Protease production by *Rhizopus oligosporus* in solid-state fermentation

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Rice bran was superior to other proteinaceous substrates for protease production by *Rhizopus oligosporus ACM 145F* in solid-state fermentation. Maximum protease yield was after 72 h. The optimal initial moisture content was 47% (a_w = 0.97). Dried, ground and resuspended fermented rice was the most practical and effective inoculum preparation, although, in the laboratory, spore suspensions prepared directly from agar slants were more convenient. Inoculum density (from 10^2 to 10^7 spores/g substrate) and age (3, 5, 7 and 9 days) had little effect on protease yield.

Key words: Protease, Rhizopus, solid-state fermentation.

Fungal proteases are of particular importance in the food industry (Kalisz 1988) *Aspergillus* and *Mucor* have been studied intensively as protease producers (Klapper et al. 1973; Narahara et al. 1982; Fukushima et al. 1989; Thakur et al. 1990; Battaglino et al. 1991), although *Rhizopus oligosporus* also produces proteases, has a high proteolytic activity in the tempe fermentation (Yokotsuka 1991) and, furthermore, does not produce toxins (Gumbira-Sa'id et al. 1991). Thakur et al. (1990) reported that *R. oligosporus* produced a satisfactory calf rennet substitute on a laboratory scale. However, industrial production of protease by this fungus has not been investigated.

Solid-state cultivation systems (Wang et al. 1974; Thakur et al. 1990; Battaglino et al. 1991; Malathi & Chakraborty 1991) and submerged liquid cultivation systems (Klapper et al. 1973; Nakadai & Nasuno 1988; Fukushima et al. 1989) have been used for protease production, although most research has used liquid culture, which allows greater control of temperature, pH etc. However, solid-state fermentation has the potential for higher protease yields (Wang et al. 1974; Lonsane & Ghildyal 1992) and therefore deserves further investigation. In addition, solid-state fermentation has advantages for low-technology applications, such as the simplicity of the techniques and the low moisture content, which can prevent bacterial contamination during fermentation.

There is no commercial enzyme production process in Indonesia and enzyme imports continue to increase (Capricorn Indonesia Consult 1989). This study explores the potential of a low-technology process for protease production, involving solid-state cultivation of *Rhizopus oligosporus*. Several solid substrates are compared, including rice bran, which is abundant in Indonesia as a by-product of rice milling. The effects of initial moisture content and inoculum preparation are also investigated.

**Materials and Methods**

**Microorganism and Maintenance**

*Rhizopus oligosporus* ACM 145F (from the Culture Collection, Department of Microbiology, The University of Queensland, Australia) was maintained on potato/dextrose/agar (PDA). Suspensions containing 10^7 spores/ml were prepared by the addition of sterile water to 3-day-old PDA slants.

**Comparison of Substrates**

Solid media for protease production were prepared by mixing 10 g of proteinaceous substrates (see Table 1) with 6 ml 0.01% (w/v) Hortico trace element fertilizer (HTEF). Media in 250-ml Erlenmeyer flasks were autoclaved at 121°C for 15 min, and each flask inoculated with 1 ml spore suspension (as prepared above). Cultures were incubated at 37°C and harvested at 72 h. Each experiment was performed twice.

**Fermentation Profile**

Rice bran and wheat bran media [6.25 g dry bran and 3.75 ml 0.01% (w/v) HTEF] in 250-ml Erlenmeyer flasks were inoculated with 1 ml spore suspension per flask, and incubated for 120 h at 37°C. Duplicate flasks were removed for sampling.

**Comparison of Initial Moisture Content**

For comparison of initial moisture content, 2, 4, 6, 8 or 12 ml 0.01% (w/v) HTEF were added to 10 g dry rice bran. These media
Table 1. Protease yield of *Rhizopus oligosporus* ACM 145F in several proteinaceous substrates.

<table>
<thead>
<tr>
<th>Substrate*</th>
<th>Protease yield (10^6 PU/g initial fresh substrate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat bran</td>
<td>2.3</td>
</tr>
<tr>
<td>Wheat bran + soy flour (7:3, w/w)</td>
<td>2.1</td>
</tr>
<tr>
<td>Wheat bran + wheat flour (7:3, w/w)</td>
<td>2.5</td>
</tr>
<tr>
<td>Wheat bran + soy oil (9:1, w/v)</td>
<td>2.1</td>
</tr>
<tr>
<td>Soy bean</td>
<td>0.2</td>
</tr>
<tr>
<td>Soy bean + rice flour (7:3, w/w)</td>
<td>0.3</td>
</tr>
<tr>
<td>Soy bean + wheat flour (7:3, w/w)</td>
<td>0.2</td>
</tr>
<tr>
<td>Rice</td>
<td>0.2</td>
</tr>
<tr>
<td>Rice + rice bran (7:3, w/w)</td>
<td>2.6</td>
</tr>
<tr>
<td>Rice + soy flour (7:3, w/w)</td>
<td>1.5</td>
</tr>
<tr>
<td>Rice bran</td>
<td>3.9</td>
</tr>
<tr>
<td>Rice bran + wheat flour (7:3, w/w)</td>
<td>1.9</td>
</tr>
<tr>
<td>Rice bran + cassava starch (7:3, w/w)</td>
<td>1.4</td>
</tr>
</tbody>
</table>

* Rice flour was from Golden Australia P/L, soy flour from Lowan Whole Foods Pty Ltd, rice bran from Rice Growers' Co-operative Ltd, wheat flour from Home Brand-Woolworths and cassava starch from Thai World Import Export Co. Ltd.

were inoculated and incubated as before. Duplicate flasks were removed at each sampling and the experiment was repeated.

**Comparison of Inoculum Types**
Rice and bran (6.25 g dry substrate and 3.75 ml water) were inoculated with 0.1 ml spore suspension per flask and incubated for 3 days at 37°C. Three methods of preparation of inocula from these rice and rice bran cultures were compared with inocula from PDA slants:

1. Fermented substrate was removed from the flask, placed in a petri dish, dried for 4 days at 37°C and then ground with a mortar and pestle. This was used as an inoculum at 0.1 g per 10 g medium.
2. Fermented substrate was dried and ground as in (1), resuspended in water (1 g/10 ml) and 1 ml of this suspension was inoculated in 10 g of medium.
3. Fresh fermented substrate was ground and 0.1 g was directly inoculated in 10 g medium.

Rice bran medium (6.25 g rice bran and 3.75 ml 0.01% HTEF) was placed in each 250-ml Erlenmeyer flask, inoculated as described in (1), (2) or (3) above, or with 1 ml of spore solution from a 3-day-old PDA slant. Cultures were incubated at 37°C. Duplicate flasks were harvested.

**Comparison of Inoculum Density**
Spore solutions containing 10^7 to 10^8 spores/ml were prepared from fermented rice (3 days' fermentation) and 3-day-old PDA slants. One ml of each inoculum was inoculated in 10 g of rice bran medium (6.25 g rice bran and 3.75 ml 0.01% HTEF). Cultures were incubated at 37°C. Duplicate flasks were harvested.

**Comparison of Inoculum Age**
Rice inoculum was incubated for up to 9 days and prepared as in (2). Duplicate flasks containing 10 g rice bran media were inoculated with 1 ml inoculum and incubated at 37°C for 72 h.

Comparison of Fermenter Types
Flasks, packed-bed (Gumbira-Sa'id et al. 1991), small tray and rolling-drum fermenters were used. These fermenters contained 10 g, 100 g, 150 g and 1 kg of rice bran media (with a composition of 6.25 g rice bran per 3.75 ml 0.01% HTEF), respectively. A spore suspension was prepared by the addition of 10 ml sterile water per 0.05 g of 5-day-old dried mouldy rice. This solution was inoculated into the rice bran media (1 ml per 10 g media). The cultures were incubated at 37°C. To reduce moisture loss during incubation, the trays were placed within a 9-l airight box. The packed-bed fermenter was aerated with 0.7 to 0.81 air/min. The rolling-drum had a capacity of 20 l. It was rotated at 15 rev/min and supplied with 2.51 dry air/min. Samples were assayed at 0, 45 and 72 h. Duplicate flasks were removed at each sampling time for flask cultures, whereas duplicate samples from the packed-bed and the trays, each of approximately 10 g, were collected from random areas within the substrate mass. For the rolling-drum fermenter, duplicate samples of approximately 10 g were taken from duplicate drums. The experiments using flask, packed-bed and tray fermenters were repeated.

Crude Enzyme Preparation
Crude protease was recovered by the addition of 100 ml 0.1 M phosphate buffer (pH 7.0) to the mouldy substrate. The mixture was homogenized with a Virtis homogenizer. Proteases were extracted by shaking this homogenate (70 to 80 oscillations/min) for 1 h. Solids were separated by centrifuging and the clear extract was used for protease assay.

Protease Assay
Protease activity was measured using a modified Anson method, as described by Adler-Nissim (1986). Each incubation (465 μl) contained 10 mg BSA/ml (dissolved in 0.1 M citrate buffer, pH 3.0) and crude enzyme preparation. The proteolytic reaction was carried out at 40°C and stopped after 45 min by adding 300 μl trichloroacetic acid (10%, w/v). The precipitate was removed by centrifugation and the amount of tyrosine in the acid-soluble hydrolys products was determined by a Folin-reaction method, as described by Hanson & Phillips (1981) except that the CuSO₄·5H₂O was dissolved in distilled water. A blank was prepared using the same steps, except that trichloroacetic acid was added prior to the addition of enzyme. One unit of protease activity (PU) was defined as 1 nmol tyrosine equivalents released per 45 min.

**Moisture Content and Water Activity Determination**
Moisture content was determined by drying the sample at 110°C for 24 h. Water activity was determined with a Novasina Humidat IC-1 hygrometer.

**Results and Discussion**

**Substrate Choice**
Protease production by *Rhizopus oligosporus* ACM 145F on a range of solid substrates was compared (Table 1). The highest protease yield (3.9 x 10⁶ PU/g solid substrate) was obtained with rice bran. This was higher than the protease yield produced by *R. oligosporus* in wheat bran (1.5 x 10⁶ PU/g solid substrate) calculated from the results of Wang et al. (1974). However, the poorest yield occurred with rice. Very poor yields were also obtained on the substrates based on soy beans. In general, supplementation of wheat bran, soy beans,