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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

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; hoice of inducer s 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,

temperature, inoculum size, aeration, and carbon source. These preliminary results showed that higher endoglucanase production was achieved by sequential cultivation (1052 ± 34 IU/L), than by conventional SmF (824 ± 44 IU/L), with maximum production after 72 h of cultivation in both cases. The sequential method therefore had a positive influence on endoglucanase production. These results can be explained by the fact that the morphology of filamentous fungi in submerged cultivations can have an effect on cellulase production (Domingues et al., 2000). Filamentous mycelia were predominant in the sequential cultivation, resulting in higher endoglucanase expression than in SmF, where the formation of pellets was observed soon after the first day of cultivation.

Given the promising results obtained in the shake flask experiments, cultivations using SF and SmF were performed in a bubble column bioreactor in order to validate the new sequential cultivation method for cellulase production.

3.2. Bubble column bioreactor cultivations

The SF and SmF experiments were performed in duplicate, and differences smaller than 20% were obtained for endoglucanase and xylanase activities, as well as for reducing sugars concentrations. In all cultivations, the minimum levels of dissolved oxygen were higher than 25% of air saturation, showing that there was no oxygen limitation. Since the sugarcane bagasse used as inducer for cellulase production was a lignocellulosic biomass that had not been pretreated, xylanase activity was also measured in the bubble column bioreactor cultivations in order to observe the lignocellulose-induced profiles of both cellulase and xylanase production.

In the sequential cultivation method, *A. niger* developed a dispersed filamentous morphological growth form. Although there was a moderate increase in broth viscosity, it was still possible to maintain good recirculation and mixing in the bioreactor. The results for sequential cultivation using the bubble column bioreactor are presented in Fig. 1. The glucose concentration (as reducing sugar) decreased rapidly during the first 12 h, and maximum enzymatic activities occurred when the carbon source was almost exhausted, as also described by Bailey and Tahtiharju (2003) for batch cultivation cellulase production with a *Trichoderma reesei* strain.

The most striking finding was that xylanase and endoglucanase production during sequential cultivation seemed to be induced by the presence of the lignocellulosic biomass (sugarcane bagasse), since the fungus first produced xylanases to degrade the hemicellulosic fraction, and then produced endoglucanase for the conversion of cellulose to fermentable sugars (Fig. 1). Xylanase production reached a peak of 1961 ± 102 IU/L after 6 h of cultivation, while endoglucanase production only reached a peak of 716 ± 89 IU/L after around 18 h of cultivation. Lignocellulose-induced xylanase and endoglucanase production was favored by the fact that in the sequential process the *A. niger* pre-culture employed solid-state fermentation with sugarcane bagasse as the only carbon source. The fungus was then able to metabolize this insoluble carbon source used to induce enzyme production, even under the highly aerated conditions that existed in the bubble column bioreactor, which causes high agitation in the fermentation broth, and consequently reduces the cell-to-substrate interaction.

Cultivations using the conventional SmF method were carried out in order to compare the results with those obtained by the proposed combined cultivation method (Fig. 2). One important difference between the endoglucanase and xylanase production profiles observed for SmF and SF was that in the SmF cultivations there was gradual production of both enzymes throughout the entire cultivation period, at much lower levels than those obtained using sequential cultivation. The maximum endoglucanase and xylanase activities of 432 ± 24 and 714 ± 97 IU/L, respectively, were reached after around 30 h of cultivation.

A possible explanation for the differences observed between the SF and SmF processes is that in SmF the fungus was not exposed to sugarcane bagasse during the pre-culture step, in contrast to the sequential procedure, where bagasse was used in the pre-culture medium. Another explanation of the difficulty of *A. niger* to assimilate sugarcane bagasse as inducer is that growth of fungal pellets occurred during pre-culture in the liquid medium, as a result of which there was insufficient contact between the fungal cells and the substrate to promote induction.

The growth morphologies of *A. niger* in submerged fermentation under different culture conditions have a strong influence on fermentation performance (El Enshasy et al., 2006). According to Gerlach et al. (1998), the morphology of filamentous fungi and product formation can be affected by the type of bioreactor used. In the present study, the type of fermentation vessel (shake flasks or bubble column bioreactor) was not responsible for the observed differences in fungal morphology, while the type of pre-culture had a strong influence. It is important to note that the results obtained using bubble column cultivations corroborated the preliminary results obtained using shake flasks, since when *A. niger* was pre-cultured using sequential method, fungal germination using SSF encouraged growth in the form of dispersed mycelia. This enhanced the interaction between the cells and the substrate, and enzyme production increased.

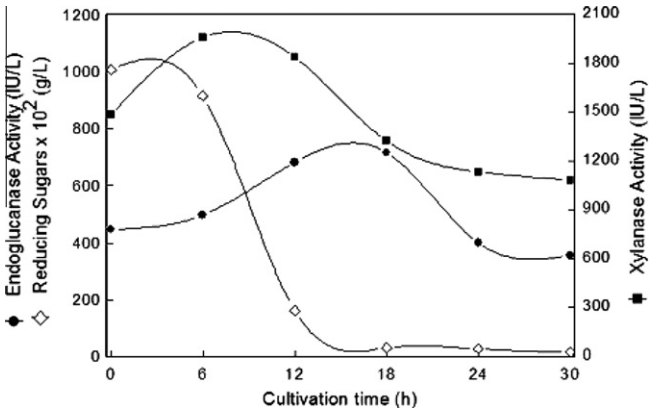

Fig. 1. Endoglucanase activity, xylanase activity, and reducing sugars concentration, as a function of time, for sequential fermentation (SF) in the bubble column bioreactor at 32 °C, pH 5.0, and an aeration rate of 4 vvm. Results are presented as means ± standard deviation.

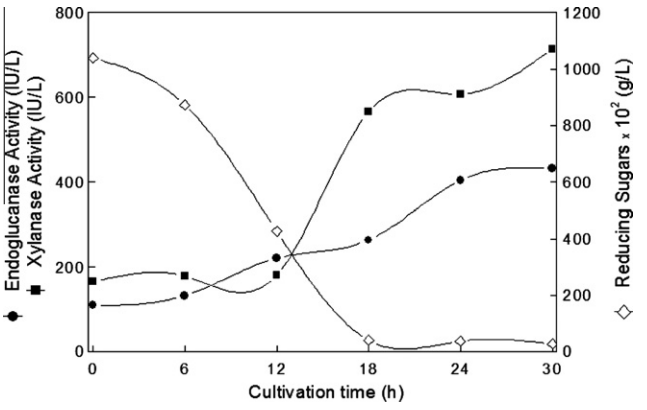

Fig. 2. Endoglucanase activity, xylanase activity, and reducing sugars concentration, as a function of time, for conventional submerged fermentation (SmF) in the bubble column bioreactor at 32 °C, pH 5.0, and an aeration rate of 4 vvm. Results are presented as means ± standard deviation.

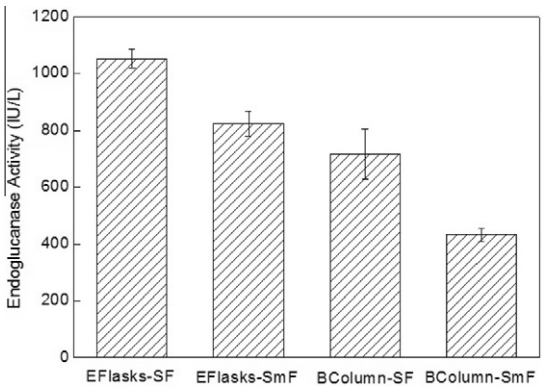

Fig. 3. Maximum endoglucanase production obtained using different cultivation methods. SmF: conventional submerged fermentation; SF: sequential fermentation; Eflasks: cultivations in shake flasks; BColumn: cultivations in the bubble column bioreactor. Results are presented as means ± standard deviation.

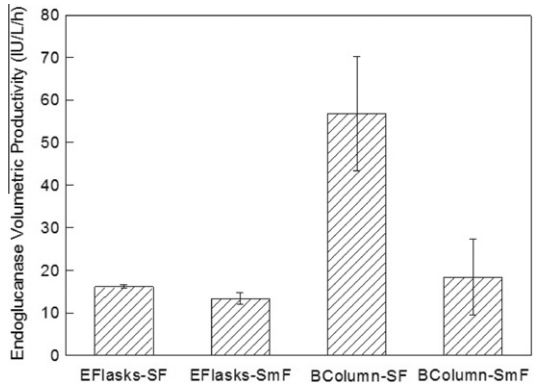

Fig. 4. Maximum endoglucanase volumetric productivity obtained using different cultivation methods. SmF: conventional submerged fermentation; SF: sequential fermentation; Eflasks: cultivations in shake flasks; BColumn: cultivations in the bubble column bioreactor. Results are presented as means ± standard deviation.

Table 1
Endoglucanase volumetric productivity for different cultivation conditions.

Organism	Carbon source	Endoglucanase volumetric productivity (IU/L/h)	Reference
A. niger A12	In natura sugarcane bagasse	57	This work
A. niger ATCC-16404	Pretreated sugarcane bagasse	1.48	Castro et al. (2010b)
A. awamori 2B.361 U2/1	Wheat bran	14.88	Gottschalk et al. (2010)
A. niger MS82	Carboxymethyl cellulose	2.65	Sohail et al. (2009)
A. niger LMA	Cellulose-yeast extract	20.14	Ahamed and Vermette (2008)

Figs. 3 and 4 illustrate endoglucanase production and volumetric productivity obtained using SF and SmF, in shake flasks and in the bubble column bioreactor. Endoglucanase activities were higher using SF than using SmF, for both systems (Fig. 3). An interesting finding was that the maximum endoglucanase activity in shake flasks (1052 ± 34 IU/L) was higher than that obtained using the bubble column bioreactor (716 ± 89 IU/L). The lower level of agitation in the shake flasks could have improved contact between the fungal hyphae and the insoluble substrate (sugarcane bagasse), resulting in greater induction and endoglucanase expression.

In terms of volumetric endoglucanase productivity (Fig. 4), the bubble column bioreactor was superior to the shake flasks, especially using the sequential process, where *A. niger* showed a dispersed growth morphology. The maximum volumetric endoglucanase productivity achieved using the bubble column bioreactor was 57 ± 13 IU/L/h, while under the similar conditions it was 16.2 ± 0.4 IU/L/h using the shake flasks. This was expected, since the pneumatic bioreactor provided high levels of mass transfer, which increased the availability of dissolved oxygen for microorganism growth, resulting in higher productivities than those achievable in shake flasks.

It is difficult to compare endoglucanase production obtained in this study with values reported in the literature, due to differences between the procedures in terms of the microorganisms and the nutritional and operational conditions. Nevertheless, the productivity obtained here, using the proposed sequential method in bubble column bioreactor cultivations, was higher than the values reported elsewhere (Table 1). In the present study, endoglucanase productivity achieved using the proposed sequential method was similar to or higher than previously reported productivities, despite using a wild-type *A. niger* strain and an insoluble inducer substrate that had not been pretreated. The new sequential cultivation method used for endoglucanase production is a promising alternative to conventional submerged cultivation and therefore should be further evaluated in terms of downstream processing and overall efficiency.

4. Conclusions

The combination of solid-state and submerged fermentation in a sequential cultivation for endoglucanase production was shown to be superior to the conventional submerged method, since endoglucanase volumetric productivity was 3-fold higher in bubble column bioreactor using sequential cultivations over conventional SmF. The sequential cultivation method improved both the assimilation of sugarcane bagasse as inducer substrate and fungal growth morphology. Germination of *A. niger* on a solid-state medium encouraged the development of a dispersed filamentous form, which resulted in superior cell-to-substrate interaction, and consequently in higher endoglucanase production.

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