Improvement of Protoplast Isolation from Lichen Mycobionts

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Lichens produce many characteristic metabolites with several biological activities.1 Few of these active substances, however, are used by mankind. This is because of the limited supply of natural lichens and their inability to grow under laboratory conditions. Slow growth and sensitivity to air pollution threaten lichens with extinction. These are reasons that gene preservation and breeding for industrial use are necessary for lichens.

A method of culture induction from lichen spores has been established2) and many papers have been published on culturing the bionts. Many lichen species, however, do not produce spores so that this method cannot be applied on these species. Yamamoto et al. (1985)3) have established in vitro culture techniques for vegetative thalli of lichens. With this method, we have obtained composite cultures of the mycobiont and photobiont from each of more than one hundred lichen species, including many with no spore. We have also established separate mycobiont and photobiont cultures by isolating each symbiont from the mixed cultures. Now we plan to enlarge our research on lichenology into the fields of cell technology. Our initial studies have focused on developing methods to improve the isolation of protoplasts from cultured lichen mycobionts.

Protoplasts of lichen mycobionts have been isolated by Ahmadjian et al. (1987).4) They isolated protoplasts from mycobiont cultures that originated from spores. We modified their method and applied it to mycobiont cultures obtained from vegetative thalli.

We collected Cladia aggregata (Cladoniaceae) at Takao, Kyoto, Japan and induced a mixed culture from vegetative thalli of that by the induction method originated by Yamamoto et al.3) A mycobiont culture of Cladia aggregata was isolated from the mixed culture. Mycobiont cells were maintained in liquid MY medium (malt extract 2% w/v, yeast extract 0.2% w/v) at 20°C in the dark with shaking (80 rpm). Cultured mycelia were subcultured every 3 weeks. We fragmented mycelia with a glass homogenizer before each subculture to prevent the mycelia from becoming entangled and compact. Mycelia from eight-day-old cultures were collected on a 150 μm nylon mesh filter and then homogenized in CP (citrate-phosphate) buffer (pH 5.8). The mycelial suspension was incubated in CP buffer containing 10mM 2-mercaptoethanol for 60 min at 25°C with shaking. Incubation in CP buffer was effective for protoplast isolation and the addition of 2-mercaptoethanol increased the isolation efficiency. After being washed at least twice with CP buffer, 0.8 g of mycelia were incubated in 80 ml of CP buffer containing 0.1% w/v NovoZym 234 (Novo Industri) and 0.6M MgSO4 for 12 hr at 25°C, 60 rpm for protoplast formation. Protoplasts were purified by filtrations of the mycelial suspension through 20, 10, and 5 μm nylon meshes. The number of protoplasts were counted with a hemocytometer by microscopy after they were washed twice with 0.6M MgCl2.

The results of our protoplast isolation method are shown in Table I. The table also includes the results of protoplast isolations from five other cultured Cladonia mycobionts that had been induced from spores. The culture source of Cladonia bellidiflora was the American Type Culture Collection (ATCC 18270) and four other

<table>
<thead>
<tr>
<th>Cladoniaceae mycobiont</th>
<th>Cladia aggregata</th>
<th>Cladonia cristatella</th>
<th>Cladonia pleurota</th>
<th>Cladonia bellidiflora</th>
<th>Cladonia stellaris</th>
<th>Cladonia boryi</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protoplasts (x 10⁴/g FW of mycelia)</td>
<td>37</td>
<td>234</td>
<td>6</td>
<td>9</td>
<td>560</td>
<td>9</td>
</tr>
</tbody>
</table>

Table II. Results of Protoplast Isolation from Cladonia cristatella Mycobiont by Cellulase, Chitinase, and Protease

<table>
<thead>
<tr>
<th>Enzyme concentration</th>
<th>Meicelase (Meiji Seika)</th>
<th>Chitinase (Sigma)</th>
<th>Ficin (Wako)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1%</td>
<td>0.02%</td>
<td>0.1%</td>
<td></td>
</tr>
<tr>
<td>1%</td>
<td>0.02%</td>
<td>0.1%</td>
<td></td>
</tr>
<tr>
<td>NovoZym 234 (Novo Industri) 0.1%</td>
<td>28</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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**Fig. 1.** Protoplasts Isolated from *Cladonia cristatella* Mycobiont by NovoZym 234.

The sizes of protoplasts ranged from 2 to 8 μm. (Left): A protoplast being released at an edge of a hypha is seen in the right photo. The bar represents 10 μm.

*Cladonia* mycobionts were from the Clark University culture collection of lichen bionts. We isolated protoplasts from the mycobiont cultures that originated from vegetative thalli as well as from the five cultures from spores. The mycobiont *Cladia aggregata* had a lower isolation efficiency than those of *Cladonia cristatella* or *C. stellaris*. We assume this was because the isolation conditions were not optimal for *C. aggregata* mycelia.

We also investigated which activity(ies) of NovoZym 234 was effective for protoplast isolation. Hamlyn et al. (1981) showed that NovoZym 234 has the activities of cellulase, chitinase, and protease. We tried to isolate mycobiont protoplasts with other enzyme(s). We isolated protoplasts with the same method mentioned above with other enzyme(s) instead of NovoZym 234. We used Meicelase (as cellulase, Meiji Seika) only or combined with Chitinase (as chitinase, Sigma) and Ficin (as protease, Wako). The mycobiont culture of *C. cristatella* from a spore was used as material. The results are shown in Table II. Protoplasts isolated with NovoZym 234 are shown in Fig. 1. All three activities were necessary for efficient isolation of protoplasts. The combination of 1% Meicelase, 0.02% Chitinase and 0.1% Ficin showed a higher isolation efficiency than NovoZym 234 only. Our study shows that we can increase the efficiency of protoplast isolations from lichen mycobionts through the improvement of enzyme combinations and concentrations.

**References**