Analytical Studies on Regeneration of Protoplasts of *Geotrichum candidum* by Quantitative Thin-Layer-Agar Plating

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A preparation of pure protoplasts of *Geotrichum candidum* became osmotically stable and colonies developed when the protoplasts were embedded in stabilizing thin-layer-agar and incubated with stabilizing basal medium. When growing protoplasts were exposed to distilled water and then reincubated with basal medium, the process of regeneration of protoplasts could be quantitatively demonstrated by counting colonies. The process was divided into three phases, lag, logarithmic, and stationary. Furthermore, the state of regeneration of protoplasts at each phase could be seen in detail by microscopic studies of protoplasts under similar growth conditions. In the lag phase, which lasted for 2 hr after inoculation, protoplasts were completely destroyed when placed in distilled water. During the logarithmic phase, from 2 to 5 hr after inoculation, protoplasts rapidly became osmotically stable and about 18% of them were growing. In the stationary phase, most protoplasts developed germ tubes within 2 hr. These results suggested that there are two main phases, although individual cells passed through three different conditions, osmotically labile, osmotically stable, and growing. No apparent structure of cell wall material could be detected by electron microscopy on the surface of the membrane of these osmotically stable cells.

The ability of protoplasts of *Bacillus megaterium* to grow (5, 23) and divide during growth (10, 11, 12) has been described, but regeneration of bacterial protoplasts into new, normal cells has not been demonstrated.

However, regeneration of yeast protoplasts into normal cells was very occasionally observed in earlier works (1, 4, 9, 13).

Recently, there has been much discussion of whether fungal protoplasts can grow and regenerate (3, 7, 14, 20, 22), and in *Saccharomyces cerevisiae* (15, 16, 17) and in *Schizosaccharomyces pombe* (18), de novo formation of cell walls during regeneration of these protoplasts was clearly demonstrated in ultrathin sections.

This study demonstrated quantitatively the process of the regeneration of protoplasts of *Geotrichum candidum*; when placed in a certain environment, they became stable against osmotic shock and developed into colonies.

**MATERIALS AND METHODS**

**Organism, growth conditions, and harvesting.** *G. candidum* was obtained from the Institute for Fermentation, Osaka, Japan and was maintained on Sabouraud's glucose-agar slopes at room temperature.

Organisms from the stock culture were inoculated into petri dishes containing the same medium, and these plates were incubated at 30 C for 48 hr.

Young hyphae were prepared by incubation at 30 C for 3 hr in 500-ml flasks containing 100 ml of Sabouraud's liquid medium inoculated with a heavy suspension of a 48-hr subculture. The hyphae were collected by centrifugation at 700 X g and washed once with distilled water.

**Preparation and purification of protoplasts.** Protoplasts were obtained by a modification of the method of Eddy and Williamson (2). Young hyphae were suspended in 0.005 M McIlvaine's sodium citrate-phosphate-buffered solution (CPBS) (pH 6.0) containing a final concentration of 0.75 M sodium chloride (stabilizing CPBS) and 10% snail gut juice. The enzyme employed was "Sue d'Helix pomatia" obtained from l'Industrie Biologique Francaise, Seine, France. To prepare protoplasts, the cells were digested by incubation with gentle shaking at 30 C for 7 to 10 hr.

The protoplast suspension was then centrifuged at 300 X g for 10 min and washed three times with stabilizing CPBS.

This suspension was contaminated with a large amount of ghosts and partially digested cells (Fig.

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FIG. 1. Protoplast fraction of G. candidum. (A) Crude; (B) purified by filtration through a sintered-glass filter. ×600.
1A). As shown in Fig. 1B, these contaminants could be removed almost completely by careful filtration through a glass filter (mean pore size, 20 µm; type Hario-F 34, Shibata Chemical Co., Tokyo, Japan). The purified protoplast suspension was stored at 4 C until used.

**Media and conditions for regeneration of protoplasts.** The basal growth medium (BGM) contained 3% Yeast Extract (Difco), 1% peptone (Difco), 2% Heart Infusion Broth (Difco), and 4% glucose; the latter was autoclaved separately. As medium for stabilizing protoplasts, BGM containing 3% sodium chloride (stabilizing BGM) was employed. Protoplasts were quite stable in stabilizing BGM or in stabilizing CPBS.

The modified and stabilizing Czapek-Dox's medium (pH 6.8) tested as synthetic medium had the following composition (in g/liter): KCl, 0.5; MgSO₄, 0.5; FeSO₄, 0.01; K₂HPO₄, 1; NaNO₃, 3; glucose, 40; NaCl, 45; and water.

For serial dilution of protoplasts, stabilizing CPBS was employed. The concentration of purified protoplast suspension was determined by measurement of the number of protoplasts with a Bürker-Türk type hemocytometer in a phase-contrast microscope. To study regeneration of protoplasts in solid medium, a sterile 2% solution of Special Agar (Noble), from Difco, dissolved in stabilizing CPBS (stabilizing agar) was prepared and kept in a water bath at 47 C before use.

**Thin-layer-agar plating method.** Protoplast suspension (0.1 ml) and approximately 0.5 ml of stabilizing agar were mixed in plastic petri dishes floating on a water bath at 40 C and spread uniformly over the bottom of the dishes by means of a sterile, bent glass rod; then the dishes were kept at room temperature. To regenerate protoplasts, two incubation steps were used. First, stabilizing BGM was used, and, after lysis of osmotically fragile protoplasts with distilled water, BGM was employed alone in the second incubation. Onto the coagulated thin layer of agar (less than 1 mm thick) containing embedded protoplasts, 15 ml of stabilizing BGM was poured in the first step of the inoculation. Plates were incubated at 30 C for the required time. Each hour during incubation, five of these plates were taken out and the stabilizing BGM in them was replaced by sterile distilled water to lyse osmotically fragile protoplasts. After about 30 min, the distilled water was replaced by BGM. Then the plates were reincubated at 30 C overnight, and the amount of regeneration of protoplasts in the solid environment was determined by counting colonies.

**Phase-contrast microscopy.** The state of regeneration of protoplasts was observed by phase-contrast microscopy under similar growth conditions. Before and after destruction of protoplasts by distilled water, the number of stable or germinating protoplasts was counted.

Regeneration of protoplasts was also studied after incubation in stabilizing BGM at 30 C with gentle agitation. The number of osmotically stable or germinating protoplasts was counted under a phase-contrast microscope.

Electron microscopy. Protoplasts embedded in stabilizing agar were incubated with stabilizing BGM at 30 C for 5 hr. Blocks of agar were placed in distilled water to burst osmotically fragile protoplasts. These blocks were fixed for 2 hr in 6% glutaraldehyde in stabilizing CPBS and then postfixed in 1% osmium tetroxide overnight at 4 C. After fixation, dehydration was carried out with an ethyl alcohol series and by embedding in a mixture of methyl- and butyl-methacrylate monomers (4:6).

Sections were made with a Porter-Blum MT-2 ultramicrotome, and electron micrographs were made with a Hitachi, Type HU-11, electron microscope.

**RESULTS**

Purified protoplasts of _G. candidum_ were osmotically hypersensitive; spherical bodies were observed under the phase-contrast microscope (Fig. 1B). In electron micrographs of thin sections of protoplasts, no apparent cell wall materials could be observed on the surface (Fig. 6A), as we previously reported (8).

In quantitative studies on regeneration of protoplasts, the correlation between the number of colonies and the protoplast concentration was determined. For this estimation, 0.1 ml of twofold dilutions of 2.5 × 10⁴ protoplasts/ml was embedded in thin layers of agar and incubated at 30 C with stabilizing BGM. Figure 2 shows the

![Fig. 2. Correlation between the dilution of protoplast suspension inoculated and the number of colonies which developed. Original suspension contained 2.5 × 10⁴ protoplasts per ml, and serial 1:2 dilutions were made. Of each diluted suspension, 0.1 ml was embedded in stabilizing thin-layer 2% agar and incubated with stabilizing BGM at 30 C.](http://jb.asm.org/)
straight-line relationship between the dilution of protoplast suspension inoculated and the number of colonies observed. This experiment indicates that individual colonies were produced by regeneration of a single protoplast. The number of colonies developed by regeneration of protoplasts reached a maximum 17 hr after inoculation.

As mentioned above, these protoplasts were hypersensitive to osmotic shock and were completely destroyed on treatment with distilled water for 3 min. However, when the protoplasts were embedded in a thin layer of stabilizing agar and treated with enriched medium, such as stabilizing BGM, they became resistant to low osmotic pressure and developed into colonies the next day.

When osmotically fragile protoplasts were treated with stabilizing BGM at 30°C, after a lag period of about two hr they became resistant to osmotic shock when exposed to distilled water (Fig. 3A). The number of osmotically stable cells reached a maximum 5 hr after the treatment.

After incubations with stabilizing BGM at 0°C (Fig. 3D), with stabilizing CPBS without nutrients at 30°C (Fig. 3C), or with stabilizing BGM containing 10% snail gut juice at 30°C (Fig. 3E), the protoplasts were completely destroyed by osmotic shock and did not produce colonies. However, when protoplasts were inoculated with stabilizing BGM containing 10% snail gut juice for 3 hr and then, with stabilizing BGM only, they became resistant to osmotic shock after a lag period of 2 hr and developed into colonies (Fig. 3B).

Furthermore, osmotically stable cells at the end of the logarithmic phase again became osmotically hypersensitive upon treatment with BGM containing 10% snail gut juice for 2 to 3 hr.

Phase-contrast microscopy. A similar experiment was done by direct observation of approximately 500 protoplasts once each hour under a phase-contrast microscope. When protoplasts were placed in stabilizing BGM, they became slightly swollen during the 2-hr lag period. As shown in Figure 4A, the number of osmotically stable cells increased following a curve identical to that in Figure 3A. On the other hand, germinating cells first became visible after 2 to 3 hr of incubation, but they were poorly resistant to distilled water (Fig. 4B). The maximal number of osmotically stable cells was seen after 5 hr of incubation, whereas osmotically stable germinating cells represented about 18% of the total protoplasts at that time. The number of osmotically stable, germinating cells rapidly increased in the next hour and represented approximately 100% of the total after 7 hr (Fig. 4B).

A single protoplast germinated after 6 hr

![Fig. 3. Appearance of protoplasts resistant to distilled water and developing into colonies during regeneration by means of the quantitative thin-layer-agar plating technique. Symbols: ○ (A), stabilizing BGM added at 30°C; ● (B), stabilizing BGM containing 10% snail gut juice added for 3 hr at 30°C and then BGM only; X (C), stabilizing CPBS added at 30°C; ○ (D) stabilizing BGM added at 0°C; ● (E), stabilizing BGM containing 10% snail gut juice added at 30°C; ↓ exposure to distilled water.](http://jb.asm.org/)

After being placed in BGM for 5 hr, the membranes of protoplasts appeared slightly more electron-dense than did those of intact protoplasts, but no particular structure could be seen

Electron microscopy. Our previous studies (8) demonstrated that the fine structure of the surface of the intact protoplasmic membrane consists of a unit membrane. Studies were made of the changes in the surface structure of osmotically stable cells after treatment with stabilizing BGM for 5 hr (Fig. 6B) and upon exposure to distilled water (Fig. 6C).

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on the surface (Fig. 6B). On the surface of protoplasts incubated with stabilizing BGM and then exposed to distilled water for 30 min, a small amount of cytoplasm-like substance which might have leaked out through the membrane was observed (Fig. 6C arrows). However, the nuclei, mitochondria, and ribosomes were well preserved (Fig. 6C).

Other factors affecting regeneration of protoplasts. Regeneration of protoplasts could not be demonstrated in broth media, such as stabilizing BGM or medium containing 20% calf serum, and, in these media, protoplasts lysed within a few hours. After 24 hr, a few protoplasts showed irregular growth forms, similar to those reported by Gascon et al. (7) for various yeast and mold protoplasts. However, most abnormal cells lysed within an incubation period of 24 hr.

Protoplasts embedded in thin layers of stabilizing agar could regenerate in stabilizing BGM of pH 5.5 to 8.0 at 20 to 30 C.

In lower concentrations of agar (0.5 to 1.0%), fewer protoplasts regenerated and resolution of colonies was not so clear as it was at higher agar concentrations (1.5 to 2.0%).

Protoplasts, and even whole cells, embedded in thick layers of agar (5 mm or more) did not show a linear relationship between dilution of the suspension of protoplasts and the number of colonies which developed, and the regenerative rate was much lower. Therefore, it seems necessary to embed protoplasts in a thin layer of agar (less than 1 mm).

To obtain a high rate of regeneration of protoplasts, enriched medium, such as stabilizing BGM, was needed. When modified and stabilizing Czapek-Dox's medium was used during the lag and logarithmic phases, no protoplasts developed into colonies, although whole cells produced colonies at a high rate. Current work shows that protoplasts are able to develop moderately well into colonies after the addition of biotin and lipoic acid to modified and stabilizing Czapek-Dox's medium. Data on this will be published soon.

DISCUSSION

Nečas (14) reported mass regeneration of protoplasts of S. cerevisiae in a medium containing a high percentage of gelatin. Using protoplasts of S. fragilis, Rost and Venner (20) confirmed these results. Later, Svoboda (22) demonstrated a high percentage (50 to 70%) regeneration of protoplasts of S. cerevisiae into new, normal cells in 2% agar medium. The percentage of regeneration of protoplasts of Neurospora crassa varied widely between 20 and 80% (1).

Furthermore, Nečas et al. demonstrated by electron microscopy the de novo formation of cell walls on the surface of regenerating protoplasts of S. cerevisiae (15, 16, 17) and Schizosaccharomyces pombe (18).

In this study, the process of regeneration of protoplasts of G. candidum was divided into three phases (lag, logarithmic, and germinating phases) from results obtained by bursting protoplasts with distilled water at intervals during incubation and counting the colonies formed from the resulting mixture the next day. However, individual cells may occur in three conditions, osmotically labile, osmotically stable, and growing; all three conditions occur simultaneously in different proportions at various times during the logarithmic phase (the results of observation under the phase-contrast microscope). Hence, there are really two main phases, although in-
individual cells pass through three different conditions.

No apparent new structure of a cell wall could be detected on the surface of protoplasts in ultrathin sections at the end of the logarithmic phase, whereas these growing cells were resistant to lysis by distilled water.

Recently, the problem of whether yeast protoplasts are true protoplasts has been raised by some workers (6, 19, 21). Ottolenghi (19) re-
FIG. 6. Electron micrographs of protoplasts embedded in stabilizing 2% agar and incubated with stabilizing BGM for 5 hr at 30 C. A, intact protoplast; B, protoplast after 5 hr; C, protoplast incubated for 5 hr and then exposed to distilled water for 30 min. Symbols: N, nucleus; m, mitochondria; er, endoplasmic reticulum; arrows, cytoplasmic substances which might have leaked out on exposure to distilled water. ×30,000.
ported that annular rings of bud scars are attached to a membranous matter of yeast cells treated with snail gut enzyme. Streiblová (21), using the freeze-etching technique, reported that at least in some cases, not all the wall substance was removed from the surface of yeast protoplasts by treatment with snail enzyme. By structural and immunological studies of the protoplast membrane of *Candida utilis*, Garcia Mendoza et al. (6) suggested that this membrane had traces of structural cell wall material which was not detectable by electron microscopy.

In this study, protoplasts of *G. candidum* were not examined by freeze-etching or immunological techniques to see whether they were completely free of cell wall material. Therefore, it is not possible to state that they were true protoplasts which regenerated and developed into colonies. Probably, these protoplast suspensions were a mixture of protoplasts and spheroplasts. Moreover, remnants of cell wall material were probably important in incorporation of substances and as barriers resisting osmotic rupture. Even so, the quantitative thin-layer-agar plating method developed in this work seems valuable for studies on regeneration of protoplasts or spheroplasts and on de novo synthesis of the cell wall.

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**LITERATURE CITED**