Biocatalysis and Agricultural Biotechnology 6 (2016) 1–8

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

journal homepage: www.elsevier.com/locate/bab

Potential use of soybean hulls and waste paper as supports in SSF for cellulase production by *Aspergillus niger*

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ARTICLE INFO

Article history:
Received 18 November 2015
Received in revised form
5 February 2016
Accepted 6 February 2016
Available online 8 February 2016

Keywords:
Endoglucanase
Exoglucanase
β-glucosidase
Aspergillus niger NRRL3
Solid-state fermentation
Soybean hulls

ABSTRACT

Cellulase has by vast applications in the biofuel, pulp and paper, detergent and textile industries. The three components of the enzyme complex (endoglucanase, exoglucanase and β-glucosidase) can effectively depolymerize the cellulose chains in lignocellulosic substrate. Solid-state fermentation (SSF) by fungi is a preferable production route for cellulase because of its low cost, among other advantages. This work describes the cellulase production by *Aspergillus niger* NRRL3 grown on SSF. SSF was carried out on soybean hulls and waste paper as supports. The effect of the support on cellulase production was assessed under a completely randomized factorial design. The support-time interaction was significant for all the variables studied. Both materials were characterized in terms of water absorption index and critical humidity point. Samples of culture were analyzed with scanning electron microscopy (SEM) to study spores and fungal growth.

Maximum endoglucanase activity was found at 96 h using soybean hulls as support (5914.29 U L^{-1}), being four times higher than that obtained using waste paper at the same fermentation time. The exoglucanase activity in soybean hulls was maximal at 96 h (4551.19 U L^{-1}), being 9.6 times higher than that obtained in waste paper at the same time. The maximum β-glucosidase activity in soybean hulls (984.01 U L^{-1}) was reached at 96 h, being 1.7 times greater than that obtained in waste paper. Besides, the use of soybean hulls provided high volumetric productivities at shorter times, which may decrease production costs considering a scaled process.

1. Introduction

Cellulase refers to an enzyme complex which hydrolyzes glycosidic linkages and which includes: endocellulase (EC 3.2.1.4.), exocellulase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21) (Jecu, 2000).

Cellulase participates in a large proportion in the world market for enzymes. Indeed, cellulase represents approximately 20% of the enzyme global market and it is the world's third largest industrial enzyme by dollar volume. The increasing demand of cellulase is related to its relevant use in the textile, food and drink, pulp and paper, biofuel, detergent and animal feed industries (Dave et al., 2013; Silva et al., 2013).

Commercially, conversion of cellulosic biomass requires the utilization of cellulases, making the process expensive. Therefore, cellulase production from a broad range of microorganisms has been studied.

Cellulases are produced by several microorganisms as they are required for their growth and development under certain conditions. *Trichoderma reesei* cellulases are characterized by a high proportion of endoglucanase and exoglucanase but a low proportion of β-glucosidase. For this reason, other microorganisms such as the genus *Aspergillus* were analyzed. *Aspergillus niger* has been widely used because it produces the three fundamental enzymes required for cellulolysis. Fermentation studies are needed to evaluate the productivity of cellulases (Sohail et al., 2009).

In previous works the use of the proven technology of submerged fermentation has been widely reported (Meinicke et al., 2012; Prajapati et al., 2014; Rustiguel et al., 2015; Vendruscolo et al., 2013) due to better monitoring and ease of handling. However, SSF (Solid-state fermentation) offers an alternative for low-cost enzyme production and it has different advantages such as greater yields, lower investment costs and lower energy demand (Gupta et al., 2015).

In order for the substrate to enable microbial growth and metabolism, it is necessary to maintain adequate moisture conditions, which imitate those of natural growth (Orzua et al., 2009). Water availability in SSF affects microbial growth and metabolism and determines water activity, a physicochemical parameter referred

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http://dx.doi.org/10.1016/j.bcab.2016.02.003
1878-8181/

to as the relationship between the water vapor pressure of the substrate and the water vapor pressure of pure water. The chemical constitution and structure of the supports employed in SSF influence the values of water activity, which can fluctuate from 0.80 to 0.99 to achieve an effective fungal metabolism (Martins et al., 2011). Different materials have been used as solid supports for SSF, such as rice and wheat bran (Khandeparkar and Bhosle, 2006), coffee by-products (Machado et al., 2012), mango peels (Buenrostro-Figueroa et al., 2010), coconut husks (Orzua et al., 2009), corn cobs (Mussatto et al., 2009b) and grape skins (Botella et al., 2007) among others. Some of these derivatives have been used as substrates and supports to obtain products of industrial interest, including antibiotics, flavor and aroma compounds, organic acids, pigments, bioactive molecules and a wide variety of enzymes (Martins et al., 2011).

In previous works, cellulase production under SSF has been evaluated by determining total cellulase activity (Latifian et al., 2007; Silva et al., 2013). However, this method does not determine the cellulase activity of the three components of the cellulase complex individually and it does not allow elucidation on whether the three components have been adequately produced.

The aim of this study was to produce cellulase enzyme by growing *A. niger* NRRL3 on SSF using different materials as carbon sources and as growth supports. An individual monitoring of each of the three components of the cellulase complex was carried out. In addition, materials were characterized in terms of water absorption index and critical humidity point. Samples of culture were analyzed with scanning electron microscopy (SEM) to study fungal growth.

2. Materials and methods

2.1. Physico-chemical characterization of the supports

The supports used in this work included: soybean hulls (acquired from agricultural regions of Argentina) and waste paper (acquired from the printing industry). The physico-chemical parameters studied were: critical humidity point (CHP) (Mussatto et al., 2009b), packing density (PD) (Santomaso et al., 2003) and water absorption index (WAI) (Orzua et al., 2009).

The CHP was assayed by adding 1 g of sample in a thermo-balance at 130 °C until a constant weight was obtained. The PD was determined by adding 10 g of sample in a graduated tube, and the sample was stirred until no volume changes were observed for 5 min.

For the analysis of WAI, 1.5 g of a sample of support and 15 mL of distilled water were placed in a centrifuge tube. The mix was homogenized at 25 °C for 1 min and centrifuged at 3000 g for 10 min. The supernatant was removed, and the WAI was determined from the weight of the remaining gel and calculated as g gel/g dry weight.

2.2. Microorganism and culture

Strains of *A. niger* NRRL3 were provided by the culture collection of Agricultural Research Service, USDA (USA for donating the strain of *A. niger*). The strain was maintained at −20 °C in 10% w/v glycerol. Spores from *A. niger* NRRL3 were activated in potato glucose agar medium at 30 °C for five days. The culture spores were collected with sterile solution of 0.1% Tween-80 and quantified through a calibration curve from *A. niger* NRRL3.

2.3. Solid-state fermentation

Cellulase production experiments were performed in 250 mL flasks considered as bioreactors, which were sterilized with an homogeneous mixture containing the following fermentable mass: 15 g of each support (soybean hulls and waste paper) was mixed with 45 mL of Mandels culture medium with the following composition (g L^{-1}): $(NH_4)_2SO_4$ (1.4), KH_2PO_4 (2.03), $CaCl_2$ (0.30), $MgSO_4 \cdot 7H_2O$ (0.30), peptone (1.00), $FeSO_4$ (0.005), $MnSO_4 \cdot 4H_2O$ (0.0016), $ZnSO_4 \cdot 7H_2O$ (0.0014), $CoCl_2 \cdot 6H_2O$ (0.02), urea (0.30), yeast extract (0.25), $CuSO_4 \cdot 5H_2O$ (0.001). The medium pH was adjusted to pH 4.6 and then autoclaved 121 °C for 15 min. The fermentable mass was aseptically inoculated with 1×10^6 spores/mL of Mandels culture medium. The SSF was carried out at 30 °C for 120 h. Enzymatic extract (EE) was obtained by adding 20 mL of 0.1 M acetate buffer (pH 4.8) to each reactor. Fermented material was compressed and filtered. Cellulase activity and soluble protein were determined on the EE. Biomass was determined from fermentable mass. Cellulase volumetric productivity (U L^{-1} h^{-1}) was calculated as the ratio among cellulase activity value and fermentation time.

2.4. Analytical methods

Protein content was estimated by the Warburg and Christian method. Fungal biomass was determined according to the glucosamine method described by Blix (1948).

The samples were subjected to acid hydrolysis in order to remove glucosamine from fungal cell wall. Glucosamine mixed with acetylacetone forms a pyrrole compound which reacts with p-dimethylaminobenzaldehyde, obtaining a product that can be measured at 530 nm. A calibration curve was performed under the same experimental conditions of the samples. The fungal biomass was expressed as mg glucosamine per gram of sample.

Assays for the activity of individual enzyme components, i.e., endoglucanase, exoglucanase and β-glucosidase are briefly described as follows:

Endoglucanase. Carboxymethylcellulose (CMC, 1%) solution was prepared in 50 mM sodium citrate buffer (pH 5.3). CMC solution was incubated with enzymatic extract at 50 °C for 10 min. A milliliter of 3,5-Dinitrosalicylic acid (DNS) reagent was added, it was incubated for 10 min at 100 °C and the absorbance was read at 560 nm. The reducing sugar concentration produced from the enzymatic reaction was then measured and used to calculate the endoglucanase activity.

Exoglucanase. Enzymatic extract was added to Whatman filter paper # 1 in 0.1 M sodium acetate buffer (pH 4.8). After incubation at 50 °C for 60 min, 1 mL of 1% DNS reagent was added, it was incubated at 100 °C for 10 min and the absorbance was read at 560 nm. The reducing sugar concentration produced from the enzymatic reaction was then measured and used to calculate the exoglucanase activity.

β-Glucosidase activity was estimated using 4-Nitrophenyl β-D-glucopyranoside (Sigma-Aldrich, Co., St. Louis, USA) as a substrate. The reaction mixture consisted of 4-Nitrophenyl β-D-glucopyranoside 9 mM and enzymatic extract in 0.2 M acetate buffer (pH 4.6). After incubation at 50 °C for 10 min, the enzyme reaction was stopped by adding 0.1 M Na_2CO_3. The p-nitrophenol liberated was measured at 400 nm. A calibration curve of p-nitrophenol was carried out at the same experimental conditions as the samples.

2.5. Scanning electron microscopy

Visualization of spore and fungal growth was done using SEM (Environmental Scanning Electron Micro-scope) FEI QUANTA 200 F Feg. Samples were dehydrated and analyzed at 5 keV under low vacuum at 0.10 mbar with a Lector LFD (large field detector). Then the samples dehydrated were coated with electrolytic gold and analyzed under high vacuum at a pressure of 6.2 10^{-4} mbar with an ETD (Everhardt Thornley Detector) and BSED (back-scattered detector).