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The influence of sorbitol on the production of cellulases and xylanases in an airlift bioreactor

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HIGHLIGHTS

- Production of cellulases and xylanases by *Penicillium echinulatum* in an airlift bioreactor.
- The use of a mixture of cellulose and sorbitol in the culture medium.
- The production of cellulases and xylanases in batch and fed-batch.

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ABSTRACT

The production of cellulases and xylanases by *Penicillium echinulatum* in an airlift bioreactor was evaluated. In batch production, we tested media with isolated or associated cellulose and sorbitol. In fed-batch production, we tested cellulose addition at two different times, 30 h and 48 h. Higher liquid circulation velocities in the downcomer were observed in sorbitol 10 g L^{-1} medium. In batch production, higher FPA (filter paper activity) and endoglucanase activities were obtained with cellulose (7.5 g L^{-1}) and sorbitol (2.5 g L^{-1}), 1.0 U mL^{-1} (120 h) and 6.4 U mL^{-1} (100 h), respectively. For xylanases, the best production condition was cellulose 10 g L^{-1}, which achieved 5.5 U mL^{-1} in 64 h. The fed-batch process was favorable for obtaining xylanases, but not for FPA and endoglucanases, suggesting that in the case of cellulases, the inducer must be added early in the process.

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1. Introduction

Cellulases form an enzyme complex that is widely used in the production of detergents, in biostoning, in paper processing, in the extraction of vegetable oils, as additives for silage and animal feed, and in the production of ethanol from lignocellulosic materials (Galbe and Zacchi, 2002; Maeda et al., 2013). These enzymes are produced by fungi and bacteria and form an enzyme complex that comprises endo-β-1,4-glycanases (EG I, EG II, EG III, EG IV, and EG V, EC3.2.1.4), which randomly hydrolyze glycosidic bonds; exo-β-1,4-glycanases (CBH I and CBH II, EC 3.2.1.91), which act on the reducing and non-reducing ends; and β-1,4-glycosidases (BG I and BG II, EC 3.2.1.21), which hydrolyze oligosaccharides and cellobiose to glucose (Mansfield et al., 1999; Martins et al., 2008).

Xylanases (1,4-β-D-xylan xylanohydrolases, EC 3.2.1.8) are a group of enzymes produced by fungi, yeasts and bacteria that are used in the production of animal feed, pulp, and food processing (Xiong et al., 2005; Vafiadi et al., 2010). These enzymes play an important role in the hydrolysis of xylan, a polysaccharide formed of xylose monomers, which is one of the major components of hemicellulose (Maciel et al., 2008).

Among the cellulase and xylanase producing microorganisms, the strain *Penicillium echinulatum* 9A01S1 is highlighted because its high levels of FPA and β-glycosidases confer to it a great potential in the production of cellulolytic complexes (Camassola and Dillon, 2007). Cellulase production may be carried out in solid-state or submerged culture (Camassola and Dillon, 2010; Singhvi et al., 2011; Prévot et al., 2013). In submerged cultivation, cellulase production is usually performed in a stirred tank reactor (STR); however, in many cases, the degree of stirring required to achieve an adequate mass transfer causes damage to the microorganisms due to high-shear areas caused by the stirring blades. In addition, stirring has been shown to consume a large amount of energy (Chisti and Jauregui-Haza, 2002; Ahamed and Vermette, 2010). As an alternative, airlift bioreactors can be used to produce cellulases (Ahamed and Vermette, 2010). The configuration of the airlift bioreactor has been well studied, especially with regard to its ability to transfer oxygen (Fontana and Silveira, 2012).

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The mechanism of action of cellulases is synergistic, and their production by microorganisms depends on various factors, such as the composition of the production medium, pH, temperature, and oxygen supply (Gabelle et al., 2012; Bai et al., 2013). During the growth phase of the microorganism, there is a biomass increase, which increases the production medium viscosity and thus affects the oxygen transfer and enzyme productivity of the process (Patel et al., 2009). Sources of insoluble cellulose such as *solka floc* (cellulose pulp) are inducers of microbial cellulases. The presence of insoluble sources in the cultivation medium also contributes to the increase in medium viscosity. The use of soluble sources may lead to an increase in the production of the enzyme complex, with an increase in oxygen transfer and a decrease in potential problems of catabolite repression by cellobiose and glucose resulting from cellulose hydrolysis (Wen et al., 2005). Several studies were performed with different inducers of cellulases and xylanases (e.g., lactose, sophorose, glucose, and sorbitol) to determine their effects (Botella et al., 2007; Ilmén et al., 1997). In this context, the present study aimed to evaluate the production of cellulases and xylanases by *P. echinulatum* in an internal circulation airlift bioreactor operated in batch and fed-batch cultivation using production media with different concentrations of cellulose and sorbitol. Additionally, the liquid circulation velocity in the downcomer was evaluated, as were various concentrations of cellulose and sorbitol, either isolated or associated, in the culture medium.

2. Methods

2.1. Microorganism

For the production of cellulases and xylanases, the mutant strain *P. echinulatum* 9A02S1 (DSM 18942) was used. This strain was obtained by exposing the wild type *P. echinulatum* strain 2HH to ultraviolet (UV) light and hydrogen peroxide (H_2O_2) (Dillon et al., 2006). These strains are stored in the culture collection of the Enzyme and Biomass Laboratory of the Institute of Biotechnology, at the University of Caxias do Sul, Brazil. The cultures were grown on C-agar slants for up to seven days at 28 °C until conidia were formed and then stored at 4 °C until use.

2.2. Culture medium

The production medium used as a basis for the remaining formulations had the following composition: commercial soybean meal, 2 g L^{-1}; KNO_3, 1.4 g L^{-1}; concentrated mineral solution, 50 mL L^{-1}; and Tween 80, 0.1 mL L^{-1}. The mineral solution described by Mandels and Reese (1957) was composed of KH_2PO_4, 20 g L^{-1}; $CO(NH_2)_2$, 3 g L^{-1}; $MgSO_4{\cdot}7H_2O$, 3 g L^{-1}; $CaCl_2$, 3 g L^{-1}; $FeSO_4{\cdot}7H_2O$, 0.050 g L^{-1}; $MnSO_4{\cdot}H_2O$, 0.0156 g L^{-1}; $ZnSO_4{\cdot}7H_2O$, 0.014 g L^{-1} and $CoCl_2$, 0.020 g L^{-1}.

The addition of different concentrations of cellulose and sorbitol, either isolated or associated, to the production medium, was evaluated under the following conditions: C10 (cellulose 10 g L^{-1}); C7.5 + S2.5 (cellulose 7.5 g L^{-1} + sorbitol 2.5 g L^{-1}); C5.0 + S5.0 (cellulose 5.0 g L^{-1} + sorbitol 5.0 g L^{-1}); C2.5 + S7.5 (cellulose 2.5 g L^{-1} + sorbitol 7.5 g L^{-1}) and S10 (sorbitol 10 g L^{-1}). The media were autoclaved at 1 atm for 15 min.

2.3. Experimental conditions

The airlift reactors used in our study had a total capacity of 4 L and a working volume of 3.5 L. The external cylinder (downcomer) was made of refractory glass, and the internal tube (riser) was made of polyvinyl chloride (PVC). The aeration system was constructed with a stainless steel tube and silicone rings with perforations to allow air to exit. The lid of the airlift reactor was made of nylon. To control the temperature, a circulating bath with an internal U-shaped tube was used (Fontana et al., 2009). This reactor was connected to a control unit system (TECNAL). The specific airflow during cultivation was between 0.5 and 2.5 vvm.

The volumetric oxygen transfer coefficients (K_La) for our airlift reactor were measured as a function of the specific airflow rate (Q: 0.3 vvm).

To obtain the inoculum, cultures were grown in 500 mL Erlenmeyer flasks, containing 100 mL of production medium inoculated with 1×10^7 spores mL^{-1} of *P. echinulatum*, kept under reciprocal shaking at a shaking frequency of 180 rpm at 28 °C for 48 h. The inoculum corresponded to 10% (v/v) of the working volume of the internal circulation airlift bioreactor.

During the cultivations, the specific airflow varied between 0.25 and 1.25 vvm. In the assays with different cellulose and sorbitol concentrations (isolated or associated), the specific airflow was 0.75 vvm. The culture temperature was controlled at 28 °C, and thee initial pH varied from 6.45 to 6.10, without control during the process.

The described production media were assessed in batch cultivation. In fed-batch cultivation, two process times for cellulose addition were evaluated, 30 and 48 h of process time, and the condition used was C7.5 + S2.5 (cellulose 7.5 g L^{-1} + sorbitol 2.5 g L^{-1}). The cultivation was initiated with a production medium free of cellulose and 2.5 g L^{-1} of sorbitol; subsequently, cellulose was added at 30 h (C7.5 + S2.5–30 h) or 48 h (C7.5 + S2.5–48 h) after the initiation of the process. The cellulose added to the medium was suspended in 200 mL of distilled water, autoclaved, and added to the cultivation medium using a peristaltic pump.

Samples were periodically collected, filtered, and stored at 4 °C for subsequent measurements of FPA, endoglucanases and xylanases.

The volumetric coefficient of oxygen transfer (K_La) in a non-inoculated medium was determined by the method described by Moo-Young and Blanch (1989).

The circulation velocity of the medium was determined according to the method described by Kawase and Moo-Young (1986), with modifications. For each airflow rate, the time required for the pellets to travel a given distance in the downcomer of the airlift bioreactor was measured. Using Eq. (1), we calculated the mean circulation velocity in the downcomer (U_{Ld}).

$$U_{Ld} = X_i/t_m \quad (1)$$

where X_i and t_m correspond to the distance traveled by the pellet and the time for each measurement, respectively. Speed determination was performed in triplicate.

2.4. Analytical methods

The enzyme activity was analyzed with Whatman No. 1 filter paper according to Camassola and Dillon (2012). The total endoglucanase activity was determined according to Ghose (1987), using 2% carboxymethyl cellulose in 0.05 M sodium citrate buffer (pH 4.8). The total xylanase activity was determined according to Bailey et al. (1992). The concentrations of reducing sugars were estimated using dinitrosalicylic acid according to Miller (1959). One unit (U) of enzyme activity was defined as the amount of enzyme required to release 1 μmol of reducing sugar from the appropriate substrates per minute under assay conditions.

Cell growth was determined by DNA dosing using perchloric acid, according to Lin et al. (2000). The viscosities of the media were measured with a viscometer; model DV-II$^+$ (BROOKFIELD ENGINEERING, USA).

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