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Effect of stirring on growth and cellulolytic enzymes production by *Trichoderma harzianum* in a novel bench-scale solid-state fermentation bioreactor

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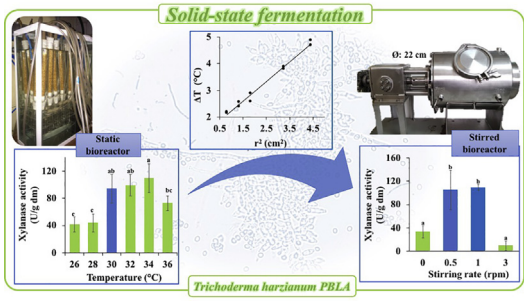
GRAPHICAL ABSTRACT



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ABSTRACT

A novel bench-scale stirred bioreactor for solid-state fermentation was used to determine the effect of the stirring rate on growth and enzymes production by *Trichoderma harzianum* PBLA. Lab-scale static tubular bioreactors were first used to assess the effect of bioreactor diameter on heat accumulation, growth, and production of cellulases and xylanases. The increased diameters (1.8–4.2 cm) led to increases in temperature up to 36 °C (at a rate of 1.08 °C/cm), which negatively affected the growth and enzyme production. Afterward, in the bench-scale bioreactor operated at rates up to 3.0 rpm, maximum xylanases production (107 ± 0.3 U/g dm) was attained at rates of 0.5 and 1.0 rpm, reaching a maximum of 34 ± 0.3 °C. Cellulases production was reduced (up to 79%) due to stirring. Therefore, the production of xylanases by *T. harzianum* can be performed in this cross-flow stirred SSF bioreactor at rates up to 1.0 rpm, avoiding heat accumulation and damage on metabolic activity.

1. Introduction

Cellulases and xylanases are hydrolytic enzymes involved in the breakdown of β-1,4 glycosidic bonds present in cellulose and hemicellulose polysaccharides, respectively (Ang et al., 2015). These enzymes have numerous applications in various industries, including chemical, fuel, textile, food, brewing and wine, as well as in laundry, pulp and paper, and agriculture, in addition to the fields of research and development (Bhat, 2000; Anwar et al., 2014). They are produced by several microorganisms, including fungi and bacteria; however, fungi

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have been emphasized because of their ability to produce large amounts of cellulases and hemicellulases, which are secreted to the culture medium, facilitating the extraction and purification steps.

The cellulolytic enzymes produced by fungi include endoglucanase (endo-1,4 β-D-glucanase, EC 3.2.1.4), exo-glucanase (1,4 β-D-glucan-cellobiohydrolase, EC 3.2.1.91), and β-glucosidase (β-D-glucoside glucanohydrolase, cellobiase, EC 3.2.1.21). Among the hemicellulolytic enzymes, fungi can produce endo-xylanase (endo-1,4-β-xylanase, EC 3.2.1.8), β-xylosidase (xylan-1,4-β-xylosidase, EC 3.2.1.37), α-glucuronidase (α-glucosiduronase, EC 3.2.1.139), α-arabinofuranosidase (α-L-arabinofuranosidase, EC 3.2.1.55) and acetylxylan esterase (EC 3.1.1.72). The synergy between both types of enzymes (cellulolytic and hemicellulolytic) is very useful in the saccharification of lignocellulosic materials (Brijwani et al., 2010). In particular, the cellulases produced by *Trichoderma harzianum* are the most efficient system for the complete hydrolysis of cellulosic substrates into glucose (Ahmed et al., 2009). Most commercial xylanolytic preparations are produced by strains of *Trichoderma* and *Aspergillus* (Mussatto and Teixeira, 2010). Although about 90% of all commercial enzymes are produced by submerged fermentation (SmF), often using genetically modified microorganisms, most of them can be produced by solid state fermentation (SSF) using native-type microorganisms (Hölker et al., 2004). Moreover, several studies have shown that the use of SSF is a promising alternative to obtain higher enzyme titers compared to SmF (Hansen et al., 2015). The higher productivity of SSF leads to a more effective production technique whose costs and energy requirements imply advantages compared to SmF (Hölker et al., 2004; Farinas et al., 2011). The use of SSF favors the growth of filamentous fungi since they can easily colonize the solid matrices, which allows the use of solid waste as support (Kilikian et al., 2014; Hansen et al., 2015). The use of heterogeneous solids as a support, such as agro-industrial wastes and low-value raw materials, in many cases, allows the solid substrate to act both as a carbon source and as an inducer for the enzyme production (Brijwani et al., 2010; Pirota et al., 2013). Also, since the SSF process is carried out in the absence of a free aqueous phase, the water consumption is minimal, which leads to a low generation of aqueous effluents (Hansen et al., 2015).

However, some operating conditions of SSF have limited its industrial application, such as the difficulty of controlling the substrate moisture level, and the accumulation of metabolic heat, among others (Pirota et al., 2013; Figueroa-Montero et al., 2011). Several studies indicate that SSF can become a highly competitive method for the production of cellulolytic enzymes from agricultural waste as a substrate, using improved bioreactor designs and adequate operating controls (Nigam and Singh, 1996). Several types of bioreactors have been traditionally used in SSF processes, which can be classified into two groups: stirred and static. The first category comprises rotary drum, gas-solid fluidized bed, rocking drum, and horizontal paddle mixers; while the static bioreactors include packed-bed and tray bioreactors (Mitchell and von Meien, 2000). The most commonly used bioreactors for SSF systems have been tray, packed-bed, rotary drum, fluidized-bed bioreactors. Particularly, SSF processes for the production of cellulolytic enzymes have frequently been carried out in a tray (Brijwani et al., 2010), rotary drum (Alam et al., 2009) or deep tank type bioreactors (Behera and Ray, 2016).

Temperature is one of the key process variables affecting SSF, because of the release of metabolic heat under aerobic growth conditions. At extreme levels, heat accumulation can cause enzyme denaturation, as well as other deleterious effects on microbial growth, and metabolite production (Figueroa-Montero et al., 2011; Farinas 2015). Heat removal in SSF bioreactors can be favored by stirring. However, since most SSF processes involve the use of filamentous fungi, the cellular integrity of the hyphae can be affected by continuous stirring due to the shearing forces between the moving particles (Mitchell et al., 2000). While some studies indicate that stirring prevents fungal growth (Desgranges et al., 1993), others report good results in stirred systems (Marsh et al., 1998). Microbial sensitivity to shear stress depends on the type of microorganism, the hyphae morphology, and the type of substrate used (Oostra et al., 2000). Nevertheless, geometry and intensity of the stirring device play an important role in mycelia disruption.

The novel bench-scale bioreactor with cross-flow stirring proposed in this work, can operate continuously at low stirring rates preventing the formation of solid aggregates, and damage of mycelium, as well as improving the heat removal, and the mass transfer of CO_2 and O_2 in the system. The aim of this work was to evaluate the stirring effect of a novel bench-scale bioreactor with cross-flow stirring on growth and production of cellulases and xylanases by *Trichoderma harzianum* PBLA.

2. Materials and methods

2.1. Microorganism

The strain *Trichoderma harzianum* PBLA was provided by Dr. Alfredo Martinez-Jimenez from the Instituto de Biotecnología at the Universidad Nacional Autónoma de México (UNAM). The strain was conserved in cryo-protect beads (Technical Services Consultant LTD, England) at −20 °C.

2.2. Inoculum preparation

Inoculum production was carried out in two steps. First, a 250 mL Erlenmeyer flask containing 50 mL of potato dextrose agar (PDA) medium (BD Bioxon) was inoculated with one cryo-protected bead containing spores and incubated at 30 °C for 7 days. The produced spores were harvested from the surface by adding 20 mL of sterile 0.1% (v/v) Tween 80 solution and scraping with a sterile magnetic stirrer. In the second step, the spore suspension obtained was used to inoculate ($1x10^6$ spores/mL) lab-scale static tubular bioreactors (TB) and bench-scale stirred bioreactors (SB) with 30 or 200 mL, respectively, of liquid medium with the following composition (in g/L): yeast extract, 20; polypeptone, 40; glucose, 40 (pH 6). The inoculated medium was incubated on a rotatory shaker at 30 °C, and 150 rpm for 72 h.

2.3. Solid-state fermentation conditions

Pine sawdust with a particle size between 0.42 and 3.3 mm was used as solid support for SSF. The support was washed with hot water, rinsed with distilled water, and oven-dried at 60 °C for 48 h. A liquid medium with the following composition (g/L) was used to moisten and inoculate the solid support: glucose, 50; KH_2PO_4, 5; NH_4NO_3, 5; urea, 2; $MgSO_4$ $7H_2O$, 0.42; $CaCl_2$, 1; peptone, 5; yeast extract, 5; 1 mL/L of trace elements solution. The trace elements solution composition was (g/100 mL): $FeSO_4$ $7H_2O$, 0.5; $MnSO_4$ $7H_2O$, 0.061; $ZnSO_4$ $7H_2O$, 0.1; $CoCl_2$ H_2O, 0.036 (Mekala et al., 2008). The initial pH of the medium was adjusted to 5.5. Half of the liquid medium was mixed with the pine sawdust and the other half was placed in a flask before autoclaving at 120 °C for 15 min. Then, the culture medium placed in the flask was mixed with the inoculum obtained from the second step of the inoculum preparation stage, at a ratio of 10 mL of inoculum to 90 mL of fresh liquid medium. The sterilized pine sawdust moistened with the fresh culture medium was then mixed with the content of the inoculated flask. Thus, the final moisture obtained for sawdust was about 65%. This inoculated SSF medium was used to fill both the lab-scale tubular bioreactors and the bench-scale stirred bioreactor (SB).

2.3.1. Effect of temperature on CO_2 and enzyme production

Assays were carried out in TBs (2.3 cm of internal diameter and 21 cm height with a packing length of 16 cm). TBs were packed with 25 g of inoculated SSF medium and incubated in water baths at temperatures from 26 to 38 °C for 48 h. Water-saturated air was passed through the TB at a flow rate of 0.8 VKgM (volume of aeration rate, in L/min, per mass of wet solid, in Kg) (Rodríguez-Fernández et al., 2011).