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Simplex centroid mixture design to improve L-asparaginase production in solid-state fermentation using agroindustrial wastes

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ABSTRACT

Agroindustrial wastes, such as wheat bran, soybean meal, cottonseed meal and orange peel, were used individually or in mixtures as fermentation feedstock for L-asparaginase production from *Aspergillus niger* LBA 02 under solid-state fermentation using a simplex centroid mixture design. At 24 h of fermentation, most of the interaction effects observed were antagonistic and the highest L-asparaginase activity was obtained with cottonseed meal as the feedstock. At 48 h of fermentation, synergistic effects were found for two binary formulations and one quaternary formulation and increase in the L-asparaginase activity ranged from 0.69 to 73.44 fold. At 72 h of fermentation, synergistic effects were also found for two binary and one quaternary formulation and increase ranged from 0.47 to 4.26 fold. At 96 h of fermentation, synergistic effects were found for four binary and one quaternary formulation and increase ranged from 0.44 to 17.84 fold. The enzyme production reached the highest value after 96 h of fermentation, and the optimum conditions predicted for L-asparaginase production were found to be a ternary mixture of wheat bran (1/3), soybean meal (1/3) and cottonseed meal (1/3) with increases of 71.53, 13.53 and 13.53 fold in L-asparaginase production as compared to the individual feedstocks, respectively. The predicted activity was 94.21 U g^{-1}, which showed no significant difference ($p < 0.05$) from the experimental L-asparaginase activity of 89.22 U g^{-1}. Therefore, mixtures contained wheat bran (1/3), soybean meal (1/3) and cottonseed meal (1/3) may be added to improve the L-asparaginase production with low cost.

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1. Introduction

L-asparaginase (EC 3.5.1.1) is an enzyme that catalyzes the hydrolysis of L-asparagine into L-aspartic acid and ammonia (Capuano et al., 2008). Fungal L-asparaginases from recombinant *Aspergillus oryzae* and *Aspergillus niger* are used in the food industry to reduce the levels of acrylamide formed when starchy foods are fried, baked and cooked (Pedreschi et al., 2011, 2008). L-asparaginases are also used to treat lymphoblastic leukemia and inhibit tumor growth (Shaffer et al., 1988). L-asparaginases from *Escherichia coli* and *Erwinia carotova* are used to treat L-asparagine-dependent leukemia and lymphoma (Keating et al., 1993). However, these treatments can lead to immunological sensitization (hypersensitivity) to some patients receiving L-asparaginase (Narta et al., 2007). Therefore, the search for other L-asparaginase sources, like eukaryotic microorganisms, could lead to an enzyme with less adverse effects (Sreenivasulu et al., 2009; Sarquis et al., 2004). Thus, in the recent years, eukaryotic fungi have been investigated as sources of L-asparaginase. The demand for L-asparaginase has increased by several fold due to its application in the food industry, in addition to its pharmaceutical applications (Mishra, 2006; Ali et al., 1994).

Worldwide, L-asparaginase production is often performed by submerged fermentation. However, this method has disadvantages because it is a high-cost process with low product yield. In addition, it generates excess effluents and, consequently, a large volume of wastewater during downstream processes (Ashraf et al., 2004). Conversely, solid-state fermentation (SSF) is a simple, effective technique that can increase production yields with lower capital investment and energy consumption (Thomas et al., 2013, Couto and Sanroman, 2006). Agroindustrial wastes represent a potential resource for biotechnological processes mainly due to their low cost, accessibility and nutrient compositions. Their use is important for developing an economically viable bioprocess. Consequently, solid-state fermentation is a promising, environmentally-friendly technology that can comprehensively utilize renewable resources (Martinello et al., 2006).

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Table 1
Matrix of the simple centroid mixture design for L-asparaginase production by *A. niger* LBA-02 under solid-state fermentation and the results for L-asparaginase activity at 24, 48, 72 and 96 h.

Run	Independent variables				L-asparaginase activity (U g^{-1})			
	Wheat bran x_1	Soybean meal x_2	Cottonseed meal x_3	Orange peel x_4	24 h	48 h	72 h	96 h
1	1	0	0	0	0.05 ± 0.00b	6.85 ± 0.67b	3.59 ± 0.35d	1.23 ± 0.14f
2	0	1	0	0	0.01 ± 0.00b	5.87 ± 0.55b	2.70 ± 0.11d,e,f	6.14 ± 0.60e
3	0	0	1	0	4.22 ± 0.54a	1.90 ± 0.13c,d	1.83 ± 0.19g,f	6.14 ± 0.82e
4	0	0	0	1	0.03 ± 0.00b	0.16 ± 0.01d,e	3.64 ± 0.33d	1.20 ± 0.13f
5	1/2	1/2	0	0	0.17 ± 0.07b	2.50 ± 0.18c	5.28 ± 0.40c	15.30 ± 0.67b
6	1/2	0	1/2	0	0.03 ± 0.00b	6.25 ± 0.32b	0.02 ± 0.02h	10.54 ± 0.76c,d
7	1/2	0	0	1/2	0.02 ± 0.00b	5.82 ± 0.62b	2.46 ± 0.09e,f	0.01 ± 0.00f
8	0	1/2	1/2	0	0.04 ± 0.01b	11.67 ± 0.96a	7.78 ± 0.55b	8.83 ± 0.60d
9	0	1/2	0	1/2	0.00 ± 0.00b	11.91 ± 1.27a	0.05 ± 0.09h	12.28 ± 1.34c
10	0	0	1/2	1/2	0.03 ± 0.00b	0.00 ± 0.00e	3.20 ± 0.03d,e	0.02 ± 0.02f
11	5/8	1/8	1/8	1/8	0.00 ± 0.00b	8.06 ± 1.68b	0.00 ± 0.22h	3.86 ± 0.22e
12	1/8	5/8	1/8	1/8	0.02 ± 0.01b	6.01 ± 0.01b	9.63 ± 0.20a	22.61 ± 1.85a
13	1/8	1/8	5/8	1/8	0.03 ± 0.00b	11.58 ± 0.61a	1.94 ± 0.62f	6.04 ± 0.63e
14	1/8	1/8	1/8	5/8	0.06 ± 0.00b	6.67 ± 0.46b	0.00 ± 0.00h	0.31 ± 0.03f
15	1/4	1/4	1/4	1/4	0.04 ± 0.01b	0.08 ± 0.00d,e	0.86 ± 0.08g.h	0.00 ± 0.00f

The results are presented as the mean ($n=3$) ± SD, and those with different letters are significantly different by Tukey's test, with $p < 0.05$. Comparisons were made between the runs at each fermentation time (not between the different fermentation times).

Different agroindustrial wastes have been used for L-asparaginase production under SSF by *A. niger*, such as rice bran, wheat bran, cotton seed, rice flour, agrowastes from leguminous crops, and others (Vivekanandha et al., 2013; Sreenivasulu et al., 2009; Mishra, 2006). All of the current research has focused on L-asparaginase production using only individual fermentation feedstock and there is no data in the literature on the use of feedstock mixtures for the production of L-asparaginase. Moreover, the use of statistical mixture designs using individual or formulations contained binary, ternary or quaternary mixtures of different feedstocks for L-asparaginase production under solid-state fermentation has not yet been reported in the literature. Statistical mixture designs offer the possibility to use different combinations among the components for changing mixture composition and exploring how such changes will affect specific response (Rao and Baral, 2011). This statistical method has been used to evaluate the interactions among agroindustrial wastes in the production of proteases, α-amylase (Castro and Sato, 2013), cellulose (Delabona et al., 2013), and glutaminase (Sathish et al., 2008) and is effective for predicting models and maximizing enzyme production.

Developing an L-asparaginase production process based only in a mixture of agroindustrial wastes in SSF is economically attractive as it is cheap and as the agroindustrial wastes are available in abundance. Therefore, the purpose of this study was to obtain a low-cost L-asparaginase using a mixture of agroindustrial wastes. A simplex centroid mixture design was used to investigate the presence of synergistic or antagonistic effects of different agroindustrial wastes for L-asparaginase production under state-solid fermentation. This statistical tool was also used to select the best combination of solid low-cost fermentation feedstock for maximum L-asparaginase production by *A. niger* LBA 02.

2. Materials and methods

2.1. Microorganism culture

The filamentous fungi *A. niger* LBA 02 was previously selected as a 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 periodically subcultured and maintained on potato dextrose agar slants. The fungal spores were produced by inoculating the microorganism into a medium composed of 10 g wheat bran and 5 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 dispensed into sterile 0.3% Tween 80 solution to prepare the inoculum for fermentation and a Neubauer cell-counting chamber was used to determine the number of spores per milliliter in the spore suspension.

2.2. Production of L-asparaginase

Wheat bran, soybean meal, and cottonseed meal were kindly provided by Bunge Foods S/A, Campinas, São Paulo, Brazil. Orange peel was purchased from the local market of Campinas (São Paulo, Brazil), and, in order to use it as a support matrix, the orange peel was ground, washed three times with distilled water and dried at 50 °C for 24–48 h.

The agroindustrial wastes wheat bran, soybean meal, cottonseed meal and orange peel were used for L-asparaginase production by *A. niger* LBA 02. Enzyme production was performed under solid-state fermentation using the individual natural feedstock, and binary and quaternary mixtures in different ratios in 250 mL Erlenmeyer flasks containing 20 g of medium. The parameters for L-asparaginase cultivation by *A. niger* LBA 02 were defined in preliminary studies from our research group (data not shown) and the most appropriate conditions were 50.0% moisture, a temperature of 30.0 °C, and an inoculum level of 10^7 spores g^{-1}. L-asparaginase activity was assessed at 24 h intervals during the 96 h fermentation period. The enzymatic crude extract was obtained by adding 100 mL of distilled water and agitating for 1 h. The solution was filtered through a filter membrane to remove any solid material from the enzymatic extract.

2.3. Statistical mixture design

The experiment mixture design was used to achieve the optimum composition of the agroindustrial wastes for maximum L-asparaginase production and to assess the interaction effects of the components. A four component augmented simplex lattice design has been employed, in which each component is studied in six

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