Note

Preparation and Regeneration of Protoplasts from Mycelia of *Morchella esculenta*Mitsuaki MORIGUCHI and
Seiichiro KOTEGAWADepartment of Environmental Chemistry and Engineering,
Faculty of Engineering, Oita University,
Oita 870-11, Japan

Received March 18, 1985

Edible fungi are mostly basidiomycetes, with a few notable exceptions being some species of *Tuber* and *Morchella* which are ascomycetes. Most of the ascomycete species grow wild, i.e., they are uncultivated. *Morchella esculenta* Fr. is highly prized for its culinary aspects, especially in European countries.

Whereas there have been some recent reports on the regeneration of basidiomycetes protoplasts for biochemical, morphogenetical and genetical studies,¹⁻⁶⁾ there have been none on *M. esculenta*. We have examined the conditions for the preparation and regeneration of protoplasts from mycelia of *M. esculenta*.

M. esculenta used in this study has been described previously.^{7,8)} Mycelia were cultured in potato extract medium⁷⁾ containing 0.5% glucose and 0.01% yeast extract (pH 6.3) (PGY medium). The cultures were grown in test tubes (1.5 × 10.5 cm) containing 2 ml of medium for 4 to 6 days at 24°C without shaking. Mycelia were harvest-

FIG. 1. Preparation of *Morchella* Protoplasts using Various Enzymes.

The potassium phosphate buffer (0.01 M, pH 7.0) contained 0.01% 2-mercaptoethanol, 0.6 M KCl and : 1.5 mg chitinase + 1.5 mg Zymolyase (○—○), 1.5 mg chitinase + 1.5 mg Zymolyase + 1.5 mg cellulase (●—●), 1.0 mg chitinase + 1.5 mg Zymolyase (□—□), 1.5 mg chitinase + 2.0 mg Zymolyase (■—■), or 2.0 mg chitinase + 1.5 mg Zymolyase (●—●).

FIG. 2. Protoplasts from Mycelia of *M. esculenta* (A) and Regeneration into Hyphae after 5 Days Culture (B).

Bars, 10 μ m.

TABLE I. REGENERATION OF *Morchella* PROTOPLASTS

	Digestion time (min)				
	30	60	90	120	180
Number of regenerated protoplasts	8	13	16	22	40
Regeneration percent (%)	0.11	0.026	0.029	0.064	0.10
Culture days	5	5	5	6	5

Potassium phosphate buffer (0.01 M, pH 7.0) containing 0.01% 2-mercaptoethanol, 0.6 M KCl, 3.0 mg chitinase and 3.0 mg Zymolyase in a final volume of 1.0 ml was filter-sterilized. After mycelia (about 20 mg wet weight) had been incubated in 0.5 ml of this buffer for the indicated times at 30°C with gentle shaking, the reaction mixture was passed through a sterilized 12G3 glass filter to remove mycelial debris. The protoplasts were aseptically washed five times with 2-ml of PGY medium containing 0.3 M inositol plus 0.3 M KCl by centrifugation at 3000 rpm for 6 min. The protoplasts were then suspended in 0.3 ml of the same medium in test tubes (1.5 × 10.5 cm), and were cultured at 24°C without shaking.

ed by centrifugation at 3000 rpm for 8 min, washed four times with 5 ml of sterile distilled water before protoplast preparation. The number of protoplasts released was determined photomicrographically.

Various combinations of chitinase (*Streptomyces antibioticus*, Calbiochem-Behring Co.), Zymolyase-20T (*Arthrobacter luteus*, Kirin Brewery Co.) and cellulase (*Aspergillus niger*, Serva Co.) were used to release protoplasts from the mycelia. Mycelia (about 50 mg wet weight) were digested in 0.5 ml of 0.01 M potassium phosphate buffer, pH 7.0, containing 0.01% 2-mercaptoethanol, 0.6 M KCl and various enzyme preparations at 30°C with gentle shaking. The largest numbers of protoplasts (1.7×10^6 per 0.5 ml) were obtained with a mixture of 1.5 mg chitinase and 1.5 mg Zymolyase after 4.5 hr digestion (Fig. 1). When chitinase from *Streptomyces griseus* (Sigma Co.), known to be effective for several basidiomycetes,⁴⁾ was used instead of the *Streptomyces antibioticus* chitinase, very few protoplasts were produced (1.1×10^5 per 0.5 ml). Addition of cellulase to the mixture of chitinase and Zymolyase did not result in a marked increase in the number of protoplasts. Few protoplasts were formed when chitinase, Zymolyase and cellulase were used singly, or when chitinase plus cellulase or Zymolyase plus cellulase were employed. The protoplasts were 5–20 μ m in diameter (Fig. 2).

Figure 3 shows the effects of various osmotic stabilizers on the preparation of protoplasts. KCl at 0.6 M was the most effective, whereas 0.3 M inositol plus 0.3 M KCl or 0.3 M mannitol plus 0.3 M KCl were less effective. With glucose, sucrose and mannitol at 0.6 M many protoplasts burst.

Some osmotic stabilizers were also tested as to their effects on the hyphal regeneration from protoplasts. Although 0.6 M KCl proved to be the most protective, it did not support regeneration of protoplasts. Regeneration occurred in the PGY medium supplemented with 0.3 M inositol plus 0.3 M KCl (Table I, Fig. 2). Inositol at 0.6 M, reported to be the most effective for regeneration in

FIG. 3. Effects of Various Osmotic Stabilizers on the Preparation of Protoplasts.

A combination of 1.5 mg chitinase and 1.5 mg Zymolyase was used.

○—○, 0.6 M KCl; ●—●, 0.6 M inositol; □—□, 0.6 M mannitol; ■—■, 0.6 M sucrose; ×—×, 0.6 M glucose; ●—●, 0.3 M inositol + 0.3 M KCl.

Lyophyllum shimeji,³⁾ was considerably less effective than 0.3 M inositol plus 0.3 M KCl (data not shown).

This study showed that protoplasts can be isolated from an edible ascomycete species. The protoplasts regenerated into hyphae at low frequencies.

REFERENCES

- 1) D. Moore, *Trans. Br. Mycol. Soc.*, **65**, 134 (1975).
 - 2) M. Abe, H. Umetsu, T. Nakai and D. Sasage, *Agric. Biol. Chem.*, **46**, 1955 (1982).
 - 3) M. Abe, T. Nakai, H. Umetsu and D. Sasage, *Agric. Biol. Chem.*, **48**, 1635 (1984).
 - 4) S. O. Yanagi and I. Takebe, *Appl. Microbiol. Biotechnol.*, **19**, 58 (1984).
 - 5) K. Akamatsu, T. Kamada and T. Takamaru, *Trans. Mycol. Soc. Jpn.*, **24**, 173 (1984).
 - 6) T. Morinaga, M. Kikuchi and R. Nomi, *Agric. Biol. Chem.*, **49**, 523 (1985).
 - 7) M. Moriguchi, S.-I. Sada and S.-I. Hatanaka, *Appl. Environ. Microbiol.*, **38**, 1018 (1979).
 - 8) M. Moriguchi, K. Kimura and S.-I. Hatanaka, *Trans. Mycol. Soc. Jpn.*, **24**, 191 (1983).
-