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Biochemical characterization of solvent, salt, surfactant and oxidizing agent tolerant proteases from *Aspergillus niger* produced in different agroindustrial wastes

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ABSTRACT

Proteases produced by *Aspergillus niger* LBA 02 under solid state fermentation using different agroindustrial wastes had their enzyme activities tested in the presence of organic solvents, NaCl, surfactants and oxidizing agents. The results showed that according to the fermentation substrate, the microorganism was able to secrete proteases with different biochemical characteristics. When wheat bran was used as the substrate, the proteases showed the highest relative activity in 50% (v/v) petroleum ether, reaching 114.3%, while the proteases produced in soybean meal and cottonseed meal presented 94.7% and 106.1%, respectively. These enzymes also presented high relative activity values in 50% (v/v) chloroform, retaining up to 85% of their initial protease activities after 3 h at 37 °C. When surfactants and oxidizing agents were tested, the proteases produced in soybean meal proved to have remarkable and the highest activity followed by the proteases produced in wheat bran and cottonseed meal. Since the protease produced in wheat bran showed considerable tolerance at high concentrations of NaCl, with relative activity of 61.1% at 15% NaCl (w/v), while the proteases produced in soybean meal and cottonseed meal retained 48.7% and 46.3% of their initial activities, respectively. This work provides an interesting approach about the modulation of biochemical properties of enzymes in response to different substrates, what can be used for obtaining proteases with specific applications.

1. Introduction

Proteases are a large category of enzymes that catalyze the hydrolysis of proteins to polypeptides and oligopeptides to amino acids. Proteases constitute one of the commercially important groups of enzymes, accounting for nearly 60% of the whole enzyme market, in which has been estimated that microbial proteases represent approximately 40% of the total worldwide sales. These enzymes have been used in a wide variety of applications from industrial sectors such as in detergent, leather, pharmaceuticals, food and biotechnology industries (Anbu, 2013; Hsiao et al., 2014; Vijayaraghavan et al., 2014).

Solid state fermentation has gained significant attention for the development of industrial bioprocesses, particularly due to lower energy requirement, higher product yields, less wastewater production, lesser risk of bacterial contamination associated with the possibility of use of agroindustrial wastes as fermentation substrates, making it an ecofriendly process (Thomas et al., 2013).

The selection of the most suitable substrate for the production of enzymes is often based on the high production or productivity values. However, some studies have been demonstrated that the same microorganism may secrete enzymes with different biochemical properties in response to the fermentation substrate (Farnell et al., 2012; de Castro et al., 2015). Therefore, enzymes produced at high levels not necessarily will present the most attractive characteristics for industrial applications.

Due to industrial demand for proteases, researchers are continuously developing biotechnological processes for obtaining proteolytic enzymes with novel biochemical properties. Some of these properties include: high stability and activity at alkaline pH and high temperature and in the presence of surfactants, metal ions, oxidizing agents and organic solvents (Li et al., 2011; Anbu, 2013).

In this context, the main objectives of the present study were to evaluate the proteases production by *A. niger* LBA 02 under solid state fermentation using different agroindustrial wastes and to determine the biochemical properties of the proteases produced in each agroindustrial waste, with emphasis on proteolytic activity in

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the presence of different solvents, NaCl, surfactants and oxidizing agents.

2. Materials and methods

2.1. Microorganism culture

The micro-organism used in this study was *A. niger* LBA 02, previously selected as a proteolytic strain from the culture collection of the Laboratory of Food Biochemistry, School of Food Engineering, 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.

2.2. Protease production

Wheat bran, soybean meal and cottonseed meal were kindly provided by Bunge Foods S/A. Orange peel was purchased from local market of Campinas (Sao Paulo, Brazil).

These agroindustrial wastes were used for the protease production by *A. niger* LBA 02. The protease production was performed under solid state fermentation using the individual substrates in 250 mL Erlenmeyer flasks containing 40 g medium. The cultivation parameters were 50% moisture, temperature set at 30 °C, an inoculum level of 10^7 spores g^{-1} and the protease production was monitored at 24, 48, 72 and 96 h fermentation. The crude extract was obtained by the addition of 100 mL distilled water for 1 h. The solution was filtered through a filter membrane to obtain an enzyme solution free of any solid material.

The crude extracts were concentrated by precipitation with ammonium sulfate (80%), dialysis and freeze drying. For biochemical characterization, the partially purified preparations had their initial protease activities adjusted to 100 U g^{-1}.

2.3. Determination of protease activity

The protease activity was measured using azocasein as the substrate according to Charney and Tomarelli (1947) and described by de 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.

2.4. Effect of organic solvents on activity of the proteases

To study the effect of organic solvents on the protease activity, protease solutions in 50 mM acetate buffer (pH 5.0) was mixed with different organic solvents (acetone, chloroform, ethanol, hexane, isobutanol, isopropanol, methanol, petroleum ether and propanol) in equal proportion and left for 3 h at 37 °C. Residual protease activities were measured using azocasein as the substrate in acetate buffer (pH 5.0) and 50 °C and the results were expressed in percentage. The protease activity in absence of any organic solvents was considered as 100%. All determinations were performed in triplicate.

2.5. Effect of surfactants and oxidizing agents on protease activity

The protease activity was investigated in the presence of different surfactants and oxidizing agents, including sorbitan monolaurate (2.0%), tween 80 (2.0%), SDS (0.5%), triton X 100 (2.0%) and H_2O_2 (1.0%) by pre-incubation of protease solutions for 1 h at 37 °C. After pre-incubation of protease solutions with each reagent, the residual protease activity was measured as previously described. The residual activity was expressed as percentage assuming the activity of control sample (in the absence of any agent) as 100%. All determinations were performed in triplicate.

2.6. Effect of NaCl on protease activity

To study the effect of NaCl on the protease activity, enzyme solutions were assayed in the presence of different concentrations of NaCl ranging from 0 (control) to 25%. The residual protease activities were assayed using azocasein as the substrate as previously described and the results expressed as percentage considering the control reaction as 100%. All determinations were performed in triplicate.

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. The differences were considered significant when p-value ≤ 0.05.

3. Results and discussion

3.1. Protease production using different agroindustrial wastes

The protease production presented different profiles when *A. niger* LBA 02 was cultivated in each agroindustrial waste. When wheat bran was used as fermentation substrate, the highest protease activities were observed between 72 and 96 h, reaching 176.21 and 186.42 U g^{-1}, respectively. The protease produced using soybean meal, reached maximum values at 48 and 72 h fermentation, with a decreased at 96 h. For cottonseed meal, a similar profile of production was observed between 48 and 96 h, reaching protease activities above 110 U g^{-1} (Fig. 1).

Supplementary Fig. 1 shows the growth of *A. niger* LBA 02 during 0, 24, 48 and 72 h fermentation using wheat bran, soybean meal and cottonseed meal as substrates.

The crude protease extracts produced in each agroindustrial waste obtained at 72 h fermentation were concentrated by precipitation with ammonium sulfate (80%), dialysis and freeze drying and used for biochemical characterization. The enzymes produced by *A. niger* LBA 02 were previously identified as acid proteases (de Castro et al., 2014).

3.2. Biochemical properties of the proteases from A. niger LBA 02

3.2.1. Effect of organic solvents on activity of the proteases

The effect of different organic solvents on activity of the proteases produced in each agroindustrial waste was shown in Fig. 2. Accordingly, the proteases exhibited high stability in the presence of chloroform (50% v/v) and petroleum ether (50% v/v), with relative activities ranging from 85 to 114% after 3 h incubation at 37 °C. Our findings are in according to Yadav et al. (2015) who reported 115.6% activity of a protease from *A. flavus* MTCC 9952 with chloroform (25% v/v) after 1 h of incubation at 30 °C. The