Protease production by *Aspergillus oryzae* in solid-state fermentation using agroindustrial substrates

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Abstract

BACKGROUND: An inexpensive and readily available agroindustrial substrate such as rice bran can be used to produce cheap commercial enzymes by solid-state fermentation. This work investigates the production of food-grade proteases by solid-state fermentation using readily available Thai rice bran.

RESULTS: A local strain of *Aspergillus oryzae* (Ozykat-1) was used to produce proteases. Rice bran used alone proved to have poor substrate morphology (insufficient porosity) for satisfactory solid-state fermentation. A certain amount of wheat bran was necessary to improve the morphology of the substrate. The following variables affected protease production: substrate composition, initial moisture content and initial pH. A high protease activity (~1200 U g^{-1} dry solids) was obtained on a substrate that had a wheat bran to rice bran ratio of 0.33 by dry weight, a moisture content of 50%, initial pH of 7.5, and incubation temperature of 30 °C.

CONCLUSION: Nutritionally, rice bran used alone was as good a substrate as mixed bran for producing protease, but rice bran had poor morphological characteristics for consistent fermentation. A substrate that had a wheat bran to rice bran ratio of 0.33 by dry weight was best for producing protease.

Keywords: *Aspergillus oryzae*; solid-state fermentation; proteases; amylases

INTRODUCTION

Protease is one of the most important commercial enzymes, and is used in food processing, detergents, diary industry and leathermaking.[1] Proteases occur widely in plants and animals, but commercial proteases are produced exclusively from microorganism. Molds of the genera *Aspergillus*, *Penicillium* and *Rhizopus* are especially useful for producing proteases, as several species of these genera are generally regarded as safe.[2] This work reports on the use of rice bran and combinations of rice and wheat brans, for producing proteases of *Aspergillus oryzae* by solid-state fermentation. *A. oryzae* is widely used in Asiatic food fermentations, therefore, its proteases can be used in food processing and other applications. The effects of various combinations of the two brans, initial pH, and initial moisture content, on protease production are examined. Because both proteases and amylases are produced simultaneously in most fermentations, production of amylases is also studied.

Proteases are extracellular enzymes that can be produced by both submerged fermentation and solid-state fermentation. Solid-state fermentation is especially suited to growth of fungi because of their lower moisture requirements compared with bacteria.[3] Solid-state fermentations are simple, low cost, and provide high yields of appropriate enzymes.[4] The enzymes produced are more concentrated than those from submerged cultures. Problems with solid-state fermentation include incomplete utilization of the nutrients because of poor oxygen and heat transfer in the substrate.[5] Solid-state fermentations can use inexpensive and widely available agricultural residues as substrates.

Although wheat bran has been found to be a suitable substrate for producing mold proteases by solid-state fermentation,[6] wheat bran is not readily available in many regions. For example, in Thailand, 70% of the agricultural land is used for producing rice,[7] therefore, rice bran is most readily available. In many Asian countries rice bran is less expensive than wheat bran.[2]

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Brans contain fat, crude fiber, carbohydrate, and protein.[8,9] Rice bran has a moisture content in the range 7–13%, depending on the initial moisture content of the rice before milling.[10] Carbohydrate content in Thai rice bran is 34%. Rice in Thailand is of a long grain type with an oil content of 15–19% in bran. Thai rice bran typically has a protein content of 13–14%[11], comparable with the 12–17% protein of wheat bran.[10] Rice bran proteins are richer in albumin and easier to digest than endosperm proteins in wheat bran.[12] With a carbohydrate to protein ratio in the range 2–3, rice bran is potentially an excellent substrate for producing enzymes by solid-state fermentation. Rice bran contains minerals such as potassium, phosphorus and calcium in considerable amounts. Therefore, rice bran potentially can be used as a substrate without further supplementation.

MATERIALS AND METHODS

Microorganism

Commercially prepared spores of *Aspergillus oryzae* (Ozykat-1) were purchased from a made-to-order local producer (Ratree Mongkolwai, Angthong, Thailand). The spores were kept in a dry air-tight container at room temperature and used as purchased within 6 months of purchase. The spore powder contained 10^9 viable spores per gram. Spores were grown as described by Mongkolwai.[13]

Fermentation conditions

Fermentations were initiated from spores in 250 mL Erlenmeyer flasks that contained either 20 g of wheat bran or rice bran supplemented with 2 g of wheat flour as inducer. Effects of various combinations of rice bran and wheat bran on fermentations were investigated. The media were moistened to 50% (g per 100 g) moisture with tap water, autoclaved at 121 °C for 25 min, and cooled to room temperature before inoculation with 0.06 g of spores. Up to 30 identical fermentation flasks were initiated at the same time. The flasks were incubated at 30 °C for 120 h. A flask was harvested every 12 h for various measurements. Contents of the harvested flasks were thoroughly mixed with a glass rod before sampling for various analyses. All experiments were carried out in triplicate.

Assay methods

The moisture content of a freshly harvested sample (~3 g) of fermented solids was determined in accordance with the AOAC[14] method. The sample was dried at 103 ± 2 °C for 2 h, cooled in a desiccator and reweighed. The pH of fermented solid was measured in accordance with Han and Anderson.[15] The samples were dried at 105 °C (4 h), and cooled in a desiccator. A 1 g sample of dried solids was ground and soaked in 10 mL of distilled water for 1 h. The pH of the supernatant was measured.

To measure enzyme activity, 10 mL of distilled water was added to a 1 g sample of fermented solids. The resulting slurry was agitated on a rotary shaker (180 rpm, 30 min, 30 °C). The slurry was then centrifuged at 10 000 × *g* for 10 min at 4 °C.[2] The supernatant was recovered and held at 4 °C until analysis. The storage period did not exceed 5 days. Protease activity was determined as described by Agrawal *et al.*:[16] 1 mL of suitably diluted (distilled water) supernatant was mixed with a 5 mL solution of 2% (g per 100 mL) casein dissolved in 0.5 mol L^{-1} carbonate buffer, pH 10. The resulting solution was incubated at 40 °C on a gyratory shaker (300 rpm) for 30 min. An aliquot (0.5 mL) of the reaction mixture was withdrawn and the reaction was quenched by adding 1.5 mL pre-chilled trichloroacetic acid (10%). The reaction tube was immersed in an ice bath (5 min) to completely precipitate the protein. The supernatant was recovered by centrifugation (10 000 × *g*, 10 min). Tyrosine liberated during casein hydrolysis was measured in the supernatant using the method of Lowry *et al.*[17] A unit of protease activity was defined as the amount of enzyme liberating 1 μg tyrosine min^{-1} at the incubation temperature of 40 °C. The protease activity is reported per gram of dry solids used in initial extraction.

Amylase activity was measured as explained by Selvakumar *et al.*[18] Thus, the culture supernatant (500 μL), appropriately diluted with distilled water if necessary, was incubated with a solution (500 μL) of 1% (g per 100 mL) soluble starch in 0.1 mol L^{-1} citrate buffer, pH 4.0, at 60 °C, for 1 h. The mixture was quenched by boiling (water bath, 10 min). Reducing sugars were determined by 3,5-dinitrosalicylic acid method.[19] A unit of amylase activity was defined as the number of micromoles of reducing sugars released per minute by the total amount of enzyme extracted from 1 g (dry weight) of sample.[18]

The amount of glucosamine in the samples was used as an indicator of fungal growth. Glucosamine was measured as described by Van de Loo.[20] Thus, a 0.5 g sample of dry fermented solids (see above) was hydrolyzed for 14 h with 250 mL of 6 mol L^{-1} HCl in an oven at 104 °C in a caped vial. After hydrolysis, the vial was cooled, opened, frozen and placed in a freeze drier. The freeze-dried sample was mixed with 0.01 mol L^{-1} phosphate buffer (pH 7.1) such that the supernatant contained between 5 and 15 μg of glucosamine hydrochloride. A 0.5 mL sample of the filtered supernatant was mixed with 0.5 mL of freshly made acetylacetone reagent (0.75 mL acetylacetone dissolved in 25 mL of 1.25 N Na_2CO_3). The sample was then heated at 96 °C for 20 min exactly in a caped vial on a water bath. The vial was immediately submerged in an ice-cold water bath. Ethanol (5 mL, 96%) and Ehrlich reagent (5 mL) were added. (Ehrlich reagent had been prepared by dissolving 1.6 g of *p*-dimethlaminobenzaldehyde in 30 mL of concentrated HCl and adding 30 mL of 96% ethanol.) The sample was mixed and allowed to stand at room temperature

for 45 min. Absorbance was measured at 530 nm and compared with a standard curve. The latter had been prepared using solutions of pure *N*-acetylhexosamine dissolved in the above specified phosphate buffer.

Effect of process variables on protease production

The effect of the following variables on protease production was studied: the ratio of wheat bran to rice bran in the substrate used for growth, the initial pH and initial moisture content of the substrate. Process variables were varied individually in separate experiments. The effect of incubation temperature was not examined, as *A. oryzae* grows rapidly at 30 °C and is known to produce proteases at higher temperatures than other protease-producing molds.[16]

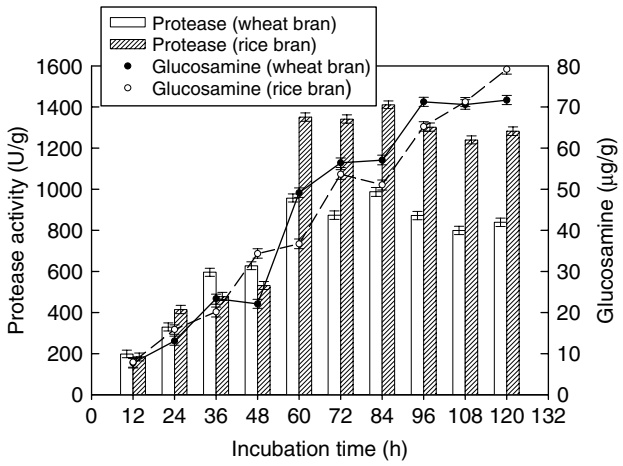

Figure 1. Effect of incubation time on glucosamine content (lines) and protease activity (bars) during fungal growth on wheat bran or rice bran as sole substrates.

RESULTS AND DISCUSSION

Effect of substrate type and composition

Mold growth, as indicated by glucosamine content of samples, and protease production during fermentation, are shown in Fig. 1 for growth on wheat bran and rice bran as sole substrates. Mold growth profiles on both substrates are comparable (Fig. 1). Rapid growth commenced almost immediately after inoculation. Absence of a lag phase indicated that fermentation conditions used were conducive to growth. Glucosamine concentration ceased to increase much beyond 96 h (Fig. 1), indicating the onset of stationary phase. Although biomass growth profiles on the two substrates were quite similar, the protease production profiles were not (Fig. 1). A high protease activity was achieved after 60 h on both substrates. Protease activity remained largely unchanged during the rest of the fermentation (Fig. 1). After the first 60 h, rice bran yielded substantially and consistently higher protease levels than wheat bran (Fig. 1). Because the fungal biomass concentration on both substrates was similar at any given time, the biomass specific production of proteases was clearly greater on rice bran after the first 60 h.

The highest protease activity on rice bran was about 1400 U g^{-1} dry solids compared with an activity of about 1000 U g^{-1} dry solids obtained on wheat bran (Fig. 1). Production of enzymes requires amino acids. Molds can produce amino acids from inorganic nitrogen sources, but a pool of amino acids provided as protein can stimulate enzyme production. Although the protein content of the two brans was similar, rice bran proteins are more easily digested than the endosperm proteins in wheat bran.[12] This probably explains the better enzyme production on rice bran.

Similar findings were reported by Ikasari and Mitchell[21] for enzyme production during fermentation of various solid substrates using the mold *Rhizopus oligosporus*. Higher levels of enzymes were observed on rice bran than on other substrates. Other reports suggest that wheat bran can be superior to rice bran for producing enzymes.[2] This discrepancy is likely associated with the differences in compositions of different varieties of rice bran. As revealed here, the typical Thai rice bran is better than the typical wheat bran for producing proteases of *A. oryzae* (Ozykat-1).

Although rice bran used as a sole substrate afforded high levels of proteases, compared with wheat bran, rice bran had poor morphological characteristics for use as a substrate in large scale solid-state fermentations. This was because the small flat particles of rice bran tended to pack together tightly (Fig. 2(a)) to form a bed of low porosity that did not allow a good circulation of air. Low bed porosity and consequent poor circulation of air adversely affects oxygen transfer and cooling in solid-state fermentations.[22] Good oxygen transfer is essential for culturing molds that are obligate aerobes. In contrast to rice bran, a bed of wheat bran had a more open structure (Fig. 2(b)). Porosity of a bed of fine, flat particles can be increased by mixing in some coarser solids that do not pack together closely. For example, Nehra *et al*.[23] suggested the use of a mixture of rice bran, rice hulk and grain hull as a fermentation substrate. However, the nutrient value of this mixed substrate is lower than that for rice bran alone. Therefore, mixtures of rice bran and wheat bran were investigated as substrates for enzyme production.

Mixtures of wheat bran and rice bran that were assessed as substrates had ratios of wheat-to-rice bran of 0, 0.33, 1 and 3 by dry weight. Protease production data on these mixtures are shown in Fig. 3. Clearly, rice bran alone was the best substrate, but a mixture with wheat-to-rice bran ratio of 0.33 attained the same final protease concentration as did rice bran used as sole substrate (Fig. 3). The presence of larger amounts of wheat bran did not improve protease production (Fig. 3). In view of the superior substrate characteristics of mixed brans and the ability of the mixed brans at a wheat-to-rice bran ratio of 0.33 to match the final proterase level observed in rice bran as sole substrate, this bran mixture was used for all

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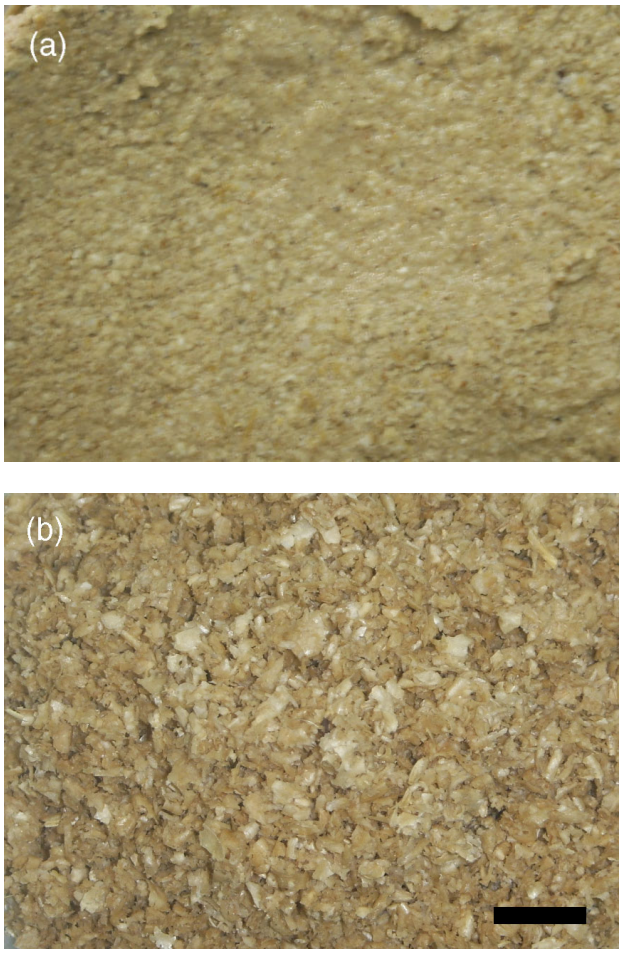

Figure 2. Low voidage in a bed of dry rice bran (a) because of a close packing of relatively flat particles compared with the more open structure of a bed of dry wheat bran (b). Scale bar = 10 mm.

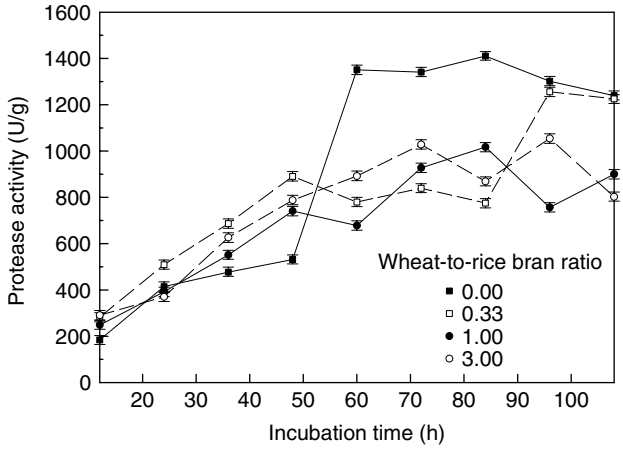

Figure 3. Effect of incubation time on protease activity during fungal growth on substrates having different combinations (w/w) of wheat bran and rice bran.

further experiments. A mixed bran with a wheat-to-rice bran ratio of 0.33 was equivalent to a mixture with 25% wheat bran and 75% rice bran by weight.

Effect of initial moisture content

Initial moisture contents of the substrate are known to critically influence mold growth and enzyme production in solid state fermentations.[22,24] Presence of water in the substrate makes the nutrients more easily accessible for mold growth. Moreover, water has an impact on physico-chemical properties of the substrate, which in turn affect enzyme production.[25] Too much water adversely affects oxygen diffusion in the substrate.[22] At about 7–13%, natural moisture in bran is too low to support mold growth; therefore, the substrate needs to be moistened during preparation. In this work, initial moisture contents of the substrate were adjusted to 45, 50, 55 and 60% in separate experiments before inoculation with spores. The effect of initial moisture on protease production is shown in Fig. 4. Clearly, the optimum initial moisture level was about 50%. Substrate moistened at this level afforded a high protease activity value of 1256 U g^{-1} dry solids at 96 h (Fig. 4). Moisture levels much above 50% reduced enzyme production as the substrate became waterlogged. High moisture content is known to reduce voidage or porosity of substrate, causes particles to stick together and adversely impacts oxygen transfer to the mold.[2,22] This explains the effects of elevated levels of moisture. In contrast, a low moisture level reduces water activity to levels that are not conducive to supporting good fungal growth.

During fermentation, the mold respires to produce carbon dioxide and water. Consequently, the moisture content of the substrate increase during fermentation. This is shown in Fig. 5. The moisture content rose from the initial optimal value of 50% to a little above 60% by around 60 h.

Effect of initial pH

As a general practice, the pH in solid-state fermentations is almost never controlled during fermentation, only the initial pH of the substrate is adjusted before inoculation. The initial pH of the substrate affected the peak protease activity, as shown in Fig. 6. The peak activity occurred at 84 h of fermentation. A moistened mixture of wheat and rice bran with 25% wheat bran had an initial pH of about 5.5. Phosphate buffer (pH

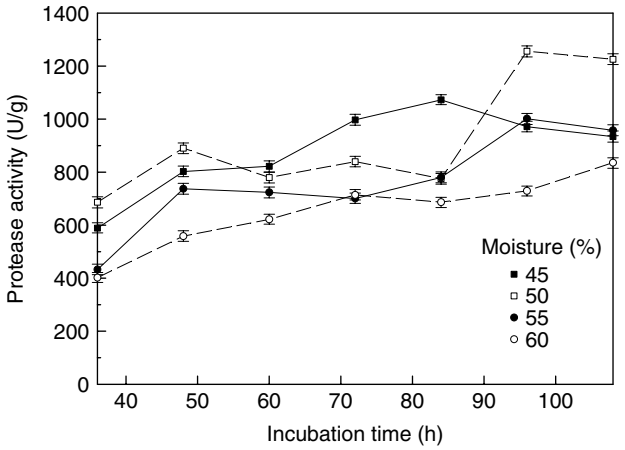

Figure 4. Effect of incubation time on protease activity during fungal growth on mixed bran substrate (25% wheat bran) with different initial moisture contents.

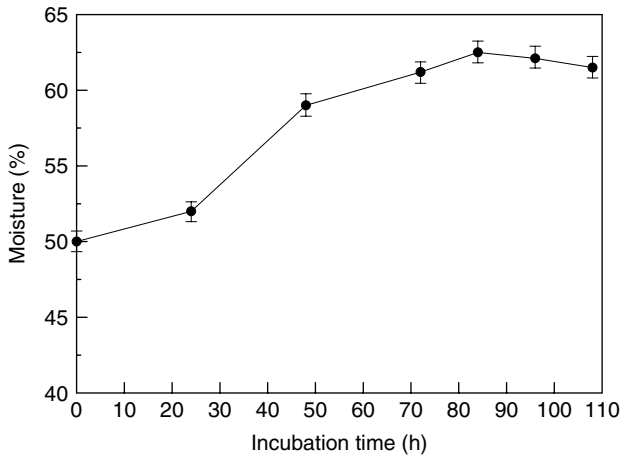

Figure 5. Effect of incubation time on moisture content of mixed bran substrate (25% wheat bran) during fungal growth.

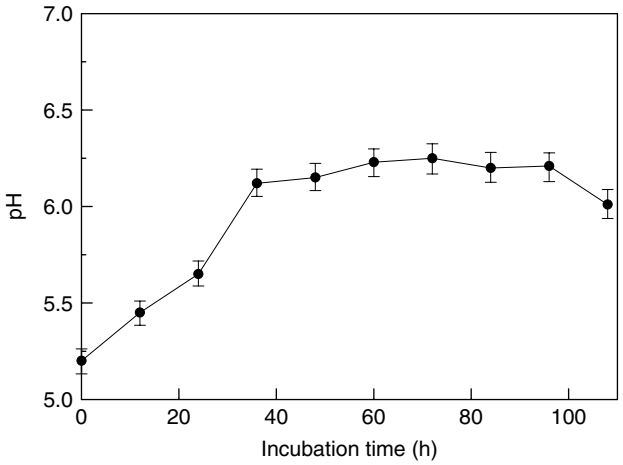

Figure 7. Effect of incubation time on pH of substrate during fungal growth on mixed substrate (25% wheat bran).

7.7) was used to adjust the initial pH to values greater than 5.5 (Fig. 6) in separate fermentations. At nearly 1000 U g^{-1} dry solids, the highest protease activity was attained on a substrate that was initially slightly alkaline at pH 7.5. Protease production at this optimal initial pH was about 30% greater than at an initial substrate pH value of 5.5.

As shown in Fig. 7, during fermentation the pH of the substrate rose from an initial value (after inoculation) of 5.2 to around pH 6 by 36 h and remained essentially constant for most of the rest of the fermentation. Most substrates used in solid-state fermentations are known to possess excellent buffering capacity[2,22] and this explains the fairly constant pH for the greater part of the fermentation (Fig. 7). The data in Fig. 7 are for unbuffered substrate with an uninoculated initial pH of 5.5. In experiments involving initial substrate pH adjustment to pH >5.5 with added phosphate buffer, the final substrate pH always converged to between 6.0 and 6.5.

Relationship between amylase and protease production

The relationship between production of amylase and protease was examined using rice bran as the sole substrate (Fig. 8). Amylase was produced during the first 12 h to digest the carbohydrates. A peak amylase concentration of about 8000 U g^{-1} dry solids was attained (Fig. 8). Protease continued to be produced throughout the fermentation and therefore its concentration continued to increase to attain a peak value of about 850 U g^{-1} dry solids. After the first 12 h, the amylase concentration continually declined possibly because of digestion by protease and lack of new production. Amylase, of course, is no longer required once the carbohydrate has been digested in the initial phase of the fermentation. Production of amylase was accompanied by an increase in glucosamine concentration, indicating rapid growth of the mold. Other fungi may be able to produce high amounts of amylase and protease at the same time. This has been reported, for example, for *Aspergillus awamori*,[1] although the peak activities of the enzymes were considerably less than those shown in Fig. 8.

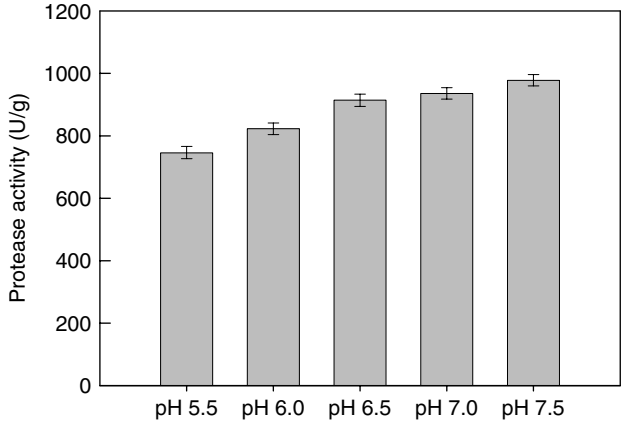

Figure 6. Effect of initial pH on the highest protease activity attained during fungal growth on mixed substrate (25% wheat bran). The peak protease activity occurred after 84 h fermentation.

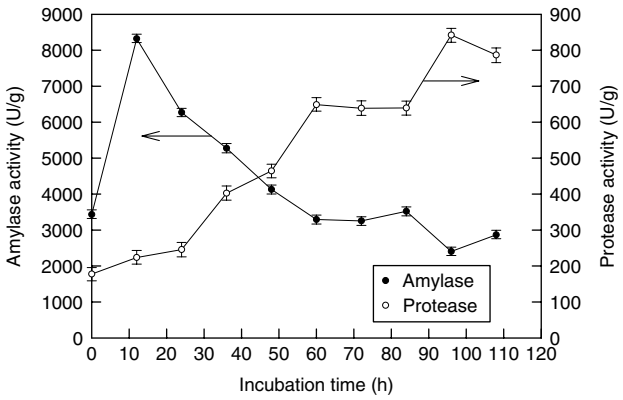

Figure 8. Effect of incubation time on amylase activity and protease activity during fungal growth on rice bran as sole substrate. Arrows indicate the appropriate y-axis for the data.

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Comparison with the literature

Enzyme biochemistry, structure and genetics of expression of *A. oryzae* proteases have been thoroughly studied in view of the commercial use of this microorganism in several widely practiced food fermentations; nevertheless, consistent data on the production of proteases by fermentation is lacking because of diversity of the modes of fermentation, fungal strains, substrates and the culture conditions that have been used by various authors. Using *A. oryzae* NRRL 1808, a supposedly high-yielding strain, Sandhya *et al*.[2] were unable to achieve a protease yield of $>8\,U\,g^{-1}$ initial dry substrate in submerged culture. A much higher final protease level of $\sim 31\,U\,g^{-1}$ dry solids was attained in solid-state fermentation using wheat bran, a relatively expensive substrate. Wheat bran proved to be the best substrate among nine different substrates that were evaluated.[2] Protease level attained in solid-state fermentation on rice bran was a minuscule $\sim 3\,U\,g^{-1}$ dry solids.[2] Compared with these results, the current study achieved a protease level of $\sim 1200\,U\,g^{-1}$ dry solids within 4 days of solid-state fermentation on a low-cost substrate mixture of 75% rice bran and 25% wheat bran. Clearly, the fungal strain used here is one of the better producers of proteases and this strain is commonly used in commercial soy sauce fermentations.

In another published study,[26] an *A. oryzae* strain that had been isolated from an Asiatic soy sauce starter, was used in submerged culture to produce proteases. The substrate was a quite expensive 1:1 by weight mixture of soy flour and wholemeal wheat flour. At a total mixed flour concentration of $26\,g\,L^{-1}$, the maximum protease level attained was $18\,U\,L^{-1}$, or about $0.7\,U\,g^{-1}$ of initial dry substrate. Compared with this, a nearly 1700-fold greater final level of proteases was obtained in this present study.

In summary, the combination of substrate, culture method and microbial strain used here provided a ~40-fold superior level of production of proteases compared with published information on protease production by *A. oryzae* in solid-state and submerged fermentations on a variety of media.

CONCLUSIONS

A local strain of *A. oryzae* (Ozykat-1) produced protease optimally using a substrate with a wheat bran to rice bran ratio of 0.33, an initial moisture content of 50%, and initial pH of 7.5. Initial moisture content had a stronger effect on protease production than did initial pH. The highest protease activity of $1200\,U\,g^{-1}$ dry solids was achieved within 4 days of fermentation. Although rice bran used alone was nutritionally as good a substrate as mixed bran for producing protease, the low porosity of beds of rice bran particles made them poorly suited to use in large-scale solid-substrate fermentations with consistent performance. Beds of low porosity do not allow sufficient penetration of oxygen needed by aerobic mold fermentations and, therefore, adversely affect fermentation. Use of a bran mixture with 25% wheat bran can greatly reduce the cost of substrate in rice producing regions such as Thailand.

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