986; Morinaga *et al.*, 1985; Park *et al.*, 1991; Peberdy, 1979 and 1989; Yoo *et al.*, 1985). Key factors for the protoplast isolation are effective lytic enzymes and osmotic stabilizers. Incubation time and pH also affect the protoplast isolation yield (Davis, 1985; Peberdy, 1989; Quigley *et al.*, 1987; Yoo *et al.*, 1985). Protoplast regeneration is influenced by a variety of factors such as culture medium, osmotic stabilizer, plating density, condition of protoplast isolation system and organism (Farkas, 1979; Gold *et al.*, 1983; Peberdy, 1989; Yoo *et al.*, 1985).

Lentinula edodes is one of the most popular fungi in Korea. It is famous for unique taste, nutritive qualities, and several pharmaco-physiological effects (Chihara *et al.*, 1970; Fujii *et al.*, 1978; Maeda and Chihara, 1971; Mizoguchi *et al.*, 1984).

In this paper we describe the optimal conditions for the protoplast isolation and regeneration of the serine-requiring auxotroph of *L. edodes*, LE207. In addition, we describe characteristics of the protoplasts including nucleus number, DNA content and ultrastructure.

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MATERIALS AND METHODS

Reagents

Growth supplements were purchased from Difco Laboratories (Detroit, MI, USA). Novozyme 234 and Cellulase Onozuka R10 were purchased from Novo Industries (Bagsvaerd, Denmark) and Yakult (Japan), respectively. Glucose, sucrose, mannitol, sorbitol, K_2HPO_4, KH_2PO_4, $MgSO_4 \cdot 7H^2O$ were obtained from Sigma Chem. Co. (St. Louis, MO, USA).

Strain and culture condition

Ultraviolet ray was used to induce auxotroph LE207 (Ser^-) of *L. edodes* (Berk.) (Kim *et al.*, 1996). Media consisted of glucose 20 g, yeast extract 2.0 g, peptone 2.0 g, K_2HPO_4 1.0 g, KH_2PO_4 0.46 g, $MgSO_4 \cdot 7H_2O$ 0.5 g per liter, and different concentrations of agar (pH 6.2). Mycelium was inoculated on the media covered with cellophane or in the liquid medium and

cultured for 6-8 days at 30°C.

Formation of protoplasts

To determine the optimum conditions for the release of protoplasts from *L. edodes*, mycelia were harvested and washed with distilled water. The mycelia were treated with a mixture of Novozyme 234 and Cellulase Onozuka R10 in 0.6 M osmotic stabilizers at 30°C with shaking at 100-120 rpm. The lytic enzyme treated mycelia were sampled half hourly for 5 hrs to determine the formation of protoplasts. The remaining hyphal fragments were removed by filtration over sintered glass filter (porosity 1), and the filtrate was collected and centrifuged at 1000 rpm for 5 min. The supernatant was sedimented by centrifugation at 1000 rpm for 15 min and the protoplasts were washed twice with 0.6 M sucrose.

Regeneration of cell wall

Protoplast regeneration was carried out as described by Choi *et al.* (1987). Briefly, 0.5 ml of protoplast suspension in 0.6 M mannitol was plated on the regeneration medium which contained suitable osmotic stabilizers. And the plate was covered with 5 ml of the regeneration medium containing 0.75% agar and incubated at 25°C. The percentage of protoplast regeneration was calculated based on the ratio of the number of colonies to the number of nucleated protoplast inoculated.

Nucleus staining and DNA content

For nucleus staining, protoplasts were fixed with Helly's fixing solution (5% $HgCl_2$, 3% $K_2Cr_2O_7$, 6% formalin) for 15 min and washed with 70% methanol. After serial incubation in 1% NaCl for 1 hr and 1N HCl for 15 min at 60°C, they were washed with phosphate buffer (pH 7.0) and stained with Giemsa solution for 45 min. DNA content was estimated by a modified method of Labarca *et al.* (1980). Briefly, protoplasts were homogenized in ice cold PBS (pH 7.4), sonicated to complete break of protoplast membrane and nucleus membrane and stained with H33258 in PBS at final concentration of 1 μg/ml (Cesarone *et al.* 1979). As a standard, Na-DNA Type III from salmon testis (Sigma, USA) was used. After keeping in cold and dark place, fluorescence was determined using a fluorometer (FP-777, Jasco, Japan) at Ex 330 nm and Em 470 nm.

Electron microscopy

Protoplasts were fixed in 3% (v/v) glutaraldehyde in sodium phosphate buffer (pH 7.6) containing 0.6 M sucrose for 5 hrs. For scanning electron microscopy, samples were washed with 0.6 M sucrose, dehydrated in ethanol (30, 50, 60, 70, 80, 90, 95, 100%) and acetone. Gold particle coated samples were scanned using SEM (JSM-T200, Jeol, Japan). For transmission electron microscopy, glutaraldehyde fixed samples were followed by post fixation in 1% osmium tetroxide for 2 hrs at 4°C. The samples were washed with 0.5 M sucrose, dehydrated in an ethanol and propylene oxide, and embedded and polymerized in epon. Ultrathin sections (50-70 nm) were stained with uranyl acetate and lead citrate and scanned using TEM (JEM-1200 EXII, Jeol, Japan).

RESULTS

Liquid culture and protoplast formation

Protoplast formation from the mycelia of *L. edodes* grown on the cellophane covered over agar medium was very difficult. Hardly any protoplast release was observed from the mycelia grown on the cellophane covered over agar plate. The large number of the protoplasts were obtained from the mycelia which grew in liquid media. The best yield was obtained when the eight-day-old mycelia were incubated in 0.6 M mannitol (pH 4.0) containing Novozyme 234 (15 mg/ml) and Cellulase Onozuka R10 (10 mg/ml) for 3-4 hrs (Fig. 1).

Regeneration

About 0.18% of the protoplast was capable of regenerating to give visible colonies when plated on the medium containing 0.6 M sucrose. With mannitol, 0.08% of the protoplast was regenerated. But hardly any regeneration was observed in the media containing NaCl, KCl, or $MgSO_4$. Colonies were visible in 25°C incubator after 10-15 days (Fig. 2).

Nucleus number and DNA content

The protoplasts stained with Giemsa or 4,6-diamidino-2-phenylindole (DAPI), a specific dye for nucleus, proved that more than 90% of them contained nucleus varing in number one to three. The average nucleus number per protoplast was 1.1 and none of the protoplasts contained more than three nuclei (Table I). DNA content of 10^6 cells was determined after purifying DNA and being treated with Hoescht 33258. The DNA content per nucleus was 5.1 pg.

Ultrastructure

Both scanning and transmission electron micrographs of the protoplasts were shown in Fig. 3. The mean diameter was about 3-5 μm, but the protoplasts which were smaller than 2 μm and larger than 15 μm were also observed. Cell organelles, nuclei, mitochondria, lipid granules and various granules, were well defin-

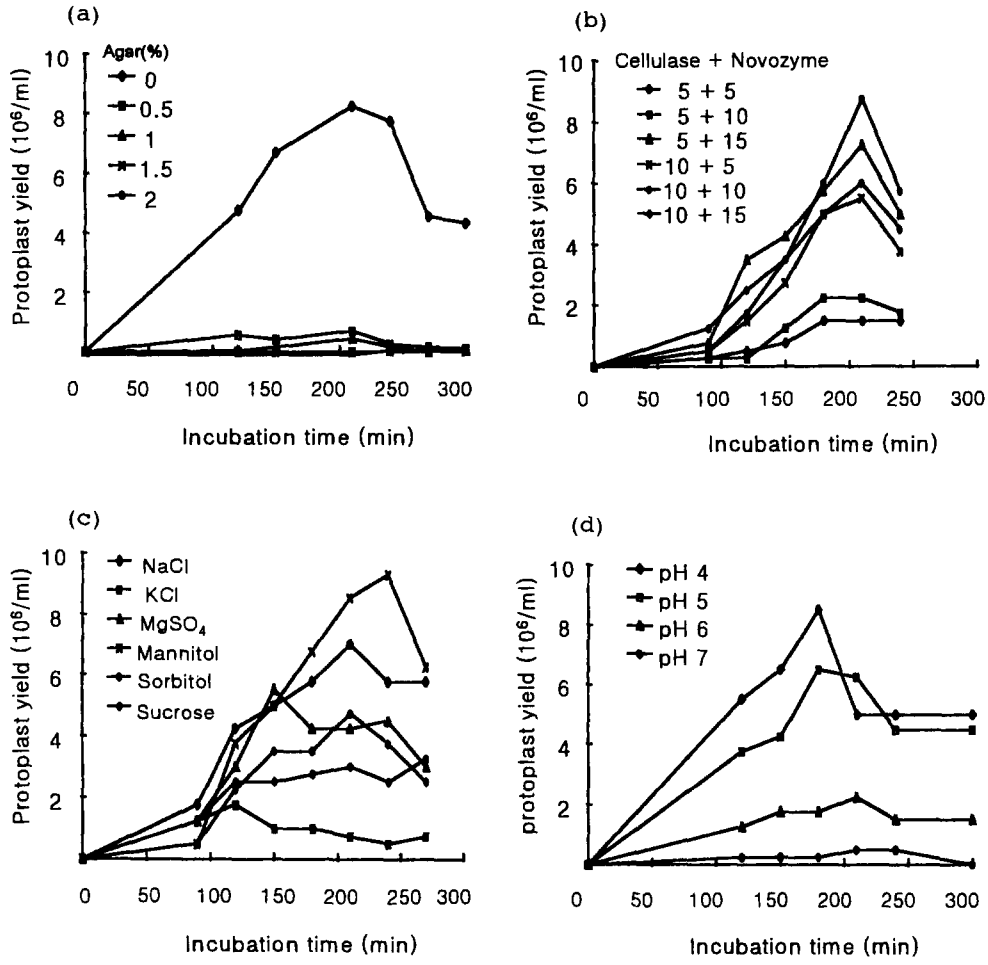

Fig. 1. Formation of protoplasts of auxotroph of *L. edodes* (Ser) in different conditions; a) agar concentration (%) of culture media, b) concentrations (mg/ml) of lytic enzymes, c) 0.6 M osmotic stabilizers, d) pH of the enzyme solution.

ed in the cytoplasm.

DISCUSSION

The isolation of protoplast from fungi is a very useful technique for fusion and transformation. Here we described studies on protoplast formation, regeneration, genetic characteristics and ultrastructure of auxotroph of *L. edodes*. In general, the effect of the media on protoplast release was not so extensive. The low yields of protoplast formation in liquid media was reported in filamentous fungi (Yoo *et al.*, 1985; Ferenczy *et al.*, 1975). But the protoplast isolation from *L. edodes* cultured on agar media revealed low yield (Kim, C. and Kim, B. K., unpublished observations). Here we used the mycelia grown in liquid media. The results in Fig. 1 indicated that liquid culture significantly enhanced protoplast release. This suggests that swelling of the mycelia may be important for the protoplast release.

Protoplast isolation frequency is affected by a suitable lytic enzyme for cell wall digestion. Novozyme 234 which has β-1,3-glucanase, chitinase and protease activities, showed higher yield than Cellulase Onozuka R10. A combination of Novozyme 234 and Cellulase Onozuka R10 showed the maximum yield. Combination of enzymes gives high yield of protoplasts in filamentous fungi (Lee *et al.*, 1986; Yoo *et al.*, 1985).

The osmotic stabilizers varied in their ability to release and maintain protoplasts. In general, inorganic salts are suitable osmotic stabilizer for the filamentous fungi, and sugars and sugar alcohols for the yeasts (Peberdy, 1979). In this experiment, the maximum isolation was obtained with 0.6 M mannitol. The use of NaCl, $MgSO_4$, and sucrose was less effective, and sorbitol and KCl had little effect.

The optimal incubation time for protoplast isolation was 3.5-4 hrs. The prolonged incubation with digestion enzymes decreased the protoplast number for it may cause the degradation of early formed protoplasts. It has been shown in some fungi that 3-4 hours incubation gives the best protoplast yield (Yamada *et al.*,

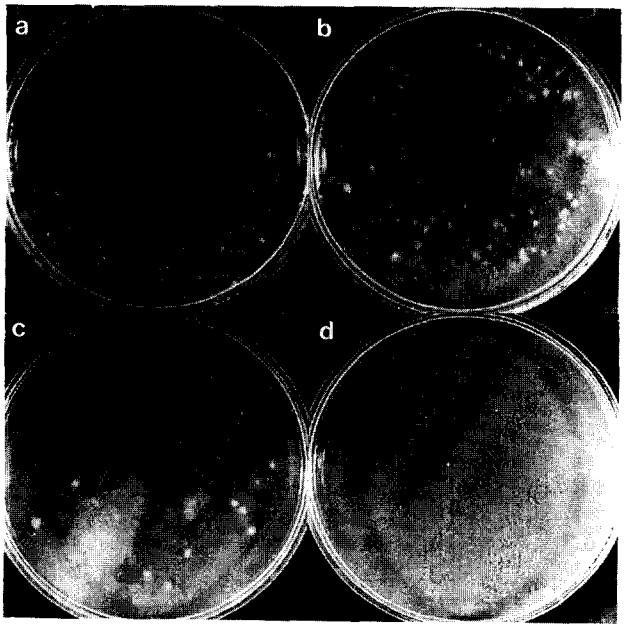

Fig. 2. Regeneration of the protoplasts of *L. edodes* on various regeneration media. Protoplasts were incubated at 25°C for 10-15 days on 0.6 M sucrose (a), mannitol (b), $MgSO_4$ (c), NaCl (d).

Table I. The distribution of nucleus in the protoplast from *Lentinula edodes*

Strain	% Nucleus No.*					Nucleus No. per cell
	0	1	2	3	4	
LE207	0.5	88.5	10.3	0.7	0.0	1.12

*Data were expressed as the percentage of 1000 count.

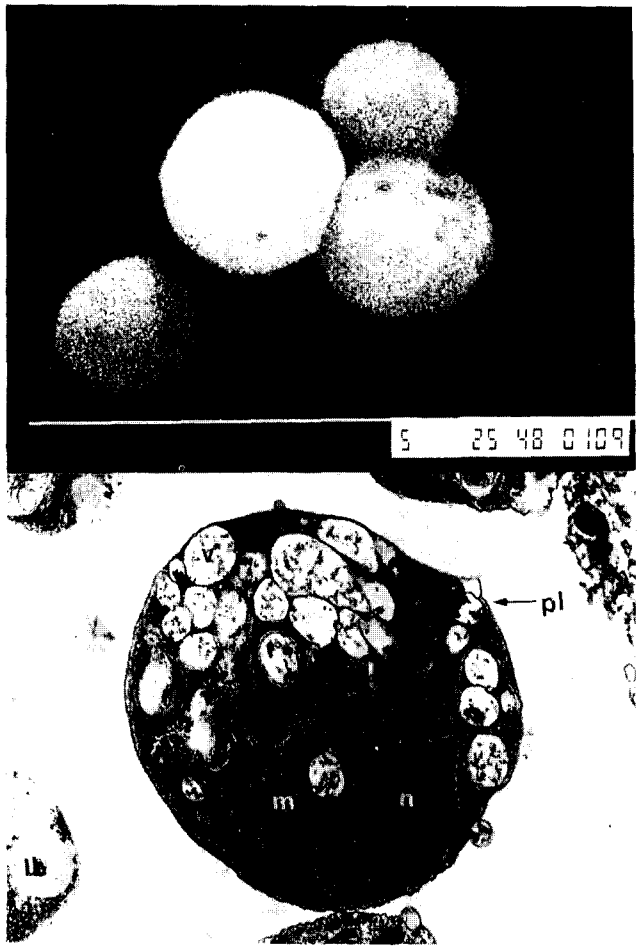

Fig. 3. Electron micrographs of the protoplasts from the auxotrophs of *L. edodes* (Ser). The mycelia were treated with 10 mg/ml Novozyme and 10 mg/ml Cellulase Onozuka R10 for 2-6 hrs. a) Scanning electron micrograph shows smooth surface round shape protoplast (×13000). b) Transmission electron micrograph represents well defined cell structure; n, nucleus; v, vacuole; lb, lipid body; m, mitochondria; pl, plasma membrane (×15000).

1983). Mycelial growth phase affect the efficiency of the protoplast formation. Mycelia from 6-8 days culture were adequate for protoplast formation and they were in exponential phase of growth (data not shown). The optimal pH was 4, and the release was decreased prominently over pH 6. Usually the optimum pH for the filamentous fungi was 5.5-6.0 (Quigley *et al.*, 1987).

Culture of the protoplasts on a hypertonic medium induces regeneration of new cell wall and their subsequent reversion to the normal hyphal form. The highest regeneration yield of the protoplasts of LE207 (Ser) was 0.18%, when supported by 0.6 M sucrose instead of 0.6 M mannitol (0.08%). The difference of osmotic stabilizers between formation and regeneration was not clearly understood, but it suggests that osmotic stabilizers may regulate lytic enzymes as well as cell wall synthesis. The reasons why sucrose and mannitol showed higher protoplast reversion than inorganic salts was not clear. However, it was assumed that ionic interactions involving the dissociated stabilizer affected the wall synthesis (Peberdy, 1980). One hundred percent of reversion to normal hyphae has never been reported, and the frequency can be quite variable depending on regeneration conditions. Regeneration frequency for *Lyophyllum shinen* and *Coprinus macrorhizus* were 0.003% and 39%, respectively (Abe *et al.*, 1984; Yanagi *et al.*, 1985). Generally low regeneration frequency is due to the absence of nuclei or the defection of organelles.

The nucleus number per protoplast was 1.1 and more than 90% of the protoplasts contained nucleus. The DNA content per nucleus was 5.1 pg. The reported DNA content per gram of the protoplasts of *Microsporium canis* and *M. gypseum* were 96.7 μg and 102.5 μg, respectively (Robinow *et al.*, 1966).

The availability of pure protoplasts allowed protoplast fusion and nuclear transfer. The protoplast isolation yield from the mycelia of *L. edodes* grown on agar media was not enough for the protoplast fusion,

but the isolation yield from the liquid cultured mycelia increased remarkably. The conditions reported here should be useful for the future studies. In addition, the ultrastructure of the protoplast gave us detailed understanding of fungal cell structure.

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