Formation and Regeneration of Protoplasts from Blastospores of an Entomogenous Fungus, *Beauveria bassiana*

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Formation and regeneration of protoplasts from blastospores of *Beauveria bassiana* were studied. When blastospores from 7-day-old culture were treated with Zymolyase 5000 for 1 hr at 30°C, high yield of protoplasts of good quality was obtained. Sodium chloride and potassium chloride plus magnesium sulfate were effective as osmotic stabilizers for the preparation of protoplasts. Sodium chloride was also effective for regeneration of protoplasts. Mannitol, sorbitol, and sucrose were not effective as osmotic stabilizers for preparation of protoplasts. Sucrose inhibited the formation of protoplasts but it was necessary for their regeneration. The highest frequency of regeneration was 60%. When protoplasts were inoculated in regeneration medium and incubated at 25°C, two morphologically distinct regeneration types were observed: i) protoplasts producing one or two germ tubes and ii) protoplasts producing a mass of spherical abnormal cells which sometimes failed to grow to a normal hypha.

INTRODUCTION

Protoplast fusion is regarded as an effective tool for genetic study and breeding of fungi and yeasts (Kevei and Peberdy, 1977; Sipiczki and Ferenczy, 1977; Van Solingen and Van der Plaats, 1977; Stahl, 1978; Svoboda, 1978; Peberdy, 1980). However, little is known about the protoplast fusion (Paris, 1977; Ribb, 1978) and protoplast production (Kawula and Lingg, 1984; Pendland and Boucias, 1984) of the entomogenous fungi of Hyphomycetes including *Beauveria bassiana*, a promising agent for microbial control of insect pests.

The objective of this study was to determine the optimal conditions for the preparation and regeneration of protoplasts from blastospores (yeast-like cells formed in hemolymph or culture media) of *B. bassiana*.

MATERIALS AND METHODS

Fungus and growth conditions. *Beauveria bassiana* F-8 strain was used throughout this study. Sakaguchi flasks containing 100 ml of Sabouraud's dextrose broth plus 0.5% yeast extract, pH 6.4, were inoculated with 1×10^8 blastospores from 7-day-old cultures and were incubated at 25°C with continuous shaking.

Formation and isolation of protoplast. Blastospores were separated from hyphae of 7-day-old culture of *B. bassiana* by filtration through sterilized cotton. The filtrate was then centrifuged at 3,000 rpm for 20 min at 20°C and the pellet of blastospores were
Fig. 1. Light micrograph of Beauveria bassiana blastospores and protoplasts. A: blastospores from 7-day-old culture in Sabouraud's dextrose broth plus 0.5% yeast extract at 25°C on reciprocal shaker. B: protoplasts suspended in 0.1 M potassium phosphate buffer, pH 7.6, containing 0.7 M KCl and 5 mM MgSO₄. Bar = 10 μm.

Washed twice with 0.1 M potassium phosphate buffer, pH 7.6, by centrifugation at 3,000 rpm for 20 min at 20°C. The blastospores (1 × 10⁸ blastospores/ml) were treated with the cell wall lytic enzyme Zymolyase 5000 (Kirin Brewery Co., Tokyo) (8 mg/ml), in 0.1 M potassium phosphate buffer, pH 7.6, containing osmotic stabilizers for 60 min at 30°C with gentle shaking.

After treatment with Zymolyase 5000, protoplasts were washed twice with 0.1 M potassium phosphate buffer, pH 7.6, containing 0.7 M KCl and 5 mM MgSO₄ by centrifugation at 1,000 rpm for 10 min at 4°C. Protoplasts were purified by differential centrifugation at 400 rpm for 8 min and 800 rpm for 7 min at 4°C. Yields of protoplasts were determined by counting in a hemocytometer. The final preparation of protoplasts was more than 99% pure, with less than 1% blastospores.

Regeneration of protoplasts. The rate of protoplasts capable of regenerating was estimated by plating a known number of protoplasts on a solid medium containing osmotic stabilizers. Number of colonies formed was scored after incubation at 25°C for 5 days. For morphological studies, protoplasts were inoculated in a complete medium containing 0.6 M NaCl as the osmotic stabilizer and incubated at 25°C on a glass slide in the moist chamber.

Complete medium (CM) used in regeneration studies was modified Sabouraud's dextrose agar containing (g/l): glucose, 20; peptone, 10; yeast extract, 10; agar, 15; NaCl, 35; in distilled water. Minimal medium (MM) used was Czapek solution agar (Difco) containing 0.6 M NaCl. The following substances were used as osmotic stabilizers: KCl, NaCl, NH₄Cl, mannitol, sorbitol, and sucrose.
RESULTS

The lytic enzyme Zymolyase 5000 liberated protoplasts from blastospores of *B. bassiana* in 1 hr (Fig. 1). The protoplasts were osmotically sensitive and burst on transfer to water. Several osmotic stabilizers were tested for their effect on the stability of protoplasts. These osmotic stabilizers varied in their ability to stimulate the release and to maintain the stability of protoplasts (Fig. 2). Inorganic salts were more effective than sugars as osmotic stabilizers. The most effective stabilizer was 0.7 M KCl with 5 mM MgSO₄ and the least effective was NH₄Cl. Sugars were not effective as osmotic stabilizers in lytic system and sucrose inhibited protoplast formation. On the basis of these results, 0.7 M KCl and 5 mM MgSO₄ was used as osmotic stabilizer in subsequent experiments.

The treatment of the blastospores with 2-mercaptoethanol stimulated protoplast formation (Table 1). Pretreatment of the blastospores with 2-mercaptoethanol was more effective than its addition during the enzyme treatment.

The blastospores from batch cultures of different ages were tested for protoplast
Table 1. Effect of 2-mercaptoethanol (2-ME) on protoplast formation

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Washing</th>
<th>Enzyme treatment</th>
<th>Protoplasts formed (no. of protoplasts/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>+</td>
<td>With 2-ME</td>
<td>$4.2 \times 10^7$</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>Without 2-ME</td>
<td>$4.1 \times 10^7$</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>Without 2-ME</td>
<td>$5.3 \times 10^7$</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>With 2-ME</td>
<td>$1.3 \times 10^7$</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>Without 2-ME</td>
<td>$1.2 \times 10^6$</td>
</tr>
</tbody>
</table>

- Treatment with 1% 2-ME for 20 min at 30°C.
- Washing with 0.1 M potassium phosphate buffer, pH 7.6, before enzyme treatment.
- Enzyme treatment with or without 0.1% 2-ME for 60 min at 30°C.

Age of culture (days)

Fig. 3. Effect of age of culture of *Beauveria bassiana* on the yield of protoplasts. Concentration of the blastospores in Zymolase 5000 solution was $1 \times 10^8$ blastospores/ml. The number of protoplasts and blastospores was counted in a hemocytometer using phase-contrast microscope after enzyme treatment for 1 hr at 30°C.
yield. The yield of protoplasts was found variable depending on the age of the culture (Fig. 3). The yield of protoplasts increased and the proportion of blastospores remaining in the lytic mixture decreased with an increase in the age of the culture. When the size of protoplasts derived from 7-day-old culture was compared with that from 8-day-old culture, the former was apparently bigger than the latter. Based on the formation of protoplasts of good quality and the percentage of protoplasts formed, blastospores from 7-day-old culture were used for the preparation of protoplasts.

Protoplasts of *B. bassiana* regenerated on a solid medium containing osmotic stabilizers. The frequency of regeneration was less than 60% (Fig. 4). Inorganic salts and sucrose were the effective stabilizer for regeneration of protoplasts, while no stabilizing effect was observed in mannitol and sorbitol. Sodium chloride gave the highest frequency of protoplast regeneration (60%). There was no significant difference in regeneration frequencies in complete and minimal media.

As shown in Fig. 5, the morphologically distinct types of regeneration were observed; (1) protoplasts germinated directly and produced one or two germ tubes (Fig. 5A and C), and (2) protoplasts produced a mass of spherical abnormal cells (Fig. 5B and D). These abnormal cells in the latter sometimes failed to grow to a normal hypha.
Our present study suggests that the blastospores of *B. bassiana* are a good source of protoplasts. The results showed that inorganic salts were more effective than sugars as osmotic stabilizer in the lytic enzyme system. Paris (1977) reported that magnesium ion was important for the release of protoplasts in the protoplast formation from hyphae of *Beauveria tenella* using digestive juice of a snail, however, in our present study, this was not essential.

The use of 2-mercaptoethanol is known to enhance the release of protoplasts from yeasts or fungi (Davies and Elvin, 1964; Doeevaard-Kloosterziel et al., 1973). We also observed a similar enhancing effect of 2-mercaptoethanol in this study.

Besides β-1, 3-glucanase, Zymolyase 5000 contains mannanase, protease, and acid phosphatase. When Zymolyase 60000, which contains a trace of protease, was used for the release of protoplasts from blastospores of *B. bassiana*, the yield of protoplasts was very low. This suggests that the release of protoplasts from blastospores of *B. bassiana*
is due to the combined action of β-1, 3-glucanase and protease and it is likely that the protease plays a very important role. In *Candida albicans*, Torres-Bauza and Riggsby (1980) reported that the pretreatment with dithiothreitol and protease was necessary to produce the protoplasts. They showed that the outer layer of the cell wall was removed by the pretreatment.

The yield of protoplasts varied depending on the age of culture. This suggests that the susceptibility of the cell wall to lytic substances alters with the age of culture. It is of interest to note that the yield of protoplasts suddenly increased on the 8th day. The size of protoplasts from blastospores of 8-day-old culture was apparently smaller than that of 7-day-old culture. This may indicate that the unicellular blastospores became bicellular on the 8th day.

Kawula and Lingg (1984) showed that protoplasts of *Beauveria bassiana* regenerated on the surface of Sabouraud’s dextrose agar containing 0.4 M KCl as the osmotic stabilizer. In our study, protoplasts of *B. bassiana* regenerated not only on the complete medium containing 0.6 M NaCl but also on the minimal medium containing 0.6 M NaCl. On the contrary, when the suspension of protoplasts was plated on complete or minimal medium without osmotic stabilizer, no colony was formed.

In this study, two morphologically distinct types of regeneration were observed. This is in agreement with types of regeneration of other filamentous fungi such as *Neurospora crassa* (Bachmann and Bonner, 1959), *Fusarium culmorum* (García Acha et al., 1966), *Aspergillus nidulans* (Peberdy and Gibson, 1971), *Pythium PRL 2142* (Sietsma and de Boer, 1973), and *Penicillium chrysogenum* (Anné et al., 1974).

When protoplasts were inoculated in regeneration medium supplemented with different concentrations of agar (0.4–2.4%) and cultured at 25°C, the ratio of direct germination increases with the increasing concentration of agar (data not shown). According to Nečas (1971), the function of the gel surrounding protoplasts is to prevent soluble cell wall material from diffusing away from the protoplast surface. This may explain the reason why the ratio of direct germination of protoplasts increased in medium at higher concentration of agar.

ACKNOWLEDGEMENTS

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REFERENCES


Kevel, F. and J. F. Peberdy (1977) Interspecific hybridization between *Aspergillus nidulans* and *Asper*