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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 flash 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

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Table 1. Protease yield of *R oligosporus* ACM 145F in several proteinaceous substrates.

Substrate*	Protease yield (10^5 PU/g initial fresh substrate)
Wheat bran	2.3
Wheat bran + soy flour (7:3, w/w)	2.1
Wheat bran + wheat flour (7:3, w/w)	2.5
Wheat bran + soy oil (9:1, w/v)	2.1
Soy bean	0.2
Soy bean + rice flour (7:3, w/w)	0.3
Soy bean + wheat flour (7:3, w/w)	0.2
Rice	0.2
Rice + rice bran (7:3, w/w)	2.6
Rice + soy flour (7:3, w/w)	1.5
Rice bran	3.9
Rice bran + wheat flour (7:3, w/w)	1.9
Rice bran + cassave starch (7:3, w/w)	1.4

* 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 0.1ml 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 10^3 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 airtight box. The packed-bed fermenter was aerated with 0.7 to 0.8 l air/min. The rolling-drum had a capacity of 20 l. It was rotated at 15 rev/min and supplied with 2.5 l 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-Nisson (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 tryosine in the acid-soluble hydrolysis products was determined by a Folin-reaction method, as described by Hanson & Phillips (1981) except that the $CuSO_4.5H_2O$ 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 (3.9×10^5 PU/g solid substrate) was obtained with rice bran. This was higher than the protease yield produced by *R. oligosporus* in wheat bran (1.5×10^5 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,

rice or rice bran with flours, starches and oils had little beneficial effect and often decreased protease yield.

In the current study, wheat bran was included because it has been widely used for protease production (Wang *et al.* 1974; Thakur *et al.* 1990; Battaglino *et al.* 1991; Malathi & Chakraborty 1991). Rice, rice bran and soy beans are available in Indonesia but wheat bran is not.

Rhizopus oligosporus produces proteolytic enzymes during the soybean *tempe* fermentation (Hesseltine *et al.* 1967; Wang *et al.* 1974; Nout & Rombouts 1990; Yokotsuka 1991). The utilization of rice as a single substrate and its combination with soy beans for the production of *tempe*-type products has been explored by Hesseltine *et al.* (1967). Rice bran supports growth of *Aspergillus flavus* but not protease production (Malathi & Chakraborty 1991) although wheat bran supplemented with rice bran is used for protease production with *Aspergillus oryzae* (Battaglino *et al.* 1991). Rice bran was chosen as the substrate for further investigation because it gave high protease yields and is readily available in Indonesia.

Fermentation Profile

The protease production profile in rice bran was followed to determine the optimal harvest time (Figure 1). Wheat bran profiles were included for comparison since, as stated above, wheat bran has been widely used for protease production.

Production of protease by *R. oligosporus* began within 10 h in rice bran, whereas in wheat bran production began after 10 h. The maximum protease yield occurred at 72 h with rice bran, and 58 h with wheat bran. After the maximum protease yield was attained, it then decreased rapidly. A similar protease profile was observed with *Bacillus subtilis* in liquid culture by Chu *et al.* (1992), who ascribed the decrease to deactivation by self-digestion (as a dominant factor), a second protease released during the stationary phase of growth and denaturation. However, Wang *et al.* (1974) claimed the rapid inactivation of protease produced by *R. oligosporus* in wheat bran after 3 days incubation at 32°C might be due to an alkaline shift in pH rather than self-digestion.

Initial Moisture Content and Water Activity

Initial moisture content significantly affects hydrolytic enzyme production in solid-state fermentation (Nishio *et al.* 1979; Narahara *et al.* 1982; Battaglino *et al.* 1991). The effects of moisture are related to the water activity (a_w) which expresses the availability of water in a medium.

The maximum protease yield occurred with an initial moisture content of 47% (a_w = 0.97) (Figure 2). Qualitatively, this moisture content led to good growth and sporulation. At 51% moisture (a_w = 0.98) growth appeared better but sporulation was poorer and the protease yield was lower. Both growth and sporulation were very poor and protease production was inhibited at the lowest initial moisture content tested (31%, a_w = 0.94); *Rhizopus oligosporus* grows poorly at this water activity (Glenn & Rogers 1988).

At the highest initial moisture content (60%), growth occurred only on the substrate surface, and the protease yield was very low. This may be due to the filling of interparticle spaces within the substrate mass with water, which limits the diffusion of O_2 (Battaglino *et al.* 1991).

Wang *et al.* (1974) obtained similar results for the growth of *R. oligosporus* on a wheat bran medium. Poor growth and low protease yields occurred at 35% moisture. At 63% moisture growth was more rapid than at 50% moisture but protease yields were lower.

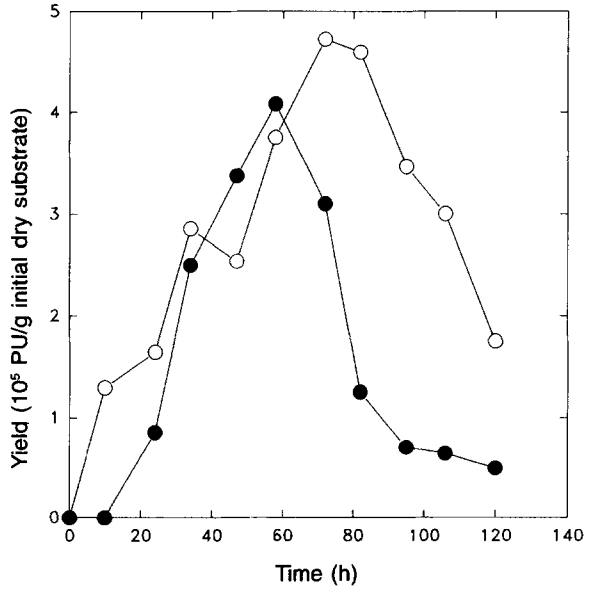

Figure 1. Protease production of *R. oligosporus* in rice bran (O) and wheat bran (●). The cultures were incubated at 37°C. Duplicate flasks were removed at sampling.

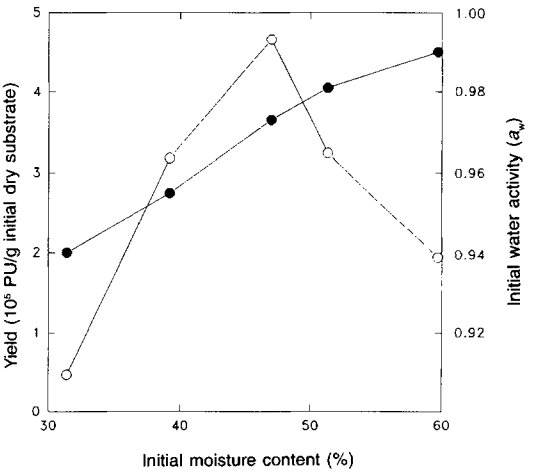

Figure 2. The influence of initial moisture content on initial water activity (●) and protease yield of *R. oligosporus* ACM 145F (O). The cultures were incubated at 37°C. Two sets of experiments were carried out and duplicate samples were taken at each sampling.

Effect of Inoculum

In protease production by fungi, either spores or mycelia can be used as inoculum. Spores are preferable because of the convenience of preparation, their stability during storage and tolerance of mistreatment during harvesting (Mitchell 1992). Different methods of preparation of spore inocula were investigated to identify the most practical and effective method for large-scale production.

Besides the fermentations inoculated with the spore suspension prepared from agar slants, the best protease yields were obtained using inocula prepared by drying and grinding fermented substrates and suspending the ground material in water before inoculation (Figure 3). This method led to well-distributed growth. At larger scale, this will be the most practical and effective method of inoculum preparation and cheaper than the use of agar slants. However, for small-scale fermentations, suspensions prepared from agar slants remain the most convenient (Hesseltine *et al.* 1976).

When the fermented substrates were dried and ground and then used directly for inoculation, the subsequent protease yields were relatively poor (Figure 3). The poorest protease yields were obtained in fermentations inoculated directly with fresh fermented substrates. The fresh inoculum aggregated in clumps and could not be distributed evenly throughout the substrate.

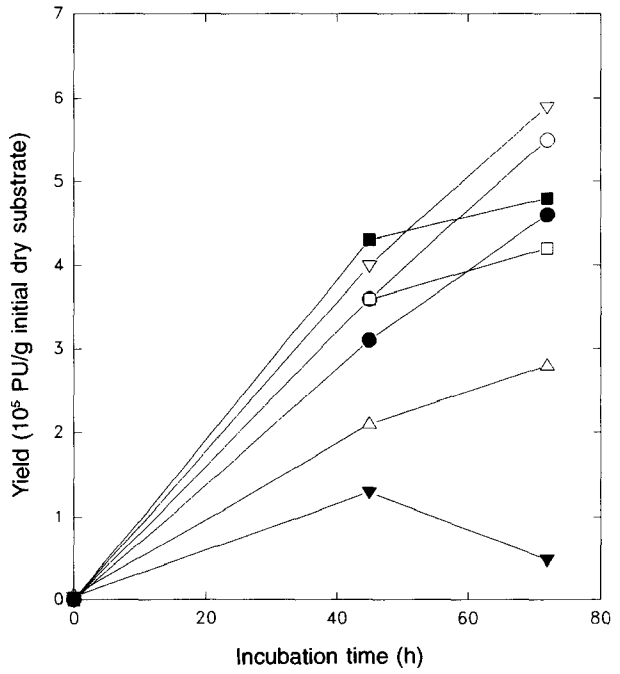

Figure 3. The influence of inoculum preparation methods on protease production of *R. oligosporus* ACM 145F. The cultures were incubated at 37°C. Duplicate flasks were harvested for protease assay. ∇—Dried, ground and suspended rice; O—PDA slant; ■—dried, ground and suspended rice bran; ●—dried and ground rice; □—dried and ground rice bran; Δ—freshly ground rice bran; ▼—freshly ground rice.

Rice was a better substrate than rice bran for sporulation of *R. oligosporus*. This may be due to better O_2 diffusion into the voids between rice grains. Rice is therefore better than rice bran for inoculum production, although the inoculum produced from rice bran and that produced from rice gave almost the same protease yield.

For inoculum densities from 10^2 to 10^7 spores/g substrate the protease yield remained between 5×10^5 and 7×10^5 PU/g initial dry substrate. This occurred for inocula prepared from either PDA slants or dried, ground and resuspended rice. Although it is difficult to produce a consistent density of inoculum, there should be little variation in process performance. Due to their cost and simplicity of preparation, lower spore densities will be preferable for protease production at larger scale. A spore density of 10^6 to 10^7 spores/g substrate was chosen for the next experiment.

Similar protease yields were achieved by *R. oligosporus* ACM 145F in rice bran media mixed with 3-, 5-, 7- or 9-day-old spore inocula prepared from PDA slants or fermented rice. Malathi & Chakraborty (1991) reported that a 5- to 7-day-old inoculum gave high protease yield with *A. oryzea*. However, the longer preparation time needed for older inocula is not convenient. For the next experiment, 5-day-old spore inoculum was chosen.

Fermenter Types

Figure 4 shows protease production in several types of laboratory-scale fermenter. The highest protease yield was achieved in flasks. The protease yield in the packed-bed was

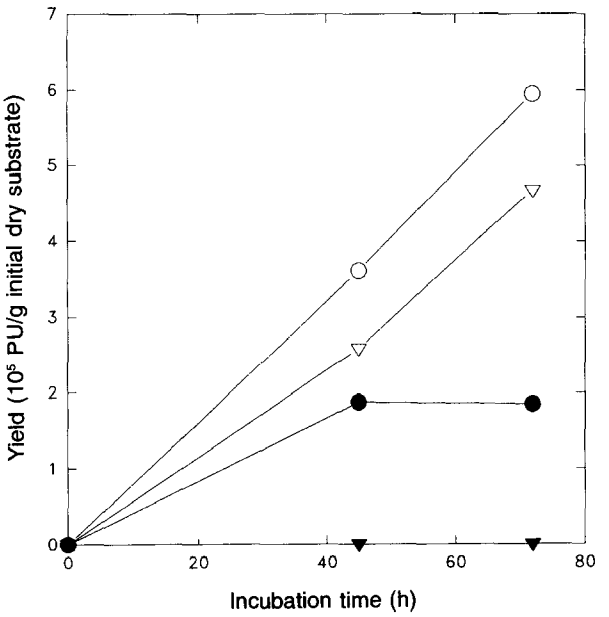

Figure 4. Protease production in several types of fermenter. The cultures were incubated at 37°C. At each sampling, duplicate flasks were removed and duplicate samples (10g) were taken from random areas in the packed bed, trays and drums. O—Flask; ∇—packed bed; ●—tray; ▼—rolling drum.

slightly lower than that in flasks, although, qualitatively, growth was similar. In trays, the mould grew poorly and produced less protease. This may be due to evaporation, which tends to dry out the media and cause growth inhibition. Protease was not produced in the rolling-drum fermenter. In this fermenter, the rice bran agglomerated into large lumps (approximately 3 to 5 cm in diameter). The shear effects on the surface of the lumps and the inadequacy of O_2 transfer within the lumps prevented growth and protease production. Flasks were chosen for continued laboratory studies because of the yield and the convenience of their preparation. Further advantages of using flasks are that each flask is identical, which means samples are representative, and that samples can be removed without disrupting future samples. In a larger bioreactor, growth can be heterogeneous and removal of samples can disrupt fungal mycelia, adversely affecting growth.

At larger scale, the use of larger flasks as well as trays may be labour-intensive and cause difficulties in control of the environment for growth and protease production. Rolling-drum fermenters for protease production using rice bran as substrate may be inappropriate due to the substrate characteristics. Packed-bed fermenters with temperature, humidity and aeration control are therefore the most promising fermenters for large-scale operations.

Solid-state cultivation has potential for low-technology protease production in Indonesia because of its simplicity. A laboratory system for studying this fermentation has now been developed. However, optimization of protease production by *R. oligosporus* using rice bran media, either at a laboratory scale or a larger scale, is still required.

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