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Performance of a fixed-bed solid-state fermentation bioreactor with forced aeration for the production of hydrolases by *Aspergillus awamori*

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ABSTRACT

In order to overcome scale-up limitations of conventional tray-type solid-state fermentation (SSF) bioreactors, a cylindrical fixed-bed bioreactor with forced aeration was investigated for the production of a pool of industrially relevant enzymes by *Aspergillus awamori* IOC-3914 using babassu cake as raw material. Significant internal radial and axial temperature gradients were observed (up to 2.4 °C cm^{-1}), but despite of this, good titers of the production of the six enzyme groups evaluated was observed in all three bed layers sampled. Maximum activities of exoamylases, endoamylases, proteases, xylanases and cellulases (CMCase) were, respectively, 73.4, 55.7, 31.8, 23.8 and 6.2 U g^{-1}. Moreover, a considerable production of isoamylases (27.0 U g^{-1}) was observed, although the production of starch-debranching enzymes by SSF is rarely reported. Productivities were highest for endoamylases (0.95 U g^{-1} h^{-1}) and exoamylases (0.67 U g^{-1} h^{-1}). The present results show that despite considerable axial and radial temperature gradients, significant levels of different enzymes can be obtained, giving evidence that fixed-bed bioreactors with forced aeration present a promising alternative in terms of instrumented bioreactors for SSF processes.

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1. Introduction

Solid-state fermentation (SSF) based on the utilization of agricultural by-products is an excellent low-cost option for enzyme production, presenting, in comparison to submerged bioprocesses, lower wastewater generation, higher product yield, lower catabolic repression and lower demand on sterility due to the low water activity [1,2]. However, some bottlenecks such as limitations in heat and mass transfer and difficulties in microbial biomass estimation [2] indicate that studies on different SSF systems are still needed. Although SSF has been industrially used for centuries, instrumented bioreactors for SSF are not yet well established, and recent publications investigating the use of various bioreactor configurations (e.g. fixed-bed bioreactors [3,4], aerated trays [5] and rotary drums [6]), different operation modes (e.g. batch [7], fed-batch [8] and continuous [9] modes), and mixing strategies (e.g. intermittent mixing [10]) are available in literature.

We have previously demonstrated that the filamentous fungus *Aspergillus awamori* IOC-3914 is a promising strain for the production of enzyme extracts containing multiple industrially relevant hydrolases (amylases, cellulases, xylanases and proteases), but our previous works were carried out in simple SSF tray bioreactors with no forced aeration, which present several scale-up bottlenecks [11]. The raw enzyme pools were shown to efficiently promote cold hydrolysis of complex agroindustrial raw materials such as canola (*Brassica napus*), sunflower (*Helianthus annus*) and castor bean (*Ricinus communis*) cakes [12], as well as of babassu (*Orbygnia phalerata*) flour [13,14]. Thus, the development of scalable SSF bioreactors can be useful for a future industrial production of these hydrolases.

The objective of this work was thus to investigate the use of an instrumented fixed-bed bioreactor designed in house for the production of an enzyme pool rich in hydrolases by *A. awamori* IOC-3914.

2. Materials and methods

2.1. Raw materials

Babassu cake (kernel residue), containing approximately 24% protein and 66% carbohydrates (dry basis) [15], was kindly supplied

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Nomenclature:

L	packed bed height (cm)
μair	viscosity of air at 22.9 °C (g cm^{-1} s^{-1})
ρair	density of air at 22.9 °C (g cm^{-3})
G	volumetric air flow (cm^3 min^{-1})
A_T	cross sectional bioreactor area (cm^2)
NRE	Reynolds number
X	microbial biomass based on *N*-acg content (mg *N*-acg g^{-1})
Xm	maximum microbial biomass based on *N*-acg content (mg *N*-acg g^{-1})
$X0$	initial microbial biomass based on *N*-acg content (mg *N*-acg g^{-1})
μ	specific growth rate (h^{-1})

by TOBASA Bioindustrial de Babaçu S.A. (Tocantinópolis, Brazil). The raw cake had a mean particle size of 923 ± 7 μm, as estimated using a vibratory shaker (Viatest 76773, Germany) fitted with sieves (Tyler 8–400 mesh). Prior to SSF, the cake was dried, ground, and sieved to exclude particles larger than Tyler mesh size 14 (>1200 μm).

2.2. Microorganism and inoculum propagation

Solid-state fermentation was carried out using *A. awamori* IOC-3914 (Instituto Oswaldo Cruz Culture Collection, Brazil). The fungus was kept at 4 °C in starch agar medium [11]. A two-step inoculum propagation strategy was used, based on spores propagation in starch agar medium followed by liquid propagation in malt extract medium, as described by Castro et al. [16].

2.3. Solid-state fermentation in the column bioreactor

A cylindrical glass bioreactor with a total volume of 2.3 L (30 cm height and 10 cm diameter) and a working volume of 1.8 L (23.9 cm packed bed height) was used (Fig. 1). The bioreactor had three sampling ports (H_1 = 21 cm, H_2 = 14 cm and H_3 = 7 cm) and four temperature measurement ports (T_1 = 23.6 cm, T_2 = 17.6 cm, T_3 = 11.6 cm and T_4 = 5.6 cm), in addition to a port at the gas outlet stream for temperature measurement. In each temperature measurement port, temperature was registered at two radial positions (r_1 = 0 cm and r_2 = 3.0 cm). The inlet of the bioreactor was connected to a jacketed air humidifying chamber, which was maintained at 22.9 °C, according to previous optimization studies in tray bioreactors [17]. The humidified air was injected evenly at the bed basis by using a sintered glass plate.

Babassu cake (400 g, dry basis) was sterilized at 121 °C for 20 min and inoculated with 9.1 mg g^{-1} fungal mycelium propagated, by uniformly pouring the grown cell suspension over the cake and gently mixing using a fork-type device, according to [16]. The inoculated cake was humidified to an initial moisture content of 61.9% [17] and then loaded to the cylindrical bioreactor, which was then kept at a room temperature of 22 °C for 168 h, with an air flow of 8.3–10.2 L min^{-1}.

Samples of approximately 2.5 g were withdrawn periodically from each sampling port (at r_2 = 3.0 cm) and used for enzyme extraction and assays. Extraction was carried out for 30 min in an aqueous solution, as described previously [18].

2.4. Assays

Exoamylase, endoamylase, isoamylase, protease, cellulase (CMCase) and xylanase activities were measured in the extraction supernatants using 1% (m/v) soluble starch, 0.5% (m/v) soluble starch, 0.5% (m/v) isomaltose, 0.5% (m/v) azocasein, 2% (m/v) sodium carboxymethylcellulose and 1% (m/v) Birchwood xylan, respectively. All these substrates were supplied by Sigma Aldrich (USA), except for soluble starch, which was provided by Vetec (Brazil). All activities were expressed as enzyme units (U) per dry mass at the sampling time. One U of endoamylase was defined as the amount of enzyme that catalyzes the liquefaction of 1 mg of starch per minute, under the assay conditions. One U of exoamylase, isoamylase or cellulase was defined as the enzyme amount that catalyzes the release of 1 μmole of glucose per minute, from their respective substrates, under the assay conditions. One U of xylanase was defined as the enzyme amount that catalyzes the release of 1 μmole of xylose per minute under the assay conditions, and 1 U of protease was defined as the amount of enzyme that promotes an increase of one absorbance unit (at 345 nm) per minute, under the assay conditions. Total protein content of enzyme extracts was measured by the Bradford method, using bovine serum albumin as standard. Details of the assays were described previously [17]. All analyses were done in triplicate and data are expressed as mean ± 1 standard deviation (SD) (corresponding to a 68%-confidence interval).

Water activity (Aw) and moisture content (MC) of the solid samples were determined using a water activity meter (Aqualab, USA) and a moisture analyzer (model MX-50, A&D, USA), respectively. Biomass was indirectly monitored by measuring *N*-acetylglucosamine (*N*-acg) content in solid samples according to the method by Aidoo et al. [19] with slight modifications, as described in [17].

2.5. Calculations and statistical analysis

For the analysis of results, different responses were evaluated at each sampling time. Enzyme/biomass yield ($Y_{P/X}$, U mg$_{N\text{-acg}}^{-1}$) was calculated as the ratio of each activity (U g^{-1}) by *N*-acg content (mg$_{N\text{-acg}}$ g^{-1}). Specific activity (U mg$_{ptn}^{-1}$) was calculated as the ratio of each activity (U g^{-1}) by the total protein content of the enzyme extract (mg$_{ptn}$ g^{-1}). Productivity (U g^{-1} h^{-1}) was calculated as the ratio of each activity (U g^{-1}) by fermentation time (h).

Reynolds number in the bioreactor was calculated according to Eq. (1) [5], considering the following values: L = 23.9 cm; $G = 9.3 \times 10^{+3}$ cm^3 min^{-1}; $A_T = 78.5$ cm^2; $\mu_{air} = 1.942 \times 10^{-4}$ g cm^{-1} s^{-1}; and $\rho_{air} = 1.192 \times 10^{-3}$ g cm^{-3}.

$$N_{RE} = \frac{L \times G \times \rho_{air}}{A_T \times \mu_{air} \times 60} \quad (1)$$

Cell growth data based on *N*-acg content were fitted to a logistic model [20,21], shown in Eq. (2). The solver tool in Microsoft Excel 2007 was used to estimate the model parameters.

$$X = \frac{X_m}{1 + \left(\left(X_m/X_0\right) - 1\right) e^{-\mu t}} \quad (2)$$

In order to evaluate the correlation between Aw and MC, experimental data were fitted to an empirical model (Eq. (3)). The solver tool in Microsoft Excel 2007 was used to calculate the parameters (1.18 and 12.34) and the determination coefficient (R^2).

$$A_w = \frac{1.18 \times MC}{12.34 + MC} \quad (3)$$

The correlations between total protein content and each enzyme activity were evaluated using the software Statistica 7, considering a 95% confidence interval.

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