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Sequential solid-state and submerged cultivation of *Aspergillus niger* on sugarcane bagasse for the production of cellulase

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ABSTRACT

Sequential solid-state and submerged cultivation with sugarcane bagasse as substrate for cellulase production by *Aspergillus niger* A12 was assessed by measuring endoglucanase activity. An unconventional pre-culture with an initial fungal growth phase under solid-state cultivation was followed by a transition to submerged fermentation by adding the liquid culture medium to the mycelium grown on solid substrate. For comparison, control experiments were conducted using conventional submerged cultivation. The cultures were carried out in shake flasks and in a 5-L bubble column bioreactor. An endoglucanase productivity of 57 ± 13 IU/L/h was achieved in bubble column cultivations prepared using the new method, representing an approximately 3-fold improvement compared to conventional submerged fermentation. Therefore, the methodology proposed here of a sequential fermentation process offers a promising alternative for cellulase production.

1. Introduction

The sugarcane industry generates around 186 million tons of bagasse annually in Brazil (Soccol et al., 2010). Some of this residue is currently used for energy cogeneration in sugar mills, with the surplus being stockpiled. An alternative usage for sugarcane bagasse is as a substrate for second generation ethanol production, which could greatly increase the ethanol yield per unit mass of sugarcane; however, a long-standing difficulty has been the cost of enzymatic hydrolysis of lignocellulosic material (Cardona et al., 2010), which could be mitigated by the development of new cost-effective bioprocesses for the production of cellulolytic enzymes (Singhvi et al., 2011).

Different culture conditions, including those during the inoculum preparation step, result in different fungal growth morphologies and consequently affect enzyme production (Domingues et al., 2000). The influence of pH and temperature (Krishna et al., 2000; Sohail et al., 2009), type of nutrient medium (Domingues et al., 2000), mixed culture cultivations (Ahamed and Vermette, 2008), and bioreactor design (Ahamed and Vermette, 2010; Kim et al., 1997; Wase et al., 1985) have been investigated. In several studies, cellulase inducers such as cellulose, lactose, or various lignocelluloses were added to the cultivation medium from the inoculum preparation step onwards for pre-induction of cellulase production (Aiello et al., 1996; Ahamed and Vermette, 2010; Ahamed and Vermette, 2008; Bailey and Tahtiharju 2003; Gottschalk et al., 2010; Wase et al., 1985). The choice of inducer substrate, in both pre-culture and culture media, is one of the main factors influencing cellulase production and studies of enzymatic hydrolysis of biomass using different enzymatic cocktails indicated that the use of lignocellulosic substrates, in place of other commercial inducers such as carboxymethyl cellulose or lactose, can contribute to the specificity of the enzymatic pool and improve hydrolysis yields (Castro et al., 2010a; Maeda et al., 2011; Singh et al., 2009).

Cellulases are currently produced using either submerged fermentation (SmF) or solid-state fermentation (SSF) (Farinas et al., 2011; Kang et al., 2004; Singhania et al., 2010). The great advantage of SSF is that lignocellulosic waste can be used as substrate. In addition, it provides growth conditions that are similar to the environment to which filamentous fungi are naturally adapted. Better interaction between the microorganism and the substrate is achieved using SSF, which results in higher enzyme concentrations (Singhania et al., 2010). The main drawbacks of SSF are temperature, pH, and nutrient gradients in the bioreactor, which complicate process monitoring, control and scale-up. Although more dilute products are generated, an advantage of homogeneous submerged systems is the availability of well-established bioreactor monitoring and control techniques (Howard et al., 2003; Singhania et al., 2010).

Previous studies have described various aspects of cellulase production; however, to the best of our knowledge, there have been no reports concerning cellulase production in submerged

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culture after first using a solid-state fermentation pre-culture step. The aim of the present work was therefore to evaluate and validate a novel sequential cultivation method for cellulase production by an *Aspergillus niger* strain. The technique was based on sugarcane bagasse as the inducer substrate for pre-culture preparation, using a solid-state fermentation step followed by a transition to submerged fermentation. The systems employed were shake flasks and a bench-scale bubble column bioreactor.

2. Methods

2.1. Microorganism

A. niger A12, originally isolated from black pepper (Couri and Farias, 1995), was obtained from the Embrapa Food Technology collection (Rio de Janeiro, Brazil). The culture was kept on dry sand under freezing conditions (−18 °C). Microorganism activation was carried out in potato dextrose agar medium slants incubated for 7 days at 32 °C. The spores were suspended by the addition of 10 mL of Tween-80 (0.3% v/v) to the slant. A 1-mL volume of the spore suspension was inoculated into a 250-mL Erlenmeyer flask containing ground corn cob and nutrient medium, according to the procedure used by Couri and Farias (1995), and incubated for 5 days at 32 °C. The spore concentration was determined by counting in a Newbauer chamber, after addition of the Tween-80 (0.3% v/v).

2.2. Inducer substrate

Sugarcane bagasse was kindly provided by Edra Ecossistemas (Ipeúna, Brazil) and used without any pretreatment. Dry material was sieved using 10 and 18-mesh sieves in order to select a fraction with particle size between 1 and 2 mm.

2.3. Pre-culture conditions

2.3.1. Nutrient medium

The nutrient medium used in the pre-culture and during cellulase production was adapted from the medium described by Mandels and Sternberg (1976), and contained (w/v): 0.14% $(NH_4)_2SO_4$, 0.20% KH_2PO_4, 0.03% $CaCl_2$, 0.02% $MgSO_4{\cdot}7H_2O$, 0.50% peptone, 0.20% yeast extract, 0.03% urea, 0.10% Tween 80, and 0.10% of salt solution (5 mg/L $FeSO_4{\cdot}7H_2O$, 1.6 mg/L, $MnSO_4{\cdot}H_2O$, 1.4 mg/L $ZnSO_4{\cdot}7H_2O$, and 2.0 mg/L $CoCl_2$).

2.3.2. Pre-culture for sequential fermentation (SF)

In the sequential cultivation method, the pre-culture was initiated as SSF, using sugarcane bagasse as the solid substrate. In the SSF cultivation step, the moisture content was adjusted to 70% (w/w) by the addition of 12 mL of nutrient medium into a 500-mL Erlenmeyer flask containing 5 g of dry bagasse. A volume of spore suspension resulting in a concentration of 10^7 spores per gram of dry bagasse was added, and cultivation was maintained as SSF under static conditions for 24 h at 32 °C. A volume of nutrient medium enriched with 30 g/L of glucose was added (40 parts of nutrient medium per gram of dry solid), and the cultivation was continued as SmF in an orbital shaker incubator (Solab, Brazil) for 48 h at 32 °C, with continuous agitation at 200 rpm. A volume of pre-culture suspension corresponding to 10% (v/v) was transferred to culture medium to initiate cellulase production in either shake flasks or a 5-L bubble column reactor (Thomasi et al., 2010).

2.3.3. Pre-culture for submerged fermentation (SmF)

In the conventional submerged fermentation process, the pre-culture was initiated in 500-mL Erlenmeyer flasks by adding a volume of spore suspension, calculated to give a concentration of 10^7 spores per mL, to the nutrient medium enriched with 30 g/L of glucose. The incubation was carried out for 50 h in an orbital shaker incubator, at 32 °C and 200 rpm. A volume of pre-culture suspension corresponding to 10% (v/v) was transferred to the culture medium in order to initiate cellulase production in either shake flasks or the bubble column reactor.

2.4. Enzyme production

The culture medium used for cellulase production was similar to that used for the pre-culture (Section 2.3.1), except that it was supplemented with 10 g/L of glucose and 1% (w/v) of sugarcane bagasse.

The proposed sequential fermentation process was first evaluated by enzyme production in shake flask cultures. The results obtained were then validated using bubble column bioreactor cultures. All experiments were carried out in duplicate, and the data were calculated as means ± standard deviation.

The preliminary cultivations were performed for 96 h in 500-mL Erlenmeyer flasks, with a 100-mL working volume, at 32 °C and 200 rpm. The pH was set at 6.0 before sterilization of the media, and was not controlled during cultivation. Samples were collected at 24-h intervals, centrifuged at 2500*g* for 10 min, and the crude enzymatic extract was used for quantification of endoglucanase activity (CMCase) and reducing sugars.

The cultivations conducted in the bubble column bioreactor (5.0-L working volume) were carried out for 30 h, at 32 °C and an air flow rate of 4 vvm. During these cultivations, the pH was controlled at 5.0 by addition of 1 mol/L HCl and 2 mol/L NaOH. Samples were collected at 6 h intervals and centrifuged at 2500*g* for 10 min. The crude enzymatic extract was used for quantification of endoglucanase (CMCase) and xylanase activities, as well as the reducing sugars concentration.

2.5. Analytical methods

The reducing sugars concentration was determined by the DNS method (Miller, 1959). Endoglucanase activity was assayed in the presence of carboxymethyl cellulose (Sigma, USA), by the standard method proposed by Ghose (1987). Xylanase activity was determined by the method described by Bailey and Poutanen (1989). One unit of endoglucanase or xylanase activity was defined as the amount of enzyme that released 1 μmol of reducing sugar per min under the assay conditions.

3. Results and discussion

3.1. Initial evaluation of the sequential cultivation method

Cellulase enzymes refer to a system of three different enzymes whose combined actions cause the degradation of cellulose. The most accepted mechanism for enzymatic cellulose hydrolysis involves synergistic actions by endoglucanase, exoglucanase or cellobiohydrolase, and β-glucosidase (Zhang et al., 2006). In fact, in the presence of plant polysaccharides, *A. niger* species are able to produce an extensive range of enzymes such as cellulases, xylanases, xyloglucanases and pectinases to promote an efficient degradation of the biomass (de Vries and Visser, 2001). Here, in order to simplify the evaluation, only endoglucanase and xylanase activities being produced by *A. niger* were used to compare the performance of the sequential cultivation method.

The first set of experiments was performed using shake flasks, and compared the efficiencies of the proposed sequential method and conventional SmF, under similar conditions in terms of pH,