Protease Production by Species of *Entomophthora*

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Ten insect-pathogenic species of *Entomophthora* showed wide variation in their ability to produce alkaline protease in surface culture. *E. coronata*, the most active producer, was selected for studies in submerged culture together with *E. virulenta*. All media tested appeared suitable for mycelial growth of these two organisms, but a liver medium was superior for the production of protease. The effect of the constituents of the liver medium upon yield was investigated. The lag between growth and the production of protease was 24 to 40 hr, and only very small amounts of protease were obtained from sonically treated mycelium. The pH values during growth rose from ranges of 4.5 to 7.5 in the initial medium to 7.2 to 7.9, and did not affect the final yields. The optimal temperature for the production of protease by *E. coronata* was 24 to 32 C, and good growth was observed at temperatures as low as 16 C. The process with *E. coronata* was scaled up to fermentors without a decrease in yield; 5 enzyme units/liter were obtained after approximately 33 hr. This corresponds to a maximal productivity of 0.45 enzyme unit per liter per hr during the protease-producing phase. The process was insensitive to changes in aeration rate. The liver in the medium was replaced by various agricultural by-products, meat scrap, rapeseed oil meal, cottonseed nutrients, milk powder, and meat hydrolysate, with approximately the same or higher yields of protease.

Members of the genus *Entomophthora* are well known as pathogens in insects, where they often appear as a hairy or velvety covering on the surface (7). This role as causative agents of insect diseases is considered to be of great importance (18). In warm-blooded animals and man, however, these infections have been observed relatively rarely (3, 5). Many of these phycomycetes grow very slowly in pure culture and have not been cultivated until recently (7, 8); others are easily grown in ordinary media. Some of the fast-growing members of this group have been used for the production of a proteolytic enzyme with a pH optimum for hydrolysis of casein at about 9.0 (A. R. Whitehill et al., U.S. Patent 2,936,265, 1960). In the present study, the production of protease by 10 species of *Entomophthora* was investigated. Two species were studied in more detail in submerged culture.

**Materials and Methods**

**Maintenance.** The strains used were supplied by Magnus Gustafsson at this college. Their origin is given in Table 1. All strains, except *E. spharosperma* and *E. thaxteriana*, have been deposited at Centraalbureau voor Schimmelcultures, Baarn, Netherlands. Organisms were cultivated at 20 C on Sabouraud agar slants, which contained (g/liter, in distilled water): glucose, 40; Neopeptone (Difco), 10; and agar (Difco), 20. Every 3 to 4 weeks a 10- to 20-mm³ portion of each culture was transferred to a fresh slant.

Two species, *E. coronata* and *E. virulenta*, survived on agar slants or under mineral oil at 2 C for 4 to 6 months; freeze-drying was unsuccessful. Cells did not survive after freezing at -20 C, and freezing was used for killing harvested mycelial cultures for further study.

**Cultivation.** A fresh Sabouraud agar slant was inoculated from the culture collection and incubated at 24 C for about 2 weeks. A small portion (10 to 20 mm³) of this culture was used for inoculation of the stationary, 200-ml Erlenmeyer flask containing 25 ml of liquid medium and a layer of glass beads, 5 mm in diameter. After 5 days at 24 C, the culture was suspended by shaking and was used to inoculate a 1-liter Erlenmeyer flask with 250 ml of medium for test in shake cultures (170 rev/min, model G10 shaker; New Brunswick Scientific Co., New Brunswick, N.J.). For pilot plant studies, 100 ml of the 5-day shake culture (24 C) was transferred to a conventional 10-liter steel fermentor (16) containing 6 liters of medium. Sabouraud glucose medium was used in all propagation steps. The various media are given under Results. The processes were run at 24 C with agitation at 260 rev/min, and 1 ml of silicon antifoam emulsion (Midland Silicon Ltd., Reading, U.K.) per liter was added before sterilization. The pH was recorded automatically.

**Disintegration.** For ultrasonic disintegration, 25-ml samples were filtered by suction, and the mycelium was suspended in 5 ml of tris(hydroxymethyl)aminomethane buffer, pH 7.5 (6). This suspension was
TABLE 1. Strains of Entomophthora

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
<th>Year of isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coronata 1</td>
<td>Soil</td>
<td>1961</td>
</tr>
<tr>
<td>E. sphaeosperma</td>
<td>Insect (Diptera)</td>
<td>1962</td>
</tr>
<tr>
<td>E. virulenta E...</td>
<td>Insect (Aphis)</td>
<td>1961</td>
</tr>
<tr>
<td>E. rhizospora 296</td>
<td>Insect (Trichoptera)</td>
<td>1962</td>
</tr>
<tr>
<td>E. conica 248 A..</td>
<td>Insect (Diptera)</td>
<td>1962</td>
</tr>
<tr>
<td>E. curvispora 2581</td>
<td>Insect (Aphis)</td>
<td>1962</td>
</tr>
<tr>
<td>E. aphidis 107...</td>
<td>Insect (Aphis)</td>
<td>1961</td>
</tr>
<tr>
<td>E. apiculata 166..</td>
<td>Insect (Psocoptera)</td>
<td>1961</td>
</tr>
<tr>
<td>E. ovispora 295...</td>
<td>Insect (Diptera)</td>
<td>1962</td>
</tr>
<tr>
<td>E. thaxteriana a</td>
<td>Insect (Aphis)</td>
<td>1960</td>
</tr>
</tbody>
</table>


FIG. 2. Conidia of Entomophthora coronata from an agar slant culture.
to consist of: liver (Difco), 8 g; KH₂PO₄, 1 g; and distilled water, 1 liter. The course of protease production in shake flasks with this medium is shown in Fig. 5. Similar yields were obtained in media that contained, in addition to KH₂PO₄, 12 g of a water-soluble liver digest (Oxo Ltd., London, U.K.) per liter, or 8 g of a liver desiccate (Oxo) per liter.

**Growth and protease production.** The relationship between growth and protease secretion by *E. coronata* was studied in a clear medium containing 12 g of water-soluble liver digest (Oxo) and 1 g of KH₂PO₄ per liter. At each incubation time, two complete parallel 250-ml cultures were harvested and assayed separately. After 24 hr of incubation, the dry weight of mycelium was almost maximal (5 to 6 g/liter) and protease production had just started (Fig. 6). After 41 hr, mycelial weight decreased slowly and the protease activity of the culture liquor was essentially constant at about 6 EU/liter. The pH of the culture, which was originally adjusted to about 6.8 with sodium hydroxide before inoculation, rose, with protease secretion, to 8.1.

Similar shake cultures of *E. coronata* were harvested at intervals after 17 to 50 hr for ultrasonic treatment. This process caused drastic disruption of the mycelium (Fig. 7). About 80%

**Fig. 3.** Effect of liver concentration on protease production. Symbols: ○, 2; ●, 4; □, 6; △, 8; ▲, 10 g of liver per liter.

**Fig. 4.** Effect of glucose concentration on protease production. Symbols: ■, *Entomophthora coronata*; □, *Entomophthora virulenta*. (Maximal yields were calculated for each species separately.)

**Fig. 5.** Protease production by *Entomophthora coronata* in a simplified liver medium.

**Fig. 6.** Protease production by *Entomophthora coronata* in a liver digest medium.
of the mycelial weight was lost into the filtrate by the ultrasonic treatment (Table 2). However, only very small amounts of protease were detected in the filtered sonic extract or in the resuspended, sonically treated mycelium at various stages of the growth and protease production phase. Ultrasonic treatment had no effect on the activity of the culture liquors.

The relationship between growth and protease production in *E. virulenta* was similar. For this study, the medium contained 30 g of glucose per liter, in addition to the other ingredients. In this species, which was a slower producer of protease than *E. coronata* (Fig. 3), the lag between mycelial growth and protease production was about 40 hr. The pH of the medium dropped to a minimum of 5, because of the glucose, before protease production started. This low pH did not seem to affect mycelial growth.

Effect of pH. The ability of *E. virulenta* and *E. coronata* to grow and to produce protease in media with pH values in the range of 4.5 to 7.5 was studied in submerged culture with liver medium. The pH values were adjusted before inoculation with hydrochloric acid and sodium hydroxide. At the time of maximal protease activity, about 50 hr after inoculation, the pH of these cultures rose to a range of 7.2 to 7.9, and the final protease yields were not affected.

Effect of temperature. The effect of temperature on growth and protease production by *E. coronata* was investigated by using the water-soluble liver digest medium in 20-ml surface cultures in Erlenmeyer flasks. The mean values from four 5-day cultures at each temperature (11 to 42°C) are given in Fig. 8. Under these conditions, the optimal temperature for protease production was 24 to 32°C and the optimal temperature for growth appeared to be lower, about 16 to 24°C. (Standard error of the mycelium weights of grown cultures was within 1 to 8% of the mean.) A temperature of 24°C was selected for further studies.

Large-scale production. Protease production by *E. coronata* in liver medium was successfully scaled up from shake flasks to 6-liter fermentors, and yields of 5 EU/liter were obtained (Fig. 9). This value corresponds to the maximal protease productivity of 0.45 EU per liter per hr measured between 22 and 33 hr. This period of protease production paralleled a decrease in the concentration of glucose, which originated from the inoculum (Fig. 9). Protease production also paralleled the increase in pH from 7.2 to about 8.0. The Kjeldahl nitrogen in the culture liquor was approximately constant throughout.

This process was remarkably insensitive to changes in aeration within the region of 0.1 to

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**TABLE 2. Ultrasonic disintegration of Entomophthora coronata**

<table>
<thead>
<tr>
<th>Incubation time (hr)</th>
<th>pH</th>
<th>Mycelium (g/liter)</th>
<th>Protease (enzyme units(^a) per liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No sonic treatment</td>
<td>After sonic treatment</td>
</tr>
<tr>
<td>17</td>
<td>6.9</td>
<td>4.5</td>
<td>0.9</td>
</tr>
<tr>
<td>23</td>
<td>7.2</td>
<td>5.7</td>
<td>1.1</td>
</tr>
<tr>
<td>29</td>
<td>7.5</td>
<td>5.7</td>
<td>1.2</td>
</tr>
<tr>
<td>40</td>
<td>8.2</td>
<td>6.0</td>
<td>1.5</td>
</tr>
<tr>
<td>50</td>
<td>8.1</td>
<td>5.2</td>
<td>1.5</td>
</tr>
</tbody>
</table>

\(^a\) See Materials and Methods for definition of "enzyme unit."
seemed to have been evacuated (Fig. 11). The amount of mycelium formed was, as in shake flask cultures, relatively small. About 2 g (dry weight) per liter was found at harvest. A part of this weight may be residues of the suspended liver. Practically no growth adhered to the walls of the fermentors.

Fig. 9. Protease production by Entomophthora coronata in 6-liter batches of liver medium. Symbols: aeration, 0.5 (○) and 1.0 (▲) liter of air per liter per min.

Fig. 10. Effect of aeration on protease yield of Entomophthora coronata.

1.0 liter of air per liter of medium per min (Fig. 10). This aeration corresponds in this equipment to 0.1 to 0.4 mmoles of oxygen per liter per min, measured by the sulfite method (2).

Mycelium from fermentor cultures of E. coronata showed no evident autolysis between 22 and 46 hr, when the protease content rose from 0 to 5 EU/liter; after 93 hr, most of the mycelium

Fig. 11. Fermentor cultures of Entomophthora coronata after cultivation for 22 hr (A), 46 hr (B), and 93 hr (C).
**TABLE 3. Low-cost media for protease production with Entomophthora coronata**

<table>
<thead>
<tr>
<th>Main componenta</th>
<th>Origin</th>
<th>Conc (g/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein hydrolysate</td>
<td>Casamino acids (Difco), other componentsb</td>
<td>10</td>
</tr>
<tr>
<td>Potato fruit water</td>
<td>Potato and water (1:1), treated in a Turmix blender and filtered</td>
<td>300</td>
</tr>
<tr>
<td>Meat scrap</td>
<td>As described (9)</td>
<td>9</td>
</tr>
<tr>
<td>Rapeseed oil meal</td>
<td>As described (9)</td>
<td>15</td>
</tr>
<tr>
<td>Cottonseed flour</td>
<td>“Proflo,” Traders Protein Division, Fort Worth, Texas</td>
<td>10</td>
</tr>
<tr>
<td>Cottonseed protein</td>
<td>“Pharmamedia,” Traders Protein Division, Fort Worth, Texas</td>
<td>11</td>
</tr>
<tr>
<td>Milk powder</td>
<td>“Famos,” Semper AB, Stockholm, Sweden</td>
<td>15</td>
</tr>
<tr>
<td>Meat hydrolysate</td>
<td>“BBCT,” Bast’s Talgsmelteri, Copenhagen, Denmark</td>
<td>12</td>
</tr>
</tbody>
</table>

a The main component was dissolved or suspended in distilled water; 1 g of KH₂PO₄ per liter was added, and the pH was adjusted to about 7.0 with HCl or NaOH. All media in flasks were autoclaved at 120°C for 20 min and in fermentors at 110°C for 1 hr.

**Development of low-cost medium.** The liver in this medium was replaced by various inexpensive products, originating predominantly from agricultural wastes or low-cost by-products (Table 3). Extracellular protease activity was found in 250-ml shake flask cultures of *E. coronata* after 1 to 3 days in these media (Fig. 12). Several products were essentially as satisfactory as liver: meat scrap, rapeseed oil meal, cottonseed nutrients, and milk powder. Cottonseed nutrients at one-half the nitrogen equivalent of liver (0.5 g of nitrogen per liter) resulted in half the yield of protease. A meat hydrolysate, BBCT (Bast Bovine Cell Tissue), surpassed liver by yielding over 13 EU/liter, but the activity in this medium seemed relatively unstable. This peak of activity was repeated in several independent cultures. Casein hydrolysate supported only low protease production. Potato fruit water also gave relatively low protease activity, but this medium contained only 0.2 g of nitrogen per liter, and this by-product may be more useful if undiluted. A medium with cottonseed protein (Pharmamedia, 11 g; and KH₂PO₄, 1 g/liter in distilled water, pH 7.0) was selected for fermentor studies. This medium, when run in a 6-liter fermentor, gave yields of 4 EU/liter (Fig. 13). Maximal productivity was 0.5 EU per liter per hour calculated during the production period of 20 to 27 hr.

**DISCUSSION**

Although most of the *Entomophthora* species are slow-growing, 6 of the 10 species tested produced measurable amounts of protease; and some of them, particularly *E. coronata*, grew rapidly enough to be useful in industrial production. In this respect, *E. coronata* may be considered distinct from many other members of the *Entomophthora* group.

The taxonomy within *Entomophthoraceae* is somewhat unclear. *E. coronata*, for example, is also known under the names of *Delacroixia coronata* or *Conidiobolus villosus*, and most recently it was included in a key to known species of the genus *Conidiobolus* with the name *Conidiobolus coronatus* (17). In the present study, we used the names given by the donor (7, 8).

Because freeze-drying of these cultures is difficult, strain maintenance is a problem. For long-term maintenance, freezing in liquid nitrogen may be the best method. The relatively frequent transfers necessary from agar slants is particularly risky when dealing with the polynucleate mycelium of these phycomycetes. In spite of the changes that are expected in such fungal populations, protease production by the strains of *E. coronata* and *E. virulenta* studied showed good consistency during this 3-yr investigation.

**Fig. 12.** Protease production by Entomophthora coronata in low-cost media. Results from liver medium are given for comparison.
protease yield (5 EU/liter) corresponds to 0.6 EU/g (dry weight). This concentration (per liter) is equivalent to about 10 Anson-hemoglobin units, as used by other workers (according to analyses by Villy Jensen, Novo Industri, A/S, Kopenhagen, Denmark). One EU corresponds to approximately 100 mg of crystalline trypsin, assayed at pH 9.5.

The process in liver medium was easily scaled up to fermentors without loss of activity. Since the organism seemed insensitive to changes in the nitrogen and carbon sources, low-cost media based on agricultural wastes and by-products were satisfactory.

The physical state of this phycomycete compares favorably with that of some of the imperfect fungi, which, in previous studies (10–12), formed viscous cultures with voluminous mycelium. In contrast, E. coronata in a suitable medium formed a relatively small amount of mycelium and never produced voluminous growth on the walls and other inner surfaces of the fermentors. The mycelium was easily separated from the culture liquor.

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LITERATURE CITED