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Original Research Paper

# Production and biochemical characterization of protease from *Aspergillus oryzae*: An evaluation of the physical–chemical parameters using agroindustrial wastes as supports

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ABSTRACT

This study reports the production optimization and the biochemical characterization of a partially purified protease obtained from *Aspergillus oryzae* LBA 01 under solid state fermentation (SSF) using different agroindustrial wastes as matrix supports. The wastes included wheat bran, soybean meal and cottonseed meal. All of them were characterized in terms of water absorption index, chemical composition and granulometric distribution. The substrates with highest water absorption index and more heterogeneous granulometric distribution have positively influenced on protease production. Some cultivation parameters were studied and the results showed that the optimum fermentation medium was composed of wheat bran, 2.0% (w/w) peptone and 2.0% (w/w) yeast extract, and the conditions for maximum protease production were an initial moisture content of 50.0%, an inoculum level of $10^7$ spores $g^{-1}$ and an incubation at 23 °C. The biochemical characterization using experimental design showed that the enzyme was most active over the pH range 5.0–5.5 and was stable from pH 4.5 to 6.0, indicative of an acid protease. The optimum temperature range for activity was 55–60 °C, and the enzyme was stable at 35–45 °C. The results showed that wheat bran have great potential as support matrix for protease production by *A. oryzae* LBA 01 in SSF.



## 1. Introduction

Proteases are multifunctional enzymes and are extremely important in the pharmaceutical, medical, food and biotechnology industries, accounting for nearly 60% of the whole enzyme market (Jinka et al., 2009; Ramakrishna et al., 2010). They can be isolated from plants, animals and microorganisms. Of these sources, the microorganisms show great potential for protease production due to their broad biochemical diversity and their susceptibility to genetic manipulation. It has been estimated that microbial proteases represent approximately 40% of the total worldwide enzyme sales (Rao et al., 1998).

*Aspergillus oryzae* (*A. oryzae*) is a filamentous fungus listed as a "Generally Recognized as Safe (GRAS)" organism by the US Food and Drug Administration. It has a long history of use in the food industry in the production of traditional fermented foods, due to its high proteolytic activity (Gotou et al., 2009; Morita et al., 2010). According to Machida et al. (2005) the molecular history of the organism shows that *A. oryzae* has the largest expansion of hydrolytic genes (135 proteinase genes).

Proteolytic enzymes can be produced by submerged and solid state fermentation. For the growth of fungi, solid state fermentation is most appropriate method because it resembles the natural habitat of the fungi. Some characteristics make solid state fermentation more attractive than submerged fermentation: simplicity, low cost, high yields and concentrations of the enzymes and the use of inexpensive and widely available agricultural residues as substrates (Chutmanop et al., 2008).

The biochemical characterization of enzymes is important to evaluate their biotechnological potential. The study of the protease properties, such as the influence of inhibitors or activators, the substrate specificity, the optimum catalytic pH conditions and the temperature and stability profiles, can be used to predict the successful application of the enzyme to particular industries or processes.

In this context, the main objectives of the present study were to optimize the production of the protease from *A. oryzae* LBA 01 by solid state fermentation using different agroindustrial wastes and to determine the biochemical characteristics of the protease, including the optimum pH and temperature for activity and stability.

## 2. Materials and methods

### 2.1. Physical–chemical characterization of the agroindustrial wastes

#### 2.1.1. Chemical composition of the agroindustrial wastes

Moisture, protein content, lipids and ash of the agroindustrial wastes were determined by Association of Official Analytical

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Chemists (AOAC) (2010) methods. The carbohydrate content was determined by difference between the total value of 100% and the sum of the other components. The tests were performed in triplicate and the results were expressed as the mean ± standard deviation.

#### 2.1.2. Determination of the water absorption index (WAI) of the agroindustrial wastes

Their water absorption index (WAI) was determined using the method of Anderson et al. (1969) with slight modifications. Briefly, the sample (1.25 g) was suspended in 15 mL of distilled water in a tared 50 mL centrifuge tube. The slurry was manually stirred for 1 min at room temperature (25 °C) and centrifuged at 8000 × *g* and 25 °C for 15 min. The supernatants were discarded, and the WAI was calculated from the weight of the remaining gel and expressed as g gel/g dry weight.

#### 2.1.3. Particle size

The particle sizes of the agroindustrial wastes were determined using AOAC method 965.22 (Association of Official Analytical Chemists (AOAC), 2010). The sieves used had the following opening values: 1.680, 0.841, 0.595, 0.250, 0.177 and 0.149 mm. An amount of 100 g of the material were transferred to top of set of sieves (opening value: 1.680 mm) assembled and fixed in a sieve shaker (Telastem, Produtest Model T, Sao Paulo, Brazil). The sieves were kept under constant shaking at 3600 VPM for 5 min to separate the fractions and the retained material on each sieve was weighed. The experiments were performed in triplicate and the results expressed as percentage.

### 2.2. Microorganism culture

The strain used in this study was *A. oryzae* LBA 01, previously selected as a proteolytic strain from the culture collection of the Laboratory of Food Biochemistry, Faculty of Food Engineering, State University of Campinas. The strain was periodically subcultured and maintained on potato dextrose agar slants. To produce fungal spores, the microorganism was inoculated into a medium composed of 10 g wheat bran and 5 mL of solution containing 1.7% (w/v) $NaHPO_4$ and 2.0% (w/v) $(NH_4)_2SO_4$ and incubated for 3 days at 30 °C. The fungal spores were dispensed into sterile Tween 80 solution (0.3%) to prepare the inoculum for fermentation. The number of spores per milliliter in the spore suspension was determined with a Neubauer cell counting chamber.

### 2.3. Protease production

Wheat bran, soybean meal, and cottonseed meal were kindly provided by Bunge Foods S/A. These agricultural residues were used to select the most appropriate substrate for the protease production by *A. oryzae* LBA 01. The protease production was performed under solid state fermentation in 500 mL Erlenmeyer flasks containing 40 g medium. For substrate selection, the initial cultivation parameters were 50% moisture, temperature set at 30 °C, and an inoculum level of $10^7$ spores $g^{-1}$. The protease activity was tested at 24 h intervals during a 120 h fermentation. The crude extract was obtained by the addition of 150 mL acetate buffer (200 mM, pH 5.0) for 1 h. The solution was filtered through a filter membrane to obtain an enzyme solution free of any solid material.

The crude extract was concentrated by precipitation with ammonium sulfate (80%), dialysis and freeze drying. The partially purified preparation was biochemically characterized.

### 2.4. Screening of the cultivation parameters: Plackett–Burman statistical design

A Plackett–Burman (PB) design was used to determine the effects of the nutrients (glucose, starch, yeast extract and peptone), initial moisture, temperature and inoculum amounts on the protease production by *A. oryzae* LBA 01. The variables were screened in 15 trials, with triplicates at the central point. The protease activity was measured after 48 and 72 h of fermentation.

The main effects of the variables were determined using the Statistica® 8.0 software package from Statsoft Inc. (Tulsa, Oklahoma, USA). The variables with confidence levels greater than 95.0% were considered to significantly influence the protease production. After evaluating the effect of each variable, the conditions that had the greatest effects on the protease production were selected. The substrate moisture variable was further evaluated over the range 40.0–70.0%.

### 2.5. Effects of pH and temperature on the activity and stability of the protease determined using an experimental design

The optimum pH and temperature for activity and stability were determined using a CCRD with three replicates at the central point and four axial points (a total of 11 runs). To study the protease stability, the enzyme was incubated for 1 h at various pH values and temperatures.

The experiments were randomized to maximize the variability in the observed responses caused by extraneous factors. A second-order model equation was used for this model, represented by Eq. (1):

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i x_i + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} \beta_{ij} x_i x_j \quad (1)$$

where $Y$ is the estimated response, $i$ and $j$ equal values from 1 to the number of variables ($n$), $\beta_0$ is the intercept term, $\beta_i$ values are the linear coefficients, $\beta_{ij}$ values are the quadratic coefficients, and $x_i$ and $x_j$ are the coded independent variables. The coefficient of determination $R^2$ and the $F$ test (analysis of variance (ANOVA)) were used to verify the quality of the fit of the second-order model equation. The relationships between the responses and the variables were determined using the Statistica® 8.0 software package from Statsoft Inc. The protease activity was determined using the azocasein method.

### 2.6. Determination of protease activity

The protease activity was measured using azocasein as the substrate according to Charney and Tomarelli (1947) and described by Castro and Sato (2013). The reaction mixture containing 0.5 mL 0.5% (w/v) azocasein (Sigma), pH 5.0, and 0.5 mL of the enzyme solution was incubated for 40 min. The reaction was stopped by adding 0.5 mL 10% TCA and the test tubes were centrifuged at 17,000 × *g* for 15 min at 25 °C. A 1.0 mL aliquot of the supernatant was neutralized with 1.0 mL 5 M KOH. One unit of enzyme activity (U) was defined as the amount of enzyme required to increase the absorbance at 428 nm by 0.01 under the assay conditions described.

### 2.7. Calculations and statistics

Values are expressed as the arithmetic mean. The Tukey test was used to check the significant differences between the groups analyzed. When $p < 0.05$, the differences were considered significant.