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Using response surface methodology to improve the L-asparaginase production by *Aspergillus niger* under solid-state fermentation

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ABSTRACT

Microbial L-asparaginase have been used as a therapeutic agent in the treatment of acute lymphoblastic leukemia and Hodgkin's disease. In addition to clinical use, L-asparaginases have been used in foods to reduce the formation of acrylamide. L- asparaginase production is carried out mainly by submerged fermentation (SmF). However, solid-state fermentation (SSF) offers potential benefits compared with submerged fermentation, such as the use of low-cost agro-industrial residues as the substrate, which is very attractive for bioprocessing. The main objective of this study was to evaluate the use of agro-industrial wastes, including wheat bran, soybean meal, rice meal, chicken feather meal, chicken viscera meal and passion fruit peel flour, for L-asparaginase production by *Aspergillus niger* LBA 02 under solid-state fermentation. Among the substrates, the highest L-asparaginase activity was obtained using passion fruit peel flour (2380.11 U/gds) after 48 h of fermentation at 30 °C. The effects of temperature, initial moisture content (%) and inoculum concentration (spores/g) on the L-asparaginase production were evaluated using passion fruit peel meal as a substrate using Central Composite Rotatable Design (CCRD) with 17 runs. An increase in L-asparaginase activity (3746.78 U/gds) was obtained using the optimized conditions of passion fruit peel flour with an initial moisture content of 60% and inoculum concentration of 2.1 10^6 spores/g after 24 h of fermentation at 25 °C. It was observed that the process optimization resulted in a 57% increase in enzyme production compared with the initial values obtained in the "one-factor-at-a-time" method.

1. Introduction

L-asparaginase (L-asparagine amidohydrolase E.C.3.5.1.1) is an enzyme belonging to the amidase group, which catalyzes the hydrolysis of the amino acid L-asparagine to L-aspartic acid and ammonia (Gurunathan and Sahadevan, 2012). This enzyme has been used for chemotherapy of lymphoid system malignancies and leukemia for over four decades (Zuo et al., 2015). Apart from its clinical usage, L-asparaginase has great potential for use in food processing, reducing acrylamide formation in foods rich in carbohydrates and L-asparagine (cereals, potatoes and coffee) subjected to high temperatures (Mottram et al., 2002). Acrylamide is classified as a probable human carcinogen (IARC, 1994).

This enzyme is widely distributed among living organisms, including animals, plants, and microorganisms (Zuo et al., 2015; Batool et al., 2016). Microorganisms are considered to be the most important source of L-asparaginase since the anti-tumor activity of asparaginase from *Escherichia coli* (Mashburn and Wriston, 1964) was first reported. However, L-asparaginase from fungal sources has gained much attention because of its stability, high productivity and easy culture conditions (Meghavarnam and Janakiraman, 2017). Fungi genera, such as *Aspergillus*, *Penicillium, Fusarium and Cladosporium*, are commonly reported in previous studies to produce L-asparaginase (Patro et al., 2014; Kumar et al., 2013; Shrivastava et al., 2012). The history of safe use for *Aspergillus niger* stems primarily from its use in the food industry for the production of many enzymes such as amylase, amyloglucosidase, cellulases, lactase, invertase, pectinases and acid proteases (Bennett, 1985).

Industrial production of L-asparaginase by fungi throughout the world is carried out mainly by submerged fermentation (SmF) (Meghavarnam and Janakiraman, 2017; Kumar et al., 2013). Solid-state fermentation (SSF) has the potential for the production of enzymes and

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has emerged as an attractive alternative to submerged fermentation. SSF has attained much relevance during the past decade to offer potential environmental benefits, as it utilizes low-cost agro-industrial residues as the substrate, which is attractive for bioprocessing (Thomas et al., 2013). These substrates act as both a source of nutrients and physical support for enzyme production. Therefore, screening and selection of an appropriate substrate is an important step for commercial production of an enzyme (Meghavarnam and Janakiraman, 2017).

Recently, many statistical experimental have been employed in bioprocess optimization. Optimization of the fermentation process can be conducted either by changing one- factor-at-a-time or by varying several factors at the same time and looking for interactions using statistical analysis. Optimization studies involving a one-factor-at-a-time approach is tedious and tend to overlook the effects of interacting factors but might lead to misinterpretations of results. In contrast, statistically planned experiments effectively tackle the problem which involves the specific design of experiments which minimizes the error in determining the effect of parameters and the results are achieved in an economical manner (Balakrishnan and Pandey, 1996).

Statistically based optimization is a proven tool for overcoming the limitations of the “one-factor-at-a-time” method. It is a more efficient technique since it can provide statistical data with a relatively small number of experiments (Uppuluri et al., 2013). Basically, this optimization process involves three major steps: performing the statistically designed experiments, estimating the coefficients in a mathematical model, predicting its response and checking the adequacy of the model (Kumar et al., 2013). Several researchers in biotechnology have applied these techniques for optimization of different parameters (El-Naggar et al., 2014; Kumar et al., 2013; Uppuluri et al., 2013; Hymavathi et al., 2009). Considering the importance of L- asparaginase, the present investigation was aimed to optimize L-asparaginase production using agro-industrial wastes by *A. niger* LBA 02 through response surface methodology.

2. Material and methods

2.1. Substrates and chemicals

Different agro-industrial wastes, such as wheat bran, rice meal, and passion fruit peel flour, were purchased from the local market of Campinas, São Paulo, Brazil. Soybean meal was provided by Bunge Foods S/A, Campinas, São Paulo, Brazil. Chicken feather meal and chicken viscera meal were provided by Ad´oro S/A, Várzea Paulista, São Paulo, Brazil. All chemicals used in the present study were of analytical grade and were obtained from Sigma–Aldrich (St. Louis, MO, USA), Merck (Darmstadt, Germany) and Difco (Kansas, USA).

2.2. Microorganism and culture conditions

The filamentous fungi *Aspergillus niger* LBA 02 was previously selected by Dias et al. (2015) as an L-asparaginase producer strain from the culture collection of the Laboratory of Food Biochemistry, School of Food Engineering, University of Campinas, Campinas SP, Brazil. The strain was maintained on slants containing potato dextrose agar (PDA) medium, incubated at 30 °C for 7 days, and stored at 4 °C.

2.3. Inoculum preparation

The fungal spores were produced by inoculating 1 mL of the microorganism spore suspension into a medium comprising 10 g wheat bran and 4 mL of a solution containing 1.7% (w/v) Na_2HPO_4 and 2.0% (w/v) $(NH_4)_2SO_4$ and incubating for 3 days at 30 °C. The fungal spores were extracted in 40 mL of the sterile 0.3% Tween 80 solution, and a Neubauer cell-counting chamber was used to determine the number of spores per mL in the spore suspension using an optical microscope.

2.4. Screening of different agro-industrial wastes for the production of L-asparaginase by the method of one-factor-at-a-time

Ten grams of each substrate (wheat bran, rice meal, passion fruit peel flour, soybean meal, chicken feather meal and chicken viscera meal) was taken separately in 125 mL Erlenmeyer flasks and moistened with water (60% moisture). The contents were thoroughly mixed, and the flasks were sterilized at 121 °C for 45 min and cooled to room temperature before use, accordance with the modified methodology of Kumar et al. (2013).

The approximate thickness of the bed formed by the agricultural wastes was averaged using a pachymeter and the measurements is presented as follows: wheat bran (9.44 ± 0.88), rice bran (6.00 mm ± 0.49), (9.41 mm ± 0.93), soybean meal (7.23 mm ± 1.23), chicken feather meal (5.01 mm ± 0.00), and viscera flour of chicken (3.60 mm ± 0.34).

The crude extract was obtained by the addition of 40 mL of distilled water. After 1 h at rest the solution was filtered through a filter membrane to obtain an enzyme solution free of any solid material. The enzyme extract was store at − 15 °C.

2.5. Determination of L-asparaginase activity

The activity of L-asparaginase was determined according to the methodology described by Imada et al. (1973) and modified by Dias and Sato (2016). The reaction mixture, composed of 0.5 mL of 0.04 M L-asparagine, 0.8 mL of 0.1 M pH 8.0 Tris-HCl buffer, and 0.2 mL of crude enzyme extract, was incubated at 40 °C for 30 min. The reaction was stopped by adding 0.5 mL of 1.5 M trichloroacetic acid. A 125-μL aliquot of the reaction mixture was diluted with 1 mL of distilled water and 125 μL of Nessler's reagent (Merck®). The absorbance was measured at 450 nm in a DU 640 spectrophotometer (Beckman Coulter, CA, USA). A standard curve with ammonium sulfate was used to quantify the released ammonia. The enzyme activity was expressed in U/gds of the dried substrate. One unit of enzyme activity (U) was defined as the amount of enzyme that liberates 1.0 μmol of ammonia per minute under standard assay conditions.

2.6. Optimization of L-asparaginase production through response surface methodology

A central composite rotational design (CCRD) for three variables was employed to determine the optimum conditions for L-asparaginase activity. Three independent variables were studied in 17 experiments: temperature, moisture, and inoculum concentration. The relationship between the coded and real values of the independent variables for the CCRD is shown in Table 1.

Upon completion of the CCRD, the models were adjusted (Eq. (1)), and the response surfaces and contour curves were determined according to the methodology recommended by Rodrigues and Iemma (2014).

Table 1
Relationship between the coded and real values of the independent variables for L-asparaginase activity by *A. niger* LBA 02.

Variables	− 1.68	− 1	0	1	1.68
Temperature (°C)	22	25	30	35	39
Moisture (%)	33	40	50	60	67
Inoculum concentration (spores / g)	10^5	2.1×10^6	5.05×10^6	7.99×10^6	10^7

The "−1.68" and the "+1.68 " sign corresponds to the α-value.