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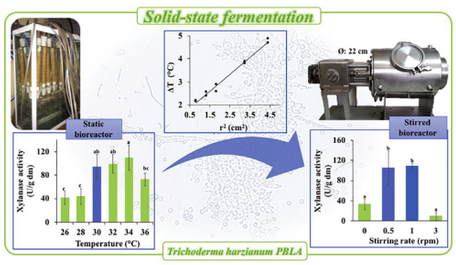
Effect of stirring on growth and cellulolytic enzymes production by *Trichoderma harzianum* in a novel bench-scale solid-state fermentation bioreactor

N. Lopez-Ramirez[a], T. Volke-Sepulveda[a], I. Gaime-Perraud[b], G. Saucedo-Castañeda[a], E. Favela-Torres[a,*]

[a] Departamento de Biotecnología, Universidad Autónoma Metropolitana, Unidad Iztapalapa, CP 09340, Iztapalapa, México, D.F., Mexico
[b] Equipe_Eco technologies et Bioremédiation, Aix Marseille Université, IMBE-UMR CNRS-7263/IRD-237, Case 421, Campus Etoile, Faculté St JérÔme, 13397 Marseille Cedex 20, France

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

* Corresponding author.
E-mail address: favela@xanum.uam.mx (E. Favela-Torres).

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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).

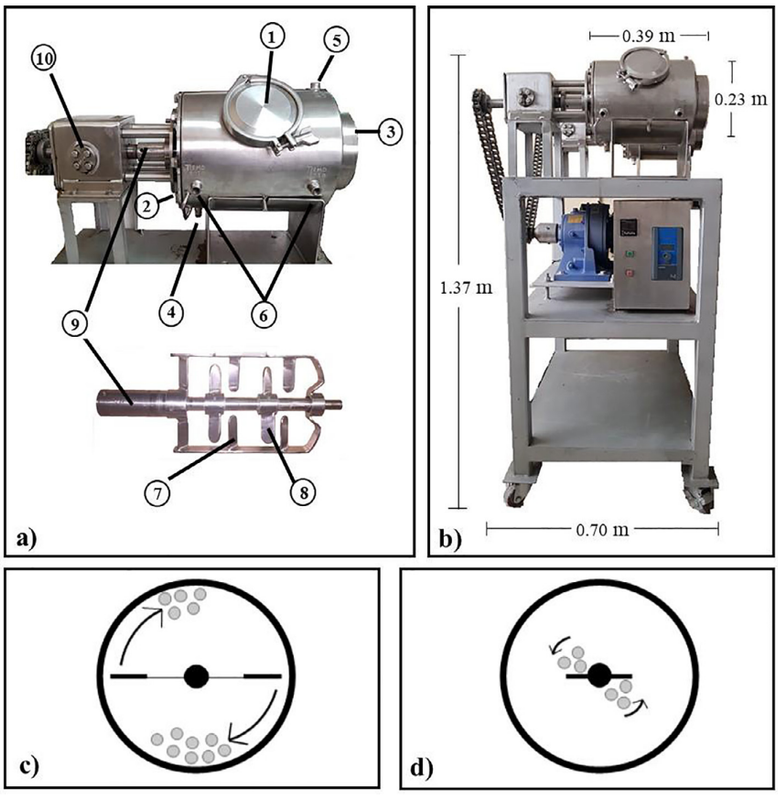

Fig. 1. Schematic diagram of the novel solid-state fermentation stirred bioreactor. (a) 1) filling and sampling door, 2) air inlet, 3) air outlet, 4) water jacket inlet, 5) water jacket outlet, 6) thermocouples, 7) external pallets, 8) centrals pallets, 9) stirring device, 10) transmission system. (b) Dimensions of the bioreactor (in m): length 0.39, external diameter 0.23, internal diameter 0.17. Stirring mechanism: (c) External pallets exert a movement of the biomass clockwise. (d) Central pallets; exerts a movement of the biomass counter clockwise, considering a lateral view of bioreactor.

For this test, two replicas were carried out for temperatures of 26 and 28 °C, four replicas for 32, 34 and 36 °C and six replicas for 30 °C.

2.3.2. Effect of TB diameter on CO_2 and enzyme production

These studies were carried out in TB with internal diameters of 1.8, 2.3, 2.7, 3.6, and 4.2 cm, and 21 cm of height with a packing length of 16 cm. TB were packed with 15, 25, 35, 58 and 82 g of inoculated SSF medium, respectively, and incubated at 30 °C for 48 h. Water-saturated air was passed through the TB at a flow rate 0.8 VKgM. Temperature was continuously registered in type-J thermocouples placed in the center of each column (at 8 cm from the bottom). For this test, two replicas were made for each diameter size.

2.3.3. Effect of stirring on O_2 uptake and CO_2 and enzyme production in bench-scale bioreactors

These assays were carried out in 6 L stirred bioreactor (SB). The SB is equipped with a cross flow mechanical stir system, water jacket, air inlet and outlet, four thermocouples (placed in the wall of the bioreactor and in the air inlet and outlet), and a port for sampling, charging and discharging the bioreactor (Fig. 1). The SB was charged at 40% of its maximum capacity with 1.3 Kg of inoculated SSF medium. Water-saturated air was supplied into the bioreactor at a flow rate of 0.8 VKgM. SSF in the SB was carried out at 30 °C for 48 h. Different stirring conditions were evaluated (0, 0.5, 1 y 3 rpm).

The SB has a double effect stirring mechanism known as cross-flow stirring. Four gears generate the movement of the stirrers. The stirring system allows mixing the wet solid with clockwise and counter-clockwise movements, considering a lateral view of the bioreactors. The anchoring system exerts upward (Fig. 1c) motion while the central forces exert a downward movement (Fig. 1d).

2.4. Measurement of O_2 uptake and CO_2 production and growth parameters estimation

The CO_2 (CO_2 sensor, Mod. K33, CO2Meter, USA), and O_2 (O_2 sensor, Mod. ZE03, WINSEN, China) concentrations, and the air-flow rate in the outlet stream of the bioreactors were continuously monitored with an on-line monitoring system (Martínez-Valdez et al., 2015) The fungal growth was indirectly estimated through the production of CO_2 as previously reported (Saucedo-Castañeda et al., 1994). The specific CO_2 production rate (μ_{CO2}) was obtained as follows, using the exponential model (Eq. (1))

$$\frac{dX}{dt} = \mu_{CO_2} X \tag{1}$$

Where X is the produced molar fraction of CO_2 at the time (t) and μ_{CO2} is the specific CO_2 production rate coefficient. The specific CO_2 production rate (μ_{CO2}) was estimated in the final zone of the *Lag* phase at the culture time where the maximum CO_2 production rate (MCO_2PR) was reached (Volke-Sepúlveda et al., 2006). *Lag* time (t_{Lag}) was estimated by the intersection of a straight line obtained from the natural logarithm function of the total CO_2 production (TCO_2P) as a function of time, considering the interval between the end time of the time *Lag* and the time of maximum CO_2 production rate (Pirt, 1975).

2.5. Enzyme extraction

The enzymatic extract was obtained after the addition of 10 mL of distilled water to 1 g of solid-state fermented material, and vortexing for 1 min; this approach allows recovering more than 80% and 95% xylanase and cellulase activity respectively. The mixture was centrifuged at 10,000 rpm for 2 min to obtain the supernatant, which was

considered as the enzymatic extract. The enzymatic extract was stored at −4 °C until enzymatic activities determination.

2.5.1. Cellulase and xylanase assays

Enzymatic activities were assayed by duplicate in at least two different biological samples. Cellulase and xylanase activities were assayed using carboxymethylcellulose (CMC) and birchwood xylan as substrates, respectively. Reaction mixtures containing 0.9 mL of 1% (w/v) substrate dissolved in sodium citrate buffer (0.1 M, pH 5.2) and 0.1 mL of enzyme extract. These were incubated at 40 °C for 30 and 15 min for cellulase and xylanase activities, respectively. The enzymatic reaction was stopped by adding 1.5 mL of 3,5-dinitrosalicylic acid (DNS), and the concentration of reducing sugars was estimated as either glucose or xylose equivalents estimated by the DNS method (Miller et al., 1960).

One unit (U) of enzyme activity is defined as the amount of enzyme required to liberate 1 μmol of reducing sugars from the corresponding substrate per minute. Enzyme activities are reported as units per gram of dry matter (U/g dm). Each sample for enzymatic activity was measured in duplicate.

2.6. Moisture content and pH determinations

The moisture content in samples was quantified in a thermogravimetric balance (Ohaus, Model MB45), and pH of the enzymatic extracts was determined in a potentiometer (Conductronic, Model pH 120).

2.7. Statistical analysis

The results were tested by one-way ANOVA and the means comparison was done using Tukey-Kramer and Duncan post hoc tests ($\alpha = 0.05$). Analyses were performed using IBM-SPSS 18.

3. Results and discussion

The effect of temperature and stirring rate on the metabolic activity of *T. harzianum* was analyzed independently in static TB or in SB, respectively. In order to evaluate the effect of temperature, SSF static cultures at isothermal conditions and different incubation temperatures were first evaluated. Afterwards, the temperature increase due to metabolic heat accumulation was measured. Once the incubation temperature range was selected and the heat accumulation in static TB was demonstrated, the novel stirred SSF bioreactor was used to evaluate the effect of the stirring rate on CO_2 and enzyme production by *T. harzianum*. Kinetic data for CO_2 production allowed the estimation of some kinetic parameters associated with fungal growth.

3.1. Effect of temperature on CO_2 and enzyme production

The effect of temperature on growth and enzyme production by *T. harzianum* under SSF static conditions was evaluated using TB with an internal diameter of 2.3 cm and a bed height of 16 cm, which were incubated in a water bath at a constant temperature. Data for the CO_2 production rate and CO_2 production (Table 1) obtained from cultures incubated at temperatures from 26 to 38 °C allowed indirect determination of the effect of temperature on growth. The maximum CO_2 production rate occurred between 17 and 20 h of culture (results not shown), and the final production of CO_2 reached values from 53 to 77 mg per gram of initial dm (mg/g idm) after 48 h of culture for incubation temperatures from 26 to 36 °C, respectively. At 38 °C no growth was registered.

The *Lag* time of cultures grown between 30 and 36 °C was significantly lower (2.1–3.8 h) compared to that registered in cultures grown at 26 and 28 °C (Table 1). Maximum CO_2 production rates were not significantly modified due to the incubation temperature in a range from 26 to 30 °C; however, this variable increased (about 2 units) at 32 and 34 °C regarding the minimum obtained (4.8 mg CO_2/g idm h) at 26 and 36 °C (Table 1). Total CO_2 production was the most sensitive variable to incubation temperature, showing a significant increase (5.3–23.5 units) as the temperature increased up to 36 °C (Table 1). Previous work has shown that *T. harzianum* has maximum growth in temperatures ranging between 25 and 30 °C (Reetha et al., 2014; Zhang and Yang, 2015). Regarding the specific CO_2 production rate (μ_{CO2}), there were no significant changes in the range of 26 to 34 °C, and a slight reduction was recorded at 36 °C (Table 1). A lower value of μ_{CO2} (0.11 h^{-1}) than those obtained in this work was recorded for *T. harzianum* cultures growing on molasses and sugar cane bagasse (Rodríguez-León et al., 1999). Based on this series of results, temperatures from 26 to 36 °C can be used for the cultivation of *T. harzianum* PBLA in SSF conditions.

The effect of the incubation temperature on xylanases and cellulases production was also evaluated (Fig. 2). Maximum production of xylanases (100 ± 8 U/g dm) was obtained at temperatures from 30 to 34 °C, which was about 2-fold higher than the values found at 26 and 28 °C. However, cellulase production was independent (9.11 ± 0.13 U/g dm) of temperature in the range from 26 to 36 °C. Xylanase and cellulase activities obtained in this work are similar to those obtained in other studies with several strains of *T. harzianum*. For instance, the maximum xylanase activity obtained in the work of Mohamed et al. (2013) was of 74 U/g of solid at 28 °C in a culture with *T. harzianum* evaluated in a temperature range between 20 and 45 °C. And in the work of Haq et al. (2006), the maximum cellulase activity was at 11 U/g of solid obtained at 28 °C, with a *T. harzianum* strain evaluated in a range between 22 and 38 °C. In both works, enzymatic activity decreased as the incubation temperature increased; above 36 °C, both activities decreased between 60 and 80%, compared to the maximum activity registered for each enzyme.

The above results showed that isothermal cultures at temperatures from 26 to 36 °C allow the growth (indirectly estimated through the production of CO_2) as well as the production of xylanases and cellulases by *T. harzianum* PBLA. No growth or enzyme production was observed in cultures incubated at 38 °C. Then, to determine the effect of the increase in temperature due to the heat accumulation by the increase in the bioreactor size, tubular bioreactors with different diameters were evaluated.

Table 1
Maximum CO_2 production rate (MCO_2PR) and total CO_2 production (TCO_2P) obtained in SSF cultures at different incubation temperature in TB of 48 h with *T. harzianum* PBLA. CO_2 data were considered for estimation of the *Lag* time and (t_{Lag}) specific CO_2 production rate (μ_{CO2}).

Temperature (°C)	MCO_2PR (mg CO_2/g idm h)	TCO_2P (mg CO_2/g idm)	t_{Lag} (h)	μ_{CO2} (1/h)
26*	4.83 ± 0.12^{a}	53.59 ± 0.18^{a}	11.12 ± 0.41^{a}	0.27 ± 0.00^{a}
28*	5.49 ± 0.02^{ab}	58.88 ± 0.81^{b}	10.18 ± 0.47^{ab}	0.30 ± 0.02^{a}
30■	5.87 ± 0.63^{ab}	68.85 ± 2.05^{c}	9.02 ± 0.78^{abc}	0.30 ± 0.02^{a}
32Δ	6.14 ± 0.52^{b}	74.60 ± 1.44^{d}	7.34 ± 1.29^{c}	0.30 ± 0.03^{a}
34Δ	6.10 ± 0.46^{b}	77.10 ± 1.53^{d}	7.95 ± 0.28^{bc}	0.29 ± 0.01^{a}
36Δ	4.81 ± 0.08^{a}	75.51 ± 4.13^{d}	8.25 ± 0.15^{bc}	0.22 ± 0.01^{b}

Data with different letter by column indicate significant differences ($p < 0.05$). Values correspond to the mean of two (*), four (Δ), and six replicates (■).

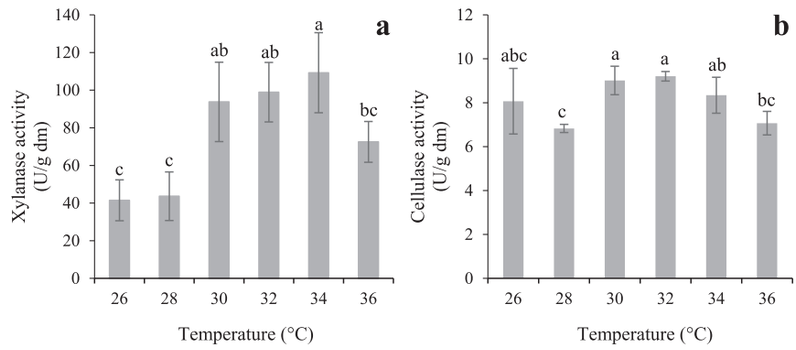

Fig. 2. Effect of the incubation temperature on the xylanases (a) and cellulases (b) production by *T. harzianum* PBLA in SSF. Samples were taken after 48 h of cultivation.

3.2. Effect of TB diameter on CO_2 and enzyme production

TBs with internal diameters of 1.8–4.2 cm and a bed height of 16 cm, incubated at 30 °C in a gaseous atmosphere, were used to evaluate the effect of bioreactor diameter on the temperature profile in the fermentation bed. Temperature profiles as a function of incubation time show that heat accumulation occurs during a short period (10 h), which is associated with the maximum CO_2 production rate (Fig. 3a and 3b). Maximum temperatures increased proportionally to the increase in the internal diameter of the tubular bioreactor (Fig. 3c). The highest recorded temperature (34.7 ± 0.01 °C) due to the accumulation of metabolic heat was observed in the tubular column with an internal diameter of 4.2 cm.

The increase in temperature in TB with a larger diameter is due to the poor removal of heat by conductive transfer (Raghavarao et al., 2003). For instance, Oostra et al. (2000) simulated temperature profiles based on different diameters, obtaining a behavior similar to that found in the present work. That is, as the diameter of the bioreactor increased from 0.2 to 1 m, the radial temperature gradient was predicted to increase from 6 to 11 °C. In general, parameters estimated from the CO_2 production data were not significantly modified due to the effect of the bioreactor diameter (Table 2). Regarding enzyme production (Fig. 4), no significant differences were found in cellulases; however, xylanase production increased significantly from 69.9 to 83.9 U/g dm by increasing the TB diameter from 1.8 to 4.2 cm.

These results show that growth and enzyme production by this *T. harzianum* strain is not negatively affected at a culture temperature of 36 °C. The above results support the conclusion that SSF process for enzyme production by *T. harzianum* PBLA can be carried out at temperatures from 30 and 34 °C. However, the use of larger bioreactors might lead to temperature values above 36 °C. To avoid metabolic heat accumulation in larger bioreactors, stirred devices can be used to improve heat removal; however, the deleterious effects of stirring on growth and enzymes production must be evaluated.

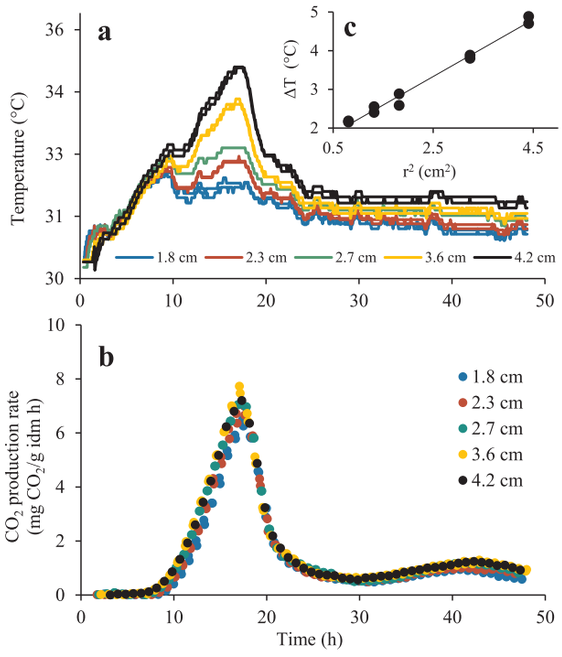

Fig. 3. Profile of temperature (a), and CO_2 production rate (b), in cultures of *T. harzianum* PBLA performed in TB incubated at 30 °C for 48 h in SSF. Internals diameters of 1.8 to 4.2 cm were evaluated, (c) effect of the radius of the TB on the maximum temperature rise (R^2 = 0.9877). The volume of TB is directly proportional to r^2 ($V = \pi r^2 h$).

3.3. Effect of stirring on the O_2 uptake and CO_2 and enzyme production in bench-scale bioreactors

To evaluate the effect of stirring on the O_2 uptake and CO_2 production, as an indirect measure of growth, and on enzymes production by *T. harzianum* PBLA, a horizontal bioreactor with cross-flow stirring was used, applying continuous stirring rates from 0 to 3 rpm. Kinetic data of O_2 uptake and CO_2 production show similar profiles at the evaluated stirring rates (Fig. 5). Both variables are reduced as a function of the stirring rate. Oxygen uptake drops from 150 to 52 mg/g dm and the CO_2 production from 140 to 41 mg/g dm, when the rate was increased from 0 to 3 rpm.

The SSF temperature in the SB increased rapidly during the exponential growth phase (12–31 h of fermentation) and, 26 h after the inoculation reached the highest temperature (34 °C) at stirring rates of 0–1 rpm (Table 3). The level of heat generation in solid cultures is directly proportional to the metabolic activity of the microorganisms (Pandey, 2003). This suggests that stirring rates of 0.5 and 1 rpm did not have a negative effect on fungal growth, since there was an increase in temperature, indicating metabolic activity. In contrast, at 3 rpm, the maximum temperature reached was 32 °C, presenting an increase of only 2 °C compared to 4 °C obtained at rates from 0 to 1 rpm, which indicates that the metabolic activity of *T. harzianum* was affected by this stirring rate (3 rpm). In fact, the growth parameters associated with CO_2 production (Table 3) were similar at stirring rates between 0 and 1 rpm, and decreased with increasing rate at 3 rpm. This indicates that *T. harzianum* can grow well at stirring rates of 0.5 to 1 rpm, but 3 rpm impairs growth. Nagel et al. (2000) used a rotary drum with continuous

Table 2
Maximum rate of CO_2 production (MCO_2PR) and total CO_2 production (TCO_2P) obtained in SSF cultures with TB from different diameter by *T. harzianum* PBLA at 30 °C for 48 h. The CO_2 data were considered for the estimation of the *Lag* time and (t_{Lag}) specific CO_2 production rate (μ_{CO2}).

Diameter (cm)	MCO_2PR (mg CO_2/g idm h)	TCO_2P (mg CO_2/g idm)	t_{Lag} (h)	μ_{CO2} (1/h)
1.8	6.37 ± 0.17^{a}	60.51 ± 1.58^{a}	10.17 ± 0.68^{a}	0.37 ± 0.01^{a}
2.3	6.83 ± 0.24^{ab}	65.48 ± 1.68^{ab}	9.74 ± 0.23^{a}	0.35 ± 0.00^{ab}
2.7	6.97 ± 0.11^{ab}	71.15 ± 0.31^{ab}	9.04 ± 0.08^{a}	0.35 ± 0.01^{ab}
3.6	7.60 ± 0.18^{b}	74.27 ± 0.93^{b}	9.13 ± 0.15^{a}	0.35 ± 0.00^{ab}
4.2	7.46 ± 0.36^{b}	69.43 ± 5.56^{ab}	11.75 ± 4.23^{a}	0.33 ± 0.01^{b}

Data with different letter by column indicate significant differences ($p < 0.05$). Values correspond to the mean of two replicate.

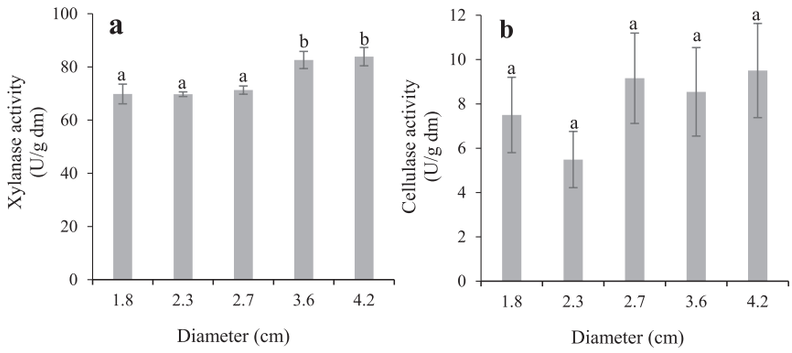

Fig. 4. Effect of TB diameter on the production of xylanases (a) and cellulases (b) by *T. harzianum* PBLA incubated at 30 °C in SSF. Samples were taken after 48 h of cultivation.

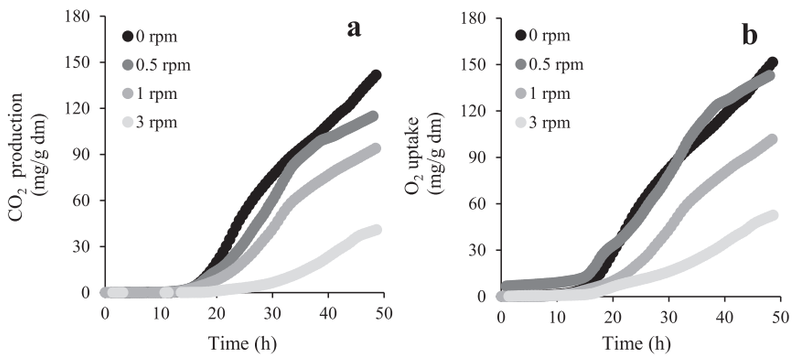

Fig. 5. Kinetics of CO_2 production and O_2 uptake by *T. harzianum* PBLA in a bench-scale bioreactor at different stirring speed rates.

Table 3
Maximum rate of CO_2 production (MCO_2PR), total CO_2 production (TCO_2P) and maxima temperature obtained in different stirring rate by *T. harzianum* PBLA in a bench-scale stirred bioreactor at 30 °C for 48 h.

Stirring rate (rpm)	MCO_2PR (mg CO_2/g idm h)	TCO_2P (mg CO_2/g idm)	t_{Lag} (h)	μ_{CO2} (1/h)	T_{max} (°C)
0	7.42	139.30	12.74	0.31	33.87 ± 0.19
0.5[1]	7.06	114.82	10.13	0.15	34.03 ± 1.09
1[2]	5.71	108.96	10.12	0.15	33.05 ± 0.14
3[1]	2.88	40.16	17.76	0.13	29.39 ± 2.27

[1] Continuous stirring for 48 h.
[2] Continuous stirring for 38 h (start of stirring 10 h after culture initiation).

stirring at 0.5 rpm, observing minimal damage to the fungal mycelium due to shear forces. They attribute such a result to the *Aspergillus oryzae* growth within the wheat grains, which was verified through the microscopic observation of cross sections of some grains; likewise, no fungal biomass was found growing on the surface of whole grains.

The CO_2 production values are similar to those (140 mg/g ds) obtained during growth of *Mucor bacilliformis* using polyurethane foam as support (Lareo et al, 2006) and those (150 mg/g ds) obtained from *A. brasiliensis* using perlite as support (Volke-Sepulveda et al., 2016). In both cases, glucose was used as carbon source and moisture content in culture media were near to 70%. Additionally, the CO_2 to glucose yields of 1.47, 1.22, 1.12 and 0.43 g/g were obtained at stirring speeds of 0, 0.5, 1 and 3 rpm respectively. Studies using glucose as the sole carbon source have shown that the CO_2 to glucose yield is up to 0.81 g/g with *A. brasiliensis* (Volke-Sepulveda et al., 2016) and 0.89 g/g with *Mucor bacilliformis* (Lareo et al., 2006). According to the CO_2 to glucose yield obtained in this study, a fraction of the pine sawdust used as support and enzyme inducer was also mineralized. This is confirmed by the maximum theoretical stoichiometric mineralization value of glucose (1 mol of CO_2 per C-mol of glucose). From the total CO_2 production, mineralization values are of 1.03, 0.95 and 0.96 mol/C-mol at stirring rates of 0, 0.5 and 1.0 respectively.

On the other hand, the xylanase production in the non-stirred

Table 4
Kinetic and yield parameters estimated from CO_2 production data, and enzyme production by *T. harzianum* PBLA in a bench-scale stirred bioreactor.

Stirring rate (rpm)	Enzymatic activity (U/g dm)	μ_{CO2} (h^{-1})*	α (U/mg CO_2)	β (U/mg CO_2 h)	R^2
Xylanases production					
0	33.04 ± 8.41	0.27	3.27E−01 ± 0.04	−1.67E−03 ± 0.00	0.99
0.5	104.86 ± 33.71	0.22	1.02E + 00 ± 0.08	−6.72E−03 ± 0.01	1.00
1	109.32 ± 4.91	0.15	1.02E + 00 ± 0.00	−3.28E−19 ± 0.00	1.00
3	9.20 ± 10.43	0.19	8.35E−01 ± 0.12	−1.27E−02 ± 0.01	1.00
Cellulases production					
0	0.73 ± 1.15	0.27	3.45E−02 ± 0.00	−1.28E−04 ± 0.00	0.99
0.5	2.14 ± 0.92	0.22	3.59E−03 ± 0.00	1.33E−04 ± 0.00	1.00
1	0.79 ± 0.07	0.15	3.16E−03 ± 0.00	1.13E−05 ± 0.00	1.00
3	3.25 ± 1.10	0.19	1.17E−02 ± 0.01	−1.14E−03 ± 0.00	0.22

* The specific CO_2 production rate (μ_{CO2}) was obtained with the logistic model; enzyme production to CO_2 production yields (α), and specific enzyme production rates (β) were estimated with the Luedeking and Piret model.

bioreactor was 33.04 U/g dm, which was three times lower than that obtained at 0.5 and 1 rpm (107.09 ± 3.15 U/g dm) (Table 4). The enzyme activity obtained at 0.5 and 1 rpm is similar to that obtained under static conditions in the TB (Fig. 2). The xylanases production at 3 rpm reached only 9.20 U/g dm, which corresponds to a loss of activity of 90% compared to the above rates. This shows that there was no detrimental effect of stirring rates below 1 rpm on the production of xylanases, but that a higher agitation rate (3 rpm) can cause damage to the fungal biomass. Regarding cellulases, their production was strongly affected by stirring, since it was three times lower than that obtained in the TB (Figs. 2 and 4). Similar results were reported for *Aspergillus awamori* (Díaz et al., 2009), and *Trichoderma reesei* (Flodman and Noureddini 2013), where a higher level of cellulase activity was obtained under static conditions (3.5 U/g dm and 12.5 U/g idm, respectively).

From the CO_2 and enzymes production data, kinetic and yield parameters associated with the logistic and Luedeking and Piret (1959) models were estimated (Table 4). For stirring rates from 0 to 1 rpm, the correlation coefficient (R^2) was close to 1. For the two enzymes studied, the αμ/β ratio was higher than 1, indicating that the production of both enzymes is associated to the CO_2 production. For all stirring rates, the xylanase production to CO_2 production yield (α) was about 60-fold higher than the yield obtained for cellulase. Although some negative values of β were found for both enzymes, they are very close to zero, indicating low specific enzyme production rates (β) not associated with CO_2 production. The negative values of β are related to the decrease in enzyme production at the end of culture. Kalogeris et al. (2003), using the Luedeking and Piret model, showed that the production of these same enzymes by SSF is growth-associated. In contrast, Gamarra et al. (2010) obtained β values of −1.5 and −13 (U/g h) for endoglucanases and xylanases in SSF, respectively; these high negative values of β are a result of the decrease of both enzyme concentrations at the end of culture.

4. Conclusions

Trichoderma harzianum PBLA grows and produces cellulases and xylanases in SSF in a range of 28–36 °C, obtaining a maximum xylanase production at 34 °C. In the static tubular bioreactors, increasing diameters lead to an increase of 1.08 °C/cm. The cross-flow stirred bioreactor operated at 0.5 and 1 rpm favors the fungal growth and the xylanases production, achieving 30% more activity than the static tubular bioreactor. Meanwhile, stirring significantly reduces the cellulases production. The production of both enzymes is associated with growth. Further studies should be conducted with pilot plant bioreactors to establish operating conditions for the production of these enzymes.

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