Note

Preparation and Regeneration of *Chalara paradoxa* Protoplasts[†]

Mitsuru MONMA and Keiji KAINUMA

National Food Research Institute, Ministry
of Agriculture, Forestry and Fisheries,
Yatabe-machi, Tsukuba-gun, Ibaraki
305, Japan

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Recently, Kainuma *et al.* reported *Chalara paradoxa* PNS-80 as a new raw starch-digesting amylase-producing fungus.⁽¹⁾ The properties of this amylase were reported in previous papers.^{2,3)} In recent years, protoplast fusion and genetic engineering have been considered as useful technologies for improving the properties of many enzyme-producing microorganisms. Isolation of protoplasts is important for serving cells without cell walls in protoplast fusion processes, or for extracting DNA's in genetic engineering. In this note, we briefly report on the isolation and regeneration of *Chalara paradoxa* mycelial protoplasts.

The organism used in this study was *Chalara paradoxa* PNS-80 which produced a highly active raw starch-

digesting amylase. The organism was maintained on a 1.5% agar slant of a medium containing soluble starch (10g), polypeptone (10g), beef extract (7g) and NaCl (3g) in one liter.

In order to isolate protoplasts, spores and oidia of hyphae obtained by washing a slant culture were inoculated into 20ml of YpG medium containing 0.1% yeast extract, 2.0% polypeptone and 1.0% glucose in 100ml conical flasks. Cultures were performed at 30°C without shaking for 14~18 hr. The cultured mycelium was harvested by filtration with a nylon bolting cloth (mesh opening, 120 μ m) and washed several times with sterilized water to exclude ungerminated and newly-formed spores or oidia. The washed mycelia were pressed gently with a spatula to remove the medium.

About 100mg of mycelia were transferred to 1 ml of enzyme solution in a short test tube, which was vortexed for a few seconds, and incubated in a water bath at 30°C with gentle shaking. The standard enzyme solution contained 0.1% (60 U/ml) Zymolyase 60000 (Kirin Brewery Co.), 0.1% (4 U/ml) chitinase (Sigma), 0.5 M mannitol and 20 mM sodium phosphate buffer (pH 6.4). Several commercial enzyme preparations were tested as to their ability to release mycelial protoplasts when used singly and in combinations, and the number of released protoplasts were counted by a haemocytometer. The results are shown in Table I. Protoplasts were not detectable with Zymolyase 60000 alone (A), and also chitinase alone yielded few protoplasts (B). The yield of protoplasts

TABLE I. RELEASE OF PROTOPLASTS FROM *Chalara paradoxa* MYCELIA USING VARIOUS ENZYME PREPARATIONS SINGLY AND IN COMBINATION^a

Enzyme solution	Number of protoplasts per ml $\times 10^{-5}$			
	Duration of treatment (min)			
	30	60	90	120
A. Zymolyase 60000 (0.1%)	ND	ND	ND	ND
B. Chitinase (Sigma 4U/ml)	10	10	10	10
C. Cellulase Onozuka R-10 (1%)	ND	ND	ND	ND
D. Zymolyase 60000 (0.1%) + chitinase (Sigma 4U/ml)	188	254	168	148
E. Zymolyase 60000 (0.1%) + Cellulase Onozuka R-10 (1%)	10	10	10	10
F. Chitinase (Sigma 4U/ml) + Cellulase Onozuka R-10 (1%)	ND	ND	ND	ND
G. Zymolyase 60000 (0.1%) + chitinase (Sigma 4U/ml) + Cellulase Onozuka R-10 (1%)	210	206	150	146

^a Mycelia of 100 mg wet weight were treated at 30°C with 1 ml of enzyme solution containing 0.5 M mannitol and 20 mM sodium phosphate buffer (pH 6.4).
ND, not detectable.

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FIG. 1. Mycelial Protoplasts of *Chalara paradoxa* and Their Regeneration. (The bar represents 15 μm .)

a: Freshly isolated protoplasts.

b: Protoplasts after 5 hr incubation with standard enzyme solution. Some protoplasts are fused to each other.

c: Normal regeneration form of a protoplast after keeping 8 hr in liquid YpG medium containing 0.5 M sucrose.

d: Bud-like regeneration of a protoplast.

was strikingly increased when these enzyme preparations were mixed (D); almost all the mycelium disappeared after 1 hr incubation, converting nearly all cells in the mycelium into protoplasts (more than 2.5×10^7 per ml). Cellulase Onozuka R-10 (Yakult Mfg. Co.) was also examined, but it was not so effective in this case.

When *C. paradoxa* mycelia were treated with the standard enzyme solution, protoplasts were released in a few minutes. After 30~60 min incubation, the number of released protoplasts reached the maximum. The diameter of the protoplasts varied considerably (5~30 μm) (Fig. 1a). This size heterogeneity perhaps reflects the varying ages of mycelia,⁴⁾ or pinching off of cytoplasm during the release of protoplasts from the half-lysed cell wall.⁵⁾ After a few hours incubation in the standard enzyme solution, the protoplasts were considerably aggregated and fused to each other spontaneously with high frequency (Fig. 1b). The fused protoplasts came to have loose structures under a microscope (Fig. 1b), and they gradually disintegrated, probably as a result of metabolic lysis.⁶⁾

Protoplasts isolated from the mycelia of *C. paradoxa* after 30 min incubation were quickly washed several times by centrifugation with a YpG medium containing 0.5 M sucrose, instead of 0.5 M mannitol, as an osmotic stabilizer. They regenerated into hyphal form after 5~10 hr

when cultured as a suspension in a liquid YpG medium, or spread on agar YpG plates containing 0.5 M sucrose. The percentage of regenerated protoplasts was about 10% on the agar medium. Since the protoplasts aggregated considerably in the liquid medium, an estimation of the ratio of regeneration in the liquid medium was difficult. The mode of regeneration from *C. paradoxa* protoplasts was similar to that of protoplasts of other filamentous fungi.^{4~7)} Apparently normal hyphae grew directly from the protoplasts (Fig. 1c); bud-like regeneration was only rarely observed (Fig. 1d).

In conclusion *Chalara paradoxa* protoplast was easily obtained with a mixture of Zymolyase and chitinase, and 10 percent of the protoplasts were regenerated into hyphal form. These observations suggest the possibility of improving the raw starch-digesting ability of *Chalara paradoxa* by protoplast fusion or by gene transfer via wall-less cells.

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