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Forced Aeration Influence on the Production of Spores by *Trichoderma* strains

Reynaldo De la Cruz-Quiroz¹ · Felipe Robledo-Padilla² · Cristóbal N. Aguilar¹ · Sevastianos Roussos³

Abstract The main purpose of the present study was to evaluate the effect of an initial aeration rate on the sporulation of six *Trichoderma* strains under solid state fermentation conditions and to evaluate the behavior of the lytic enzymes involved in the process at laboratory scale. A mixture of sugarcane bagasse, wheat bran, chitin, potato flour and olive oil were used as a substrate. Cultures were carried out using glass columns applying an initial aeration rate of 50 ml min⁻¹ (2 mL min⁻¹g⁻¹). Under the present culture conditions, chitinase and amylase activities were enhanced by the aeration with values of 30.74 and 44.54 U g⁻¹ with *T. longibranchiatum*. These values represent an increase of 226 and 167%, respectively compared with the system without air supply. For most of the strains, the forced aeration increased the endoglucanase activity, but the best value was observed with *T. yunnanense* (38.32 U g⁻¹). All strains showed low values of exoglucanase and lipase when the aeration was applied. *Trichoderma* spp. was the strain most affected negatively by the aerated process, showing the lowest enzyme activities. The forced air supply into solid-state

fermentation did not improve the production of spores from *Trichoderma* since similar results were obtained in the process without forced aeration. On this report, we reach concentrations greater than 1 × 10⁹ spores per gram of substrate at 96–120 h of culture, indicating a feasible approach of agro-industrial wastes as a substrate for the biomass production.

Keywords Solid-state fermentation · Agro-industrial wastes · Cellulases · Chitinase · Amylase · Lipase

Introduction

Trichoderma is considered as one of the most popular biological control agent (BCA) against several plant pests due to the diversity of its mechanisms of action, including the competence for nutrients and space, antibiotics production, and mycoparasitism mediated by the production of lytic enzymes [1, 2]. *Trichoderma* strains are a potential alternative to chemical pesticides applied in the food crops due to the harmful impact on the ecology and human health caused by the wrong and excessive application of pesticides, the waste generation, the destruction of non-target organisms, and induction of pests resistant to pesticides [3, 4].

The main challenge faced by BCA products is its low availability and distribution due to the limited production of fungal biomass [5]. The fungal spore is the most resistant propagule that tolerates the critical environmental conditions presented in the field crops [6, 7]. Therefore, the production of spores from BCA's is a core point to gain ground to the chemical pesticides. Several reports have been published on the topic of spore production from different BCA's, such as Loera-Corral et al. [6] evaluating *Metarhizium anisopliae*, Lopez-Perez et al. [8] with *Beauveria bassiana* or de la

Reynaldo De la Cruz-Quiroz
reynaldocq87@hotmail.com

Sevastianos Roussos
sevastianos.roussos@imbe.fr

Department of Food Research, School of Chemistry,
Universidad Autónoma de Coahuila (UAdeC), V. Carranza
s/n, 25280 Saltillo, Mexico

Tecnológico de Monterrey, Escuela de Ingeniería y
Ciencias, Ave. Eugenio Garza Sada 2501, 64849 Monterrey,
Nuevo León, Mexico

Institut Méditerranéen de Biodiversité et d'Ecologie marine
et continentale (IMBE), Aix-Marseille Université, UMR
CNRS/IRD/Univ. Avignon-FST St-Jérôme, case 421,
13397 Marseille cedex 20, France

Cruz-Quiroz et al. [9] with *Trichoderma asperellum*, among others.

Solid-state fermentation (SSF) is a microbial process with the high potential to produce fungal biomass, and also other several kinds of value-added products such as antibiotics, pigments, aromas, and enzymes of industrial interest like cellulases, chitinases, amylases, etc., [10–13]. The SSF allows the valorization of agro-industrial wastes generating a positive impact on the worldwide ecology due to this material could be used as the carbon source to the fungal development showing excellent yields for the production of fungal spores [14–16]. Sugarcane bagasse (SCB) is the major agro-industrial waste generated globally. SCB is an inexpensive material rich in cellulose (50%), hemicellulose (25%) and lignin (25%) which can be used for the generation of value added products, such as enzymes or biomass [17, 18]. Due to its low concentration of ashes, it is an excellent substrate for bioconversion process offering great approaches instead of wheat bran or rice bran, among others [19]. SSF is an aerobic process, therefore is highly needed an adequate oxygen diffusion through the fermented matter making one of the most important phenomena to support the microbial development [20]. Commonly a passive oxygen diffusion takes place in reactors such as flasks, trays or bags resulting in a high production of spores [14, 16, 21]. Arzumanov et al. [22] reported that the aeration does not always make a big difference to increase the production of spores. Nevertheless, several authors suggest that forced aeration is a key point to increase or optimize the spore production from different filamentous fungi [20, 23]. Therefore, the oxygen diffusion inside of the system, the removal of heat and CO₂ from the fermented matter caused by the forced aeration could be a possible alternative to increase the spore production using the Raimbault columns (Packed Bed Bioreactor) [23]. The aim of the present study was to evaluate the effect of an initial aeration rate of 2 mL min⁻¹g⁻¹ on the sporulation of

six *Trichoderma* strains and to evaluate the behavior of the lytic enzymes involved in the process at laboratory scale.

Materials and Methods

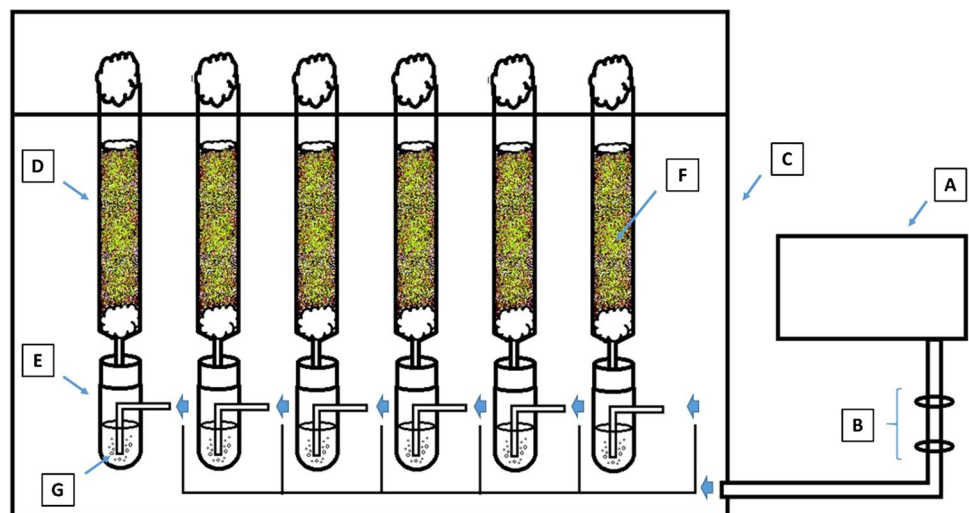
Microorganisms and Culture Conditions

The strains of *T. longibranchiatum*, *T. harzianum*, *T. yunnanense*, *T. asperellum* (T2-10 and T2-31), and *Trichoderma sp.* were proportioned by the Department of Agricultural Parasitology of UAAAN (Universidad Autónoma Agraria Antonio Narro, Coahuila, Mexico). The fungal strains were cultured and preserved in a cryogenic solution of glycerol-milk 8.5%. The strains were activated in sterilized PDA and incubated during 5 days at 29 °C to preserve at 4 °C.

Solid-State Fermentation (SSF)

The SSF was performed using the modified Raimbault columns (diameter of 2.5 cm and height of 20 cm) coupled to an air humidifier (Fig. 1). The aeration was generated by an air compressor and humidified by passing the air through distilled water. A flowmeter was used to adjust the initial aeration rate at 50 mL min⁻¹ (2 mL min⁻¹g⁻¹) [22]. The columns were placed in a water bath to maintain the temperature at 29 °C. The substrate was integrated by a mix of sugarcane bagasse (40%) as support, wheat bran (30%), chitin (10%), potato flour (10%), and olive oil (10%) on dry weight basis. Mixed substrates were sterilized at 121 °C for 30 min. After cooling the substrate was inoculated with 2 × 10⁷ spores g⁻¹ dry matter. Initial pH was adjusted to 5.6 using 1.0 M HCl [24]. Each column was packed with 20 g solids at 75% of moisture (w/v) [25]. Each treatment was done in triplicates and monitored kinetically.

Fig. 1 Schematic representation of the SSF system. **a** compressor, **b** sterile filter (0.22 μm), **c** water bath incubator, **d** packed bed column, **e** air humidifier, **f** fermented matter and **g** distilled water



Spore Determination

The fermented matter (1 g) was added into 100 mL of Tween 80 (0.01%) in an Erlenmeyer flask. A magnetic stirrer (200 rpm) was used to release the spores from the solid matter and also to homogenize the suspension. The spores were counted using an hemocytometer.

Enzyme Assays

Fermented material (5 g) was mixed with 50 mL of distilled water, the suspension was homogenized using an Ultra-turax for 1 min. Chitinase activity was determined using colloidal chitin (1%) in citrate-phosphate (50 mM, pH 5.0) at 50 °C for 30 min. The reaction was cooled into an ice bath and after centrifuged at 10,000 g for 5 min. Amylase activity was measured using soluble starch (0.7%) in sodium citrate buffer (50 mM, pH 5.9) at 50 °C for 15 min. Endoglucanase activity was determined using carboxymethyl cellulose (1%) in sodium citrate buffer (50 mM, pH 4.8) at 50 °C for 30 min, in according with Nava-Cruz et al. [26]. Exoglucanase activity was determined using filter paper Whatman No.1 (1 cm × 5 cm) in sodium citrate buffer (50 mM, pH 4.8) at 50 °C for 60 min in according with Nava-Cruz et al. [26]. The four enzyme determinations mentioned above were stopped on a bath with ice for 5 min. Sugars concentration was determined after each enzyme reaction according to the amount of resulting reducing sugars using 3,5-dinitro salicylic acid (DNS) following the assay method described by Miller [27]. An enzyme activity (U) was defined as the amount of enzyme that catalyzes the release of 1 μmol of glucose per minute. Lipase activity was determined using 0.5 mL of *p*-nitrophenyl (25 mM) in phosphate buffer (25 mM, pH 7.0) at 30 °C for 30 min. The calibration curve was done with *p*-nitrophenol (0–100 ppm) and 412 nm was the wavelength used. An enzyme activity (U) was defined as the amount of enzyme required to release 1 μmole of *p*-nitrophenol per minute.

Analysis and Experimental Design

The experimental design was integrated by the evaluation six *Trichoderma* strains under two different culture conditions (aerated and not aerated). Each treatment was done in triplicate. The analysis of variance and means comparison (Tukey) were done in all cases. Data were analyzed by using the computer software, Statistical Package for Social Sciences (SPSS) version 23.0 for Windows and Microsoft Excel 2016.

Results

Determination of the Enzyme Activities During the Solid-State Fermentation Without Aeration

The highest value of chitinase activity was showed by *T. harzianum* (24.15 U g⁻¹ at 72 h), followed by *T. yunnanense* and *T. asperellum* T2-10 with 16.01 and 15.86 U g⁻¹, respectively (Fig. 2b). *T. harzianum* also resulted in a great amylase activity (24.85 U g⁻¹ at 72 h) followed by *Trichoderma* spp. and *T. yunnanense* with 20.97 and 17.95 U g⁻¹ at 72 h, respectively (Fig. 2b). *T. yunnanense* and *T. asperellum* T2-10 showed the best endoglucanase activity (30.89 and 27.86 U g⁻¹ at 72 h, respectively), followed by *T. harzianum* with 22.03 U g⁻¹ (Fig. 2c). The maximum exoglucanase activity was showed by *T. longibranchiatum* with a value of 9.29 U g⁻¹ at 48 h (Fig. 2a). The rest of the strains showed values between 6.67 and 7.89 U g⁻¹ at 72 h. High lipase activities were showed by *Trichoderma* spp., and *T. longibranchiatum* (11.56 and 11.42 U g⁻¹ at 72 h, respectively) (Fig. 2e).

Determination of the Enzyme Activities During the Aerated Solid-State Fermentation

The great value for chitinase activity were showed by *T. longibranchiatum* (30.74 U g⁻¹ at 48 h), followed by *T. harzianum* and *T. yunnanense* with 21.42 U g⁻¹ and 21.33 U g⁻¹, respectively (Fig. 3a). These three strains also showed the best results for amylase activity. *T. longibranchiatum* started with an activity of 28.33 U g⁻¹ at 24 h, but reaching 44.54 U g⁻¹ at 48 h (Fig. 3a). *T. yunnanense* and *T. harzianum* showed values of 33.68 and 21.57 U g⁻¹, respectively at 72 h. *T. yunnanense* showed an initial endoglucanase activity of 19.31 U g⁻¹ at 24 h, 33.83 U g⁻¹ at 48 h and reaching its maximum of 38.32 U g⁻¹ at 72 h (Fig. 3c). High values of endoglucanase were also shown by *T. harzianum* (31.70 U g⁻¹ at 72 h) and *T. longibranchiatum* (22.19 U g⁻¹ at 48 h). The values of exoglucanase activity were homogeneous among all strains evaluated. The best enzyme value (4.80 U g⁻¹) was showed by *Trichoderma* spp. at 72 h (Fig. 3e). However, high values of exoglucanase were also shown by *T. yunnanense* (3.66 U g⁻¹ at 48 h) and *T. asperellum* T2-10 (3.14 U g⁻¹ at 24 h). *T. longibranchiatum* showed a lipase activity of 4.97 U g⁻¹ at 24 h, 7.41 U g⁻¹ at 48 h and reaching its maximum of 9.12 U g⁻¹ at 72 h (Fig. 3a). *Trichoderma* spp. showed an activity of 6.77 U g⁻¹ at 72 h, followed by *T. asperellum* T2-10 (5.26 U g⁻¹ at 72 h) and *T. harzianum* (5.06 U g⁻¹ at 48 h).

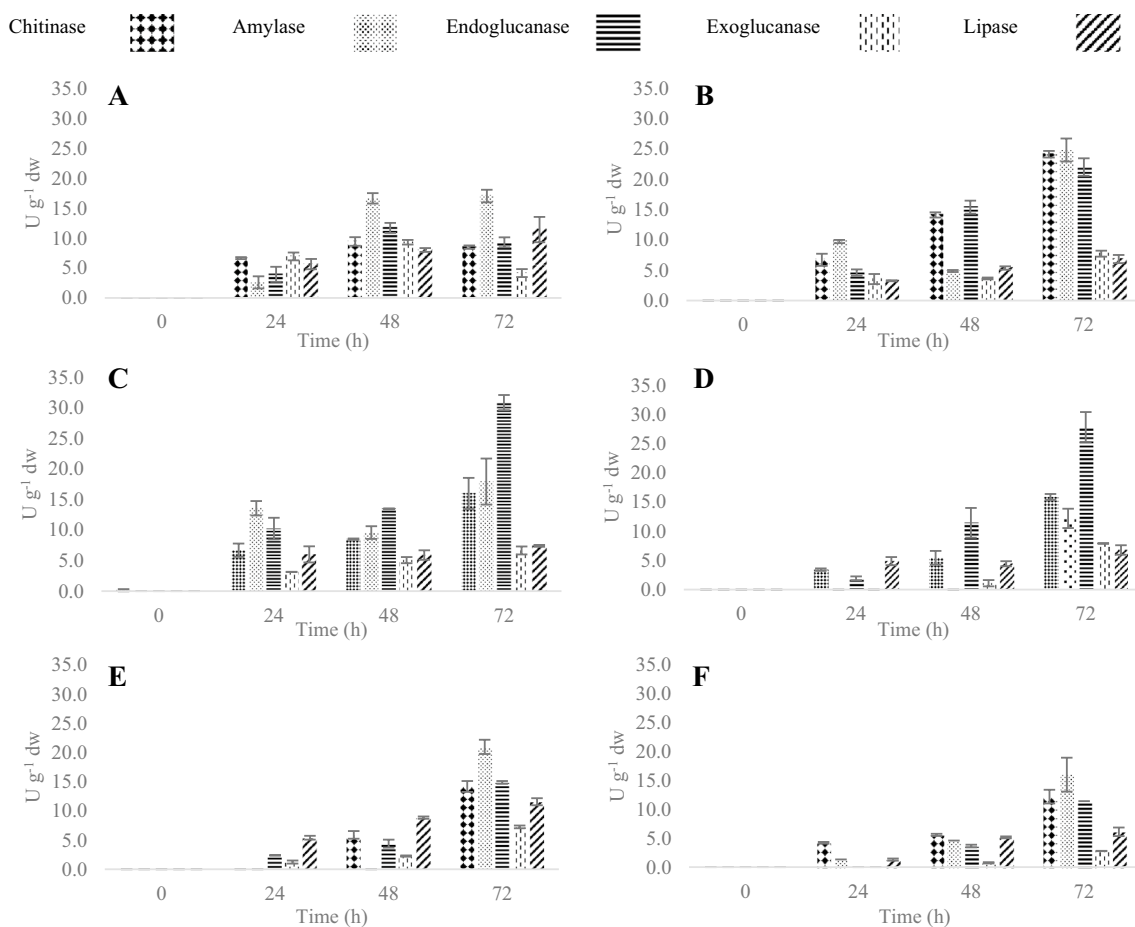


Fig. 2 Kinetic of lytic enzymes production under solid-state fermentation with passive aeration. **a** *T. longibranchiatum*, **b** *T. harzianum*, **c** *T. yunnanense*, **d** *T. asperellum* (T2-10), **e** *Trichoderma* spp., and **f** *T. asperellum* (T2-31)

Effect of Forced Air Supply on Spore Production During the Solid-State Fermentation

The maximum spore production for all six *Trichoderma* strains was achieved at 96 h of fermentation. *T. longibranchiatum* resulted in a production of 1.31×10^9 spores g^{-1} dw (aerated) and 1.11×10^9 spores g^{-1} (not aerated). Since the 120 h of culture, the sporulation was maintained without important changes (Fig. 4a). *T. harzianum* showed faster lag phase when the system was aerated, reaching its higher value of 1.08×10^9 spores g^{-1} dw at 96 h. However, at the same time the system without aeration showed a value of 1.06×10^9 spores g^{-1} dw (Fig. 4b). *T. yunnanense* and *Trichoderma* spp. showed a very similar pattern of spore production in the lag and exponential phase (Fig. 4c, e). *T. yunnanense* showed values of 1.20×10^9 spores g^{-1} dw (aerated) and 1.08×10^9 spores g^{-1} (not aerated) as well as, *Trichoderma* spp. showed values of 1.10×10^9 spores g^{-1} dw (aerated) and 1.18×10^9 spores g^{-1} (not aerated). The strains of *T. asperellum* (T2-10 and T2-31) resulted in a slow lag phase for the both systems, aerated and not aerated (Fig. 4d,

f). Both strains showed its first values of spores until the 72 h of culture. After that, they reached the maximum spore production at 96 h, like the others *Trichoderma* strains. *T. asperellum* T2-10 showed values of 1.23×10^9 spores g^{-1} dw (aerated) and 1.11×10^9 spores g^{-1} (not aerated). In the case of *T. asperellum* T2-31, the values were 1.30×10^9 spores g^{-1} dw (aerated) and 1.03×10^9 spores g^{-1} (not aerated).

Discussion

In this paper, the effect of forced air supply was evaluated on SSF for the production of spores and to determine the behavior of the lytic enzymes involved in the process at laboratory scale. Sugarcane bagasse was the major substrate used in the process to take advantage of its great characteristics to work as a source of nutrients and also as a matrix to anchor on it [12]. It is well known that sugarcane bagasse plays an important role because its high porosity allowing good water absorption, indispensable to carried out the microbial metabolism. Wheat bran, potato flour, chitin and olive

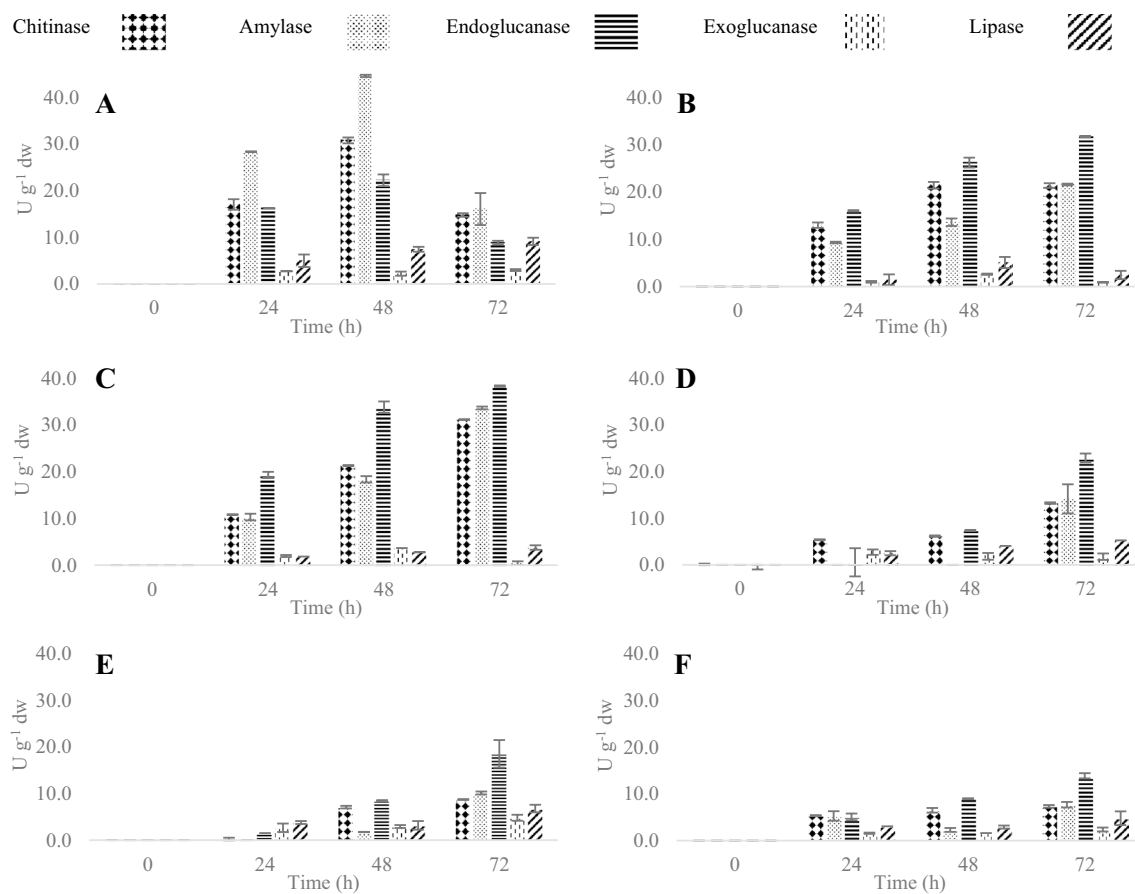


Fig. 3 Kinetic of lytic enzymes production under solid-state fermentation with forced aeration. **a** *T. longibranchiatum*, **b** *T. harzianum*, **c** *T. yunnanense*, **d** *T. asperellum* (T2-10), **e** *Trichoderma* spp., and **f** *T. asperellum* (T2-31)

oil were added as inducers of the lytic enzymes synthesis [28, 29]. The strains *T. longibranchiatum* and *T. yunnanense* were benefited by the application of aeration on the SSF system for chitinase, amylase and endoglucanase activities. Despite this, the same conditions influenced a decrease on exoglucanase and lipase activities. This pattern was almost the same for *T. harzianum*, except for chitinase and amylase which were maintained without any effect of the air. *T. asperellum* T2-10 was affected negatively for endoglucanase and exoglucanase activities were reduced by the aeration, but the values of chitinase, amylase, and lipase were maintained with any modification by the application of air. The chitinase and amylase activities resulted diminished because of the aeration applied on *T. asperellum* T2-31. For this strain, the air represented an increment for endoglucanase, but it was not observed any change for exoglucanase and lipase. *Trichoderma* spp. was the most affected strain by the aeration. The values of chitinase, amylase, exoglucanase, and lipase were decreased by the application of air into the system. It is possible to observe homogenous patterns for exoglucanase and lipase activities, but in most of the cases, these two enzymes showed better activity under SSF with

no aeration. Endoglucanase activity was higher when the air was applied which corresponds with the report of Kalođeris et al. [30]. The most significant differences between the values of enzymatic activity are shown in Table 1. The application of air into a fermentation system could provide homogeneous environmental conditions, including the regulation of temperature and moisture which allows an enhancing in the carry of nutrients, metabolites and the oxygen diffusion through the solid substrate [23, 31]. However, the dehydration on the SSF is one of the main possible detrimental result caused by aeration and therefore is an important challenge for the production of enzymes [23, 29]. The values of the enzymes were highly variables suggesting that the enzyme activities were more related to the intrinsic capabilities of the present fungal strains than the effect of the air. Oliveira et al. [29], reported an improvement in lipase activity applying an aeration rate of $2 \text{ mL min}^{-1} \text{ g}^{-1}$ using on an SSF with *Aspergillus ibericus* and wheat bran with 10% of olive oil as substrate. No fungal growth was observed when they tested the SSF without aeration. Despite this, an aeration rate above $8 \text{ mL min}^{-1} \text{ g}^{-1}$ also has a negative effect on lipase activity. A similar bioreactor was evaluated

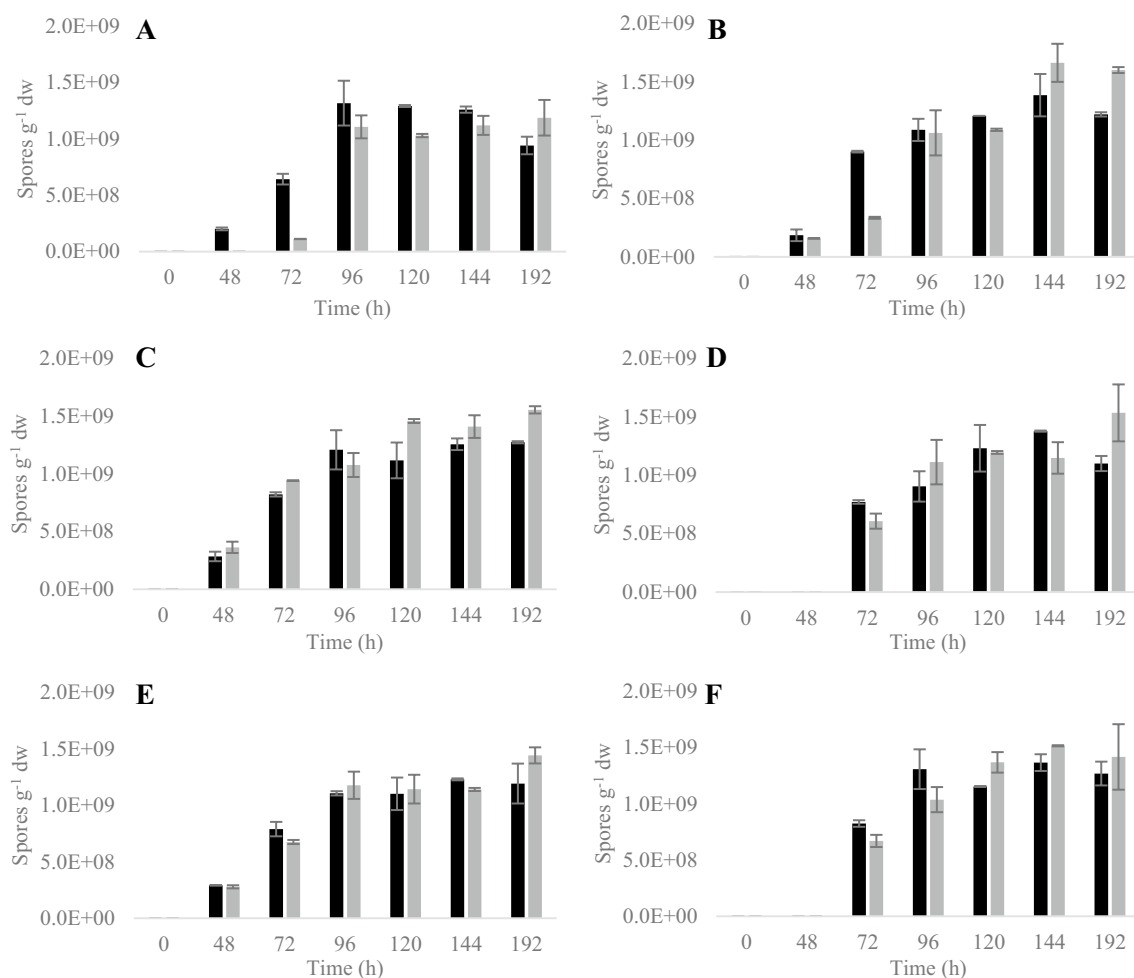


Fig. 4 Spore production under solid-state fermentation with aeration (*black shaded bar*) and without aeration (*grey shaded bar*). **a** *T. longibranchiatum*, **b** *T. harzianum*, **c** *T. yunnanense*, **d** *T. asperellum* (T2-10), **e** *Trichoderma* spp., and **f** *T. asperellum* (T2-31)

Table 1 The most significant values of the enzyme activities by *Trichoderma* strains

Enzyme activity	Fungal strain	Fermentation system	Culture time (h)	Enzyme activity (U g ⁻¹)
Chitinase	<i>T. longibranchiatum</i>	Aerated	48	30.74 ^a
	<i>T. harzianum</i>	Not aerated	72	24.15 ^b
Amylase	<i>T. longibranchiatum</i>	Aerated	48	28.33 ^a
	<i>T. yunnanense</i>	Aerated	72	33.68 ^b
Endoglucanase	<i>T. yunnanense</i>	Aerated	72	38.32 ^a
	<i>T. yunnanense</i>	Aerated	72	31.70 ^b
	<i>T. harzianum</i>	Not aerated	72	30.89 ^b
	<i>T. longibranchiatum</i>	Not aerated	48	9.29 ^a
Lipase	<i>T. longibranchiatum</i>	Not aerated	72	11.56 ^a
	<i>Trichoderma</i> spp.	Not aerated	72	11.42 ^a

Means with different letter are significantly different according to the Tukey's multiple range test ($p < 0.05$)

by Abdeshahian et al. [32] to enhance the cellulase activity from *Aspergillus niger* FTCC 5003. They used palm kernel cake as a substrate, reporting high activity when was applied an aeration rate of $15 \text{ mL min}^{-1} \text{ g}^{-1}$, but lower values at 5 and $10 \text{ mL min}^{-1} \text{ g}^{-1}$. This same values of aeration (5, 10 and $15 \text{ mL min}^{-1} \text{ g}^{-1}$) were evaluated by Kalogeris et al. [30] in an SSF process using wheat bran as substrate. They reported an improvement in the enzyme activities for endoglucanase, cellobiohydrolase, exoglucanase, xylanase, β -xylosidase and β -glucosidase from *Thermoascus aurantiacus*.

According to Motta and Santana [20], forced aeration has a high influence on the stimulation of fungal sporulation. They tested empty fruit bunches as substrate and a *Trichoderma reesei* strain evaluating an aeration rate of $16.6 \text{ mL min}^{-1} \text{ g}^{-1}$ resulting in 4.41×10^9 spores g^{-1} at 120 h of culture. However, they did not show data to support or compare that affirmation. Flodman and Nouredini [33] reported 7.50×10^8 spore g^{-1} of *T. reesei* using corncob wastes as a substrate on an Erlenmeyer bioreactor without aeration. Although the authors presented good results under their test, those values were 23 times less than the present work. The production of spores from *Trichoderma asperellum* T2-10 was evaluated in a previous work without application of aeration on the SSF [9]. The sporulation index was 1.4×10^9 spores g^{-1} using corn cob as substrate and a plastic bag as a bioreactor. In the present study, the forced aeration did not show any increase in the spore production for any of the six strains evaluated. This behavior suggests that the production of spores from the genus *Trichoderma* is not related with the air applied because of a possible minimal demand of oxygen, therefore the sporulation may not be oxygen-limited [22]. This outcome has a direct impact on the technical feasibility for the spore production of *Trichoderma* because under these culture conditions it is possible to use reactors such as flasks, trays or bags, which represents an energy saving related with the air control and also the laborious handling on the preparation of the fermentation process.

Conclusion

The chitinase, amylase and endoglucanase activities from *T. longibranchiatum* and *T. yunnanense* were enhanced through the application of forced aeration. For most of the strains, the aeration increased the endoglucanase activity. All strains showed low values of exoglucanase and lipase when the aeration was applied. *Trichoderma* spp. was the strain most affected negatively by the aerated process, showing the lowest enzyme activities. The results obtained suggest that chitinase and amylase could be more dependent on the *Trichoderma* strain than the aeration applied. The forced air supply into solid-state fermentation did not improve the production of spores from *Trichoderma* since similar production

was obtained in the process without forced aeration. On this report, we reach concentrations greater than 1×10^9 spores per gram of substrate at 96–120 h of culture, indicating a feasible approach of agro-industrial wastes as a substrate for the biomass production.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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