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Full Length Research Paper

Comparison of humic acids production by *Trichoderma viride* and *Trichoderma reesei* using the submerged fermentation of oil palm empty fruit bunch

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The remarkable properties of humic acids have generated a broad spectrum of applications in pharmaceutical, cosmetic and agricultural fields, and encouraged fermentation studies focusing on humic acids production. This work compares the humic acids production of *Trichoderma* (*viride* and *reesei*) species using empty fruit bunch as the substrate during submerged fermentation. The performance of each species was compared by examining spore production in oat medium, and the significant medium components and fermentation conditions were identified using Plackett and Burman statistical design. For both *Trichoderma* species, the results indicate that humic acids production can be enhanced by increasing the temperature, empty fruit bunch and peptone concentrations and by decreasing the $(NH_4)_2SO_4$ concentration. *T. reesei* performed better than *T. viride*, generating 3-fold more of humic acids.

Key words: Humic acids, *Trichoderma reesei, Trichoderm aviride*, submerged fermentation, empty fruit bunch.

INTRODUCTION

Trichoderma is a genus of asexually reproducing fungi with a high level of genetic diversity (Harman et al., 2004). They are frequently found growing in soil as well as on other substrates, such as wood, bark, and other fungi, demonstrating their high opportunistic potential and their adaptability to various ecological conditions (Druzhinina, 2011). These characteristics indicate that this genus could be used in many biotechnological applications (Esposito and Silva, 1998). The genus *Trichoderma* is widely used in industrial applications, because they produce extracellular lignocellulose-degrading hydrolases in large amounts (Cavaco-Paulo and Gubitz, 2003), which can be useful for recycling cellulosic waste materials as well as producing useful by-products (Samuels, 1996).

Humic acids (HA), a component of the organic matter in soil, are the soil fraction that is most resistant to microbial degradation. They are complex polymeric organic acids with a wide range of molecular weights and exist as heterogeneous mixtures of a variety of organic

compounds, including aromatic, aliphatic, phenolic, and quinolic functional groups (Aiken, 1985). They are one of the most active fractions of organic matter and affect a variety of chemical, physical, and biological reactions. Previously recognized for agricultural applications, they have received growing attention from the biomedical field primarily due to their antiviral, profibrinolytic, anti-inflammatory and estrogenic activities (Yamada et al., 1998), which are of great importance for pharmaceutical and biomedical applications (von Borstel et al., 1994). Traditionally, HA is extracted from lignite, brown coals and humified organic materials (Asing et al., 2011). However, harvesting HA from non-renewable carbon resources can be expensive and environmentally/ecologically unsustainable. Moreover, the extraction of peat leads to the destruction of peat lands, which is associated by environmental problems (Joosten et al., 2012). Thus, it is desirable to use more ecologically sustainable precursors/feedstock for HA and to develop cheaper and cleaner methods for the extraction of this valuable product.

For economic reasons, industrial fermentation involves are complex, almost indefinable substrates that are often the by-products of other industries. Empty fruit bunch (EFB), a cellulosic material source containing 43.8% cellulose, 35.0% hemicelluloses, and 16.4% lignin (Hamzah et al., 2011), is a strong candidate for use as a fermentation substrate. EFB is the product of oil palm processing and is produced in large quantities, but it is a waste product that has not been completely utilized as 23% of the fresh fruit bunch (Harun et al., 2013) used in oil palm production remains unused as EFB. Thus, there is a growing interest in making EFB useful, reducing the large volume of waste and its ensuing environmental problems (Oviasogie et al., 2010).

Although no quantitative studies have been performed previously, *Trichoderma* sp. is a suitable genus for the production of HA from EFB. These fungi have been recognized for their extreme facility in producing a large variety of extracellular enzymes and the degradation of lignocellulose (Kirk and Farrell, 1987). Furthermore, *T. viride* and *T. reesei* are the most extensively studied fungi in the field of cellulosic material degradation (Cullen and Kersten, 1992).

In our previous work, it was demonstrated that the production of HA from EFB is by using a *T. viride* strain (Motta and Santana, 2013). This work extends our previous findings by comparing the performance of *T. reesei* and *T. viride* in HA production from EFB, as well as evaluating medium components and fermentation conditions. Spores from both species were produced by submerged fermentation in oat medium as previously described for *T. viride* (Motta and Santana, 2012). The effects of medium components and fermentation conditions were compared using Plackett and Burman (PB) statistical design (Plackett and Burman, 1946).

MATERIALS AND METHODS

Microorganism maintenance

For storage, the *T. reesei* culture was grown on potato dextrose agar plates at 24°C for 10 days. After sporulation, the spores were resuspended in a sterile 20% glycerol solution. This mixture was stored in 1.2 ml cryotubes at -70°C.

Production of *T. reesei* spores in oat medium

The spore production and fermentation analysis for *T. reesei* were performed as previously described by Motta and Santana (2012) for *T. viride*. The medium was prepared by adding 30 g of oat meal (Quaker Oats Company®, thin flakes; Table 1 for detailed composition) into 1 L of distilled water and boiling the suspension for 90 min at 90°C with constant stirring. Immediately after heating, the suspension was filtered through a sieve (0.150 mm diameter holes). Distilled water was added to the filtrate to achieve a final volume of 1 L. Then, 5 g of potato peptone purchased from Sigma-Aldrich was added, and the media was adjusted to pH 6.0 (Al-Taweil et al., 2009) prior to autoclaving at 121°C for 15 min.

The cultures were grown in 500 mL Erlenmeyer flasks containing 300 mL of oat meal culture media inoculated with the spore suspension prepared as described in the item "microorganism maintenance". To obtain the same initial concentration of spores for both species, 0.4 ml of the *T. reesei* spore suspension were used. The culture flasks were incubated at 24°C at 150 rpm. During 120 h of cultivation, samples were collected every 24 h for dry biomass estimation and cellular protein quantification, which are direct and indirect methods for fungal biomass determination, respectively. Image analysis was performed every 24 h, and spore counts were performed at 0 and 120 h of fermentation.

Dry weight biomass

Based on the methodology adapted by Szijártó et al. (2004), in which the optical density was used to evaluate *T. reesei* biomass behavior in delignified pine pulp, the dry biomass concentration of *T. reesei* was evaluated by reading the optical density at 600 nm in a spectrophotometer. The absorbance at 600 nm was correlated to the cell dry weight per culture volume. To determine the dry weight, the fungal biomass was extracted from the culture medium by heating an aliquot of known volume at 85°C under constant agitation for 10 min after dilution in distilled water at a ratio of 1:10. After heating, the aliquot was filtered through coffee filter paper, and the filtrate was collected. The solids were retained, heated and filtered as described above until the green color (typical of *Trichoderma* spores) disappeared. The total volume of the filtrate was centrifuged at 10,000 g at 5°C, and the supernatant was discarded. The precipitate was dried at 105°C to achieve a constant weight and placed in a desiccator until fungal mass was determined.

Cellular protein quantification

Indirect estimation of the fungal biomass was performed by determining the protein concentration in each sample. Protein quantification was performed according to the method adapted by Callow and Ju (2012) to quantify only the cellular proteins. Culture samples (3.0 mL) were collected and centrifuged at 10,000 g for 10 min to obtain pellets. The supernatants were collected for further processing. The pellets were resuspended and washed twice with

Table 1. Composition of oatmeal thin flakes (data collected from the manufacturer's product specification sheet, Quaker Oats Company®).

Constituent	Content (g per 100 g)
Carbohydrate	56.7
Protein	14.3
Total fat	7.3
Fibers	9.7
β-glucan	4.0

deionized water. After each wash step, the biomass was centrifuged and the water was discarded. To release intracellular proteins, the pellets were suspended in 3.0 mL of 1 N sodium hydroxide and heated at 100°C for 10 min. After cooling, the digested samples were centrifuged at 10,000 g for 10 min to remove the cell debris and other solids; the supernatants were then collected, and the protein concentrations were determined by the bicinchoninic acid assay (Smith et al., 1985) using a commercial kit (BCA Protein Assay, Thermo Scientific, USA). Standard curves were generated with bovine serum albumin.

Spore counts

The spores were directly counted using a Neubauer chamber. After mycelia and conidia were observed with a Reichert-Jung Series 150 microscope (Reichert, USA), indicating that fungal sporulation had been completed at 120 h of fermentation, the spore count was performed at 0 and 120 h to compare the initial concentration of spores to the spore concentration at the end of fermentation.

Humic acids (HA) production

The HA production for both species was analyzed and compared according to the results obtained with the PB assay as well as the effect of medium components and fermentation conditions.

Submerged fermentation

EFB was provided by Oil Palm S/A - Agro-industrial OPALMA (Bahia, Brazil) and was milled to a standardized particle size between 125 and 500 µm (115 and 32 mesh in the Tyler series, respectively). The composition of the EFB particles was determined with an elemental analyzer CNH (Perkin Elmer Series II 2400, USA) which indicated that the elemental mass percentage is 48.0±0.7% carbon, 2.6±0.1% nitrogen and 6.1±0.2% hydrogen. These results are expressed as the mean of triplicates and the average deviation. Potato peptone was purchased from Fluka Analytical (France); (NH4)$_2$SO$_4$ and K$_2$HPO$_4$ were purchased from Ecibra (Brazil). *T. reesei* and *T. viride* were cultured in 500 mL Erlenmeyer flasks containing 270 mL of culture media inoculated with 30 mL of inoculum, which for both species consisted of spores produced in oat medium for both species (item "Production of *T. reesei* spores in oat medium"). The culture flasks were incubated at 150 rpm, and samples were withdrawn at 120 h of fermentation for HA quantification.

Humic acids (HA) quantification

According to the methods adapted by Badis (2010), the samples were centrifuged at 10,000 g for 15 min (Rotina 380 R Centrifuge, Hettich Zentrifugen, Tuttlingen, Germany), and the supernatant fractions were filtered using the microfilter syringe system (Thomapor®-Membranfilter, 5FP 025/1). The supernatant fractions were diluted five-fold with 0.5 M NaOH solution, and the absorbance at 350 nm at pH 4.5±0.01 was measured. Standard curves were obtained from the absorbance at 350 nm of known concentrations of commercial HA (Sigma-Aldrich, United Kingdom) in 0.5 M NaOH solution, pH 4.5±0.01.

Plackett and Burman (PB) design

The Plackett and Burman (PB) design was used for screening the selected variables, which had significant effects on HA production. Six variables were screened: EFB (g/L) as a carbon source; potato peptone (g/L) as an organic nitrogen source; pH; temperature (°C); and K$_2$HPO$_4$ (g/L) and (NH$_4$)$_2$SO$_4$ (g/L) as inorganic nitrogen sources (Table). Each factor in this experimental design was examined at three levels: low (−), high (+) and central (0) to evaluate the linear and curvature effects of the variables (Table). Table shows the design with 16 PB trials along the levels. This statistical design does not involve the interactions between the selected variables and follows a linear approach for screening the factors (Plackett and Burman, 1946).

RESULTS

Spores production in oatmeal medium

As shown in Figure 1, the biomass and protein concentration curves have the same behavior over 120 h of cultivation for both *T. viride* (Motta and Santana, 2012) and *T. reesei*. Moreover, it is possible to observe by both biomass estimation methods that there is effectively no fungal growth after 96 h of fermentation, with the largest growth rate observed between 24 and 72 h of fermentation. With regard to the relationship between *T. viride* and *T. reesei* biomass concentration and cellular protein concentration, as described by Equations 1 and 2, respectively, there is a strong correlation value (0.98) for both species.

Biomass (g/L) = 3.78 * Protein (g/L) + 0.11   (1)

Biomass (g/L) = 2.33 * Protein (g/L) + 0.23   (2)

Using image analysis with optical microscopy, it was observed that at 120 h of fermentation the *T. reesei* strain completed its sporulation phase, and only spores were present in the culture medium at 120 h, as was observed for the *T. viride* strain by Motta and Santana (2012). The initial concentration of spores in the culture medium was 4.54 x 10^4 spores/mL, and after 120 h of fermentation, the spore concentration found in the fermentation medium was 6.36 x 10^6 spores/mL.

Humic acids (HA) production

Six variables were screened using PB, as shown in Table

Table 2. Six variables screened using PB design at lower (-1), higher (+1) and central (0) levels.

Variable	Unit	Experimental value		
		-1	0	+1
EFB	g/L	10.0	20.0	30.0
Peptone	g/L	1.00	3.85	6.70
pH	-	4.0	6.0	8.0
Temperature	°C	25.0	30.0	35.0
K_2HPO_4	g/L	0.28	1.54	2.80
$(NH_4)_2SO_4$	g/L	0.24	0.77	1.30

Table 3. Experimental PB design used to screen six variables with real and code values (parentheses) for the response of HA production along with its observed values.

Trial	Experimental value						HA (mg/L)*	
	EFB (g/L)	Peptone (g/L)	pH	Temperature (°C)	K_2HPO_4 (g/L)	$(NH_4)_2SO_4$ (g/L)	Observed for *T. reesei*	Observed for *T. viride*
1	30.0 (+1)	1.00 (-1)	8.0 (+1)	25.0 (-1)	0.28 (-1)	0.24 (-1)	128.1	12.6
2	30.0 (+1)	6.70 (+1)	4.0 (-1)	35.0 (+1)	0.28 (-1)	0.24 (-1)	318.7	85.2
3	10.0 (-1)	6.70 (+1)	8.0 (+1)	25.0 (-1)	2.80 (+1)	0.24 (-1)	112.6	48.9
4	30.0 (+1)	1.00 (-1)	8.0 (+1)	35.0 (+1)	0.28 (-1)	1.30 (+1)	189.1	38.3
5	30.0 (+1)	6.70 (+1)	4.0 (-1)	35.0 (+1)	2.80 (+1)	0.24 (-1)	182.9	107.0
6	30.0 (+1)	6.70 (+1)	8.0 (+1)	25.0 (-1)	2.80 (+1)	1.30 (+1)	159.8	44.3
7	10.0 (-1)	6.70 (+1)	8.0 (+1)	35.0 (+1)	0.28 (-1)	1.30 (+1)	113.7	46.9
8	10.0 (-1)	1.00 (-1)	8.0 (+1)	35.0 (+1)	2.80 (+1)	0.24 (-1)	75.4	46.9
9	10.0 (-1)	1.00 (-1)	4.0 (-1)	35.0 (+1)	2.80 (+1)	1.30 (+1)	27.1	10.3
10	30.0 (+1)	1.00 (-1)	4.0 (-1)	25.0 (-1)	2.80 (+1)	1.30 (+1)	1.1	3.3
11	10.0 (-1)	6.70 (+1)	4.0 (-1)	25.0 (-1)	0.28 (-1)	1.30 (+1)	85.5	47.6
12	10.0 (-1)	1.00 (-1)	4.0 (-1)	25.0 (-1)	0.28 (-1)	0.24 (-1)	0.6	20.2
13	20.0 (0)	3.85 (0)	6.0 (0)	30.0 (0)	1.54 (0)	0.77 (0)	143.4	58.8
14	20.0 (0)	3.85 (0)	6.0 (0)	30.0 (0)	1.54 (0)	0.77 (0)	142.3	58.1
15	20.0 (0)	3.85 (0)	6.0 (0)	30.0 (0)	1.54 (0)	0.77 (0)	139.7	57.5
16	20.0 (0)	3.85 (0)	6.0 (0)	30.0 (0)	1.54 (0)	0.77 (0)	147.1	60.8

*HA concentration obtained after 120 hours of fermentation.

3, which reports the HA concentration for each trial and for both species. Trials 13 to 16 consisted of the center points, which were the same conditions used in our previous work in which it demonstrated the production of HA using EFB and the *T. Viride* strain (Motta and Santana, 2013). According to the values obtained for these center points, the average production of HA was 143.1±3.0 mg/L and 58.8±1.4 mg/L for *T. reesei* and *T. viride*, respectively. The largest HA production by *T. reesei* was observed in Trial 2 (318.7 mg/L), which used the highest level for EFB, peptone and temperature, and the lowest level (-1) for pH, K_2HPO_4 and $(NH_4)_2SO_4$. For *T. viride*, Trial 5 generated the highest HA production (107.0 mg/L), which used the highest level for EFB, peptone, temperature and K_2HPO_4, and the lowest level

(-1) for pH and $(NH_4)_2SO_4$.

From the summary of effects shown in Table and obtained using Statistica version 8.0 (Statsoft, Oklahoma, USA), the temperature and the concentration of EFB, peptone, $(NH_4)_2SO_4$ and K_2HPO_4 were the statistically significant variables with an effect on HA production in *T. reesei* (p-value<0.1). Among these five variables, $(NH_4)_2SO_4$ and K_2HPO_4 concentration had a negative effect on HA production, presenting similar estimated effect values (-40.3 and -46.1, respectively). The EFB concentration had the greatest positive effect on HA production, followed by peptone concentration and temperature. EFB, peptone and temperature have a highly significant effect on HA production, with a *p*-value much smaller than 0.1. For *T. viride*, four of the six
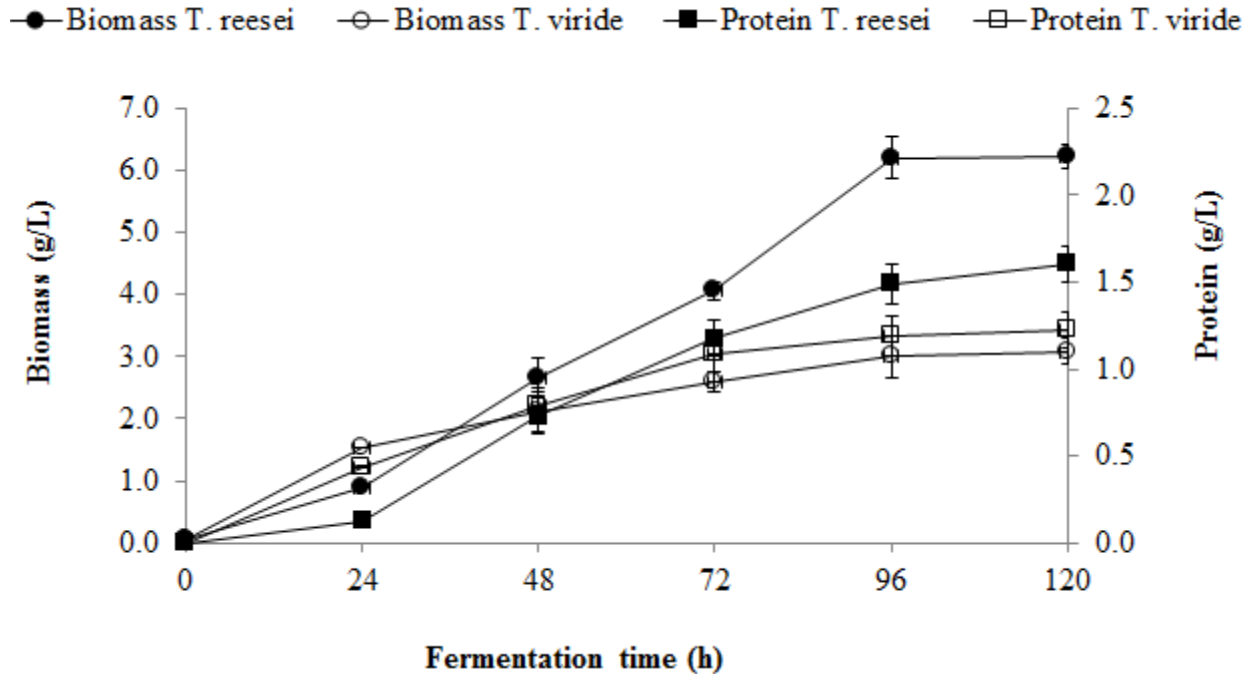
Figure 1. Biomass and cellular protein concentration over time during the submerged fermentation of oatmeal with *T. viride* (Motta and Santana, 2012) and *T. reesei.*

Table 4. Summary of estimated effects from the PB.

| Factor	*T. reesei*				*T. viride*			
	Estimated effect	Standard error	t(8)	*p*-value	Estimated effect	Standard error	t(8)	*p*-value
Mean/Interc.	116.2	10.2	11.4	<0.0001	42.6	3.1	13.6	<0.0001
Curvature	53.8	40.7	1.3	0.2229	32.3	12.5	2.6	0.0323
EFB	94.1	20.3	4.6	0.0017	11.6	6.3	1.9	0.0998
Peptone	92.0	20.3	4.5	0.0019	41.4	6.3	6.6	0.0002
pH	27.1	20.3	1.3	0.2188	-6.0	6.3	-1.0	0.3671
Temperature	69.9	20.3	3.4	0.0089	26.3	6.3	4.2	0.0030
K_2HPO_4	-46.1	20.3	-2.3	0.0530	1.7	6.3	0.3	0.7984
$(NH_4)_2SO_4$	-40.3	20.3	-2.0	0.0828	-21.7	6.3	-3.5	0.0085

variables screened were significant, because unlike *T. reesei*, K_2HPO_4 levels were not statistically significant. For *T. viride*, $(NH_4)_2SO_4$ concentration had a negative effect on HA production (-21.7), and the peptone concentration had the greatest positive effect on HA production, followed by temperature.

Using the Pareto Chart for both *Trichoderma* species (Figure 2), it is possible to identify the variables that do or do not significantly affect HA production by comparing the value of the statistic (t_{cal}) for each variable, represented by the length of the bar, and the critical value (t_{crit} = 1.860), represented by the red line. Variables with $t_{cal} > t_{crit}$ have a statistically significant effect on HA production,

and the variables with $t_{cal} < t_{crit}$ are not considered statistically significant. With respect to the curvature, it was not statistically significant for *T. reesei* (t_{calc} = 1.322 < t_{crit} = 1.860) but was statistically significant for *T. viride* (t_{calc} = 2.586 > t_{crit} = 1.860).

DISCUSSION

Biomass is a fundamental parameter in the characterization of microbial growth, and its measurement is essential for kinetic studies on fermentation. Complete recovery of fungal biomass from the substrate is very
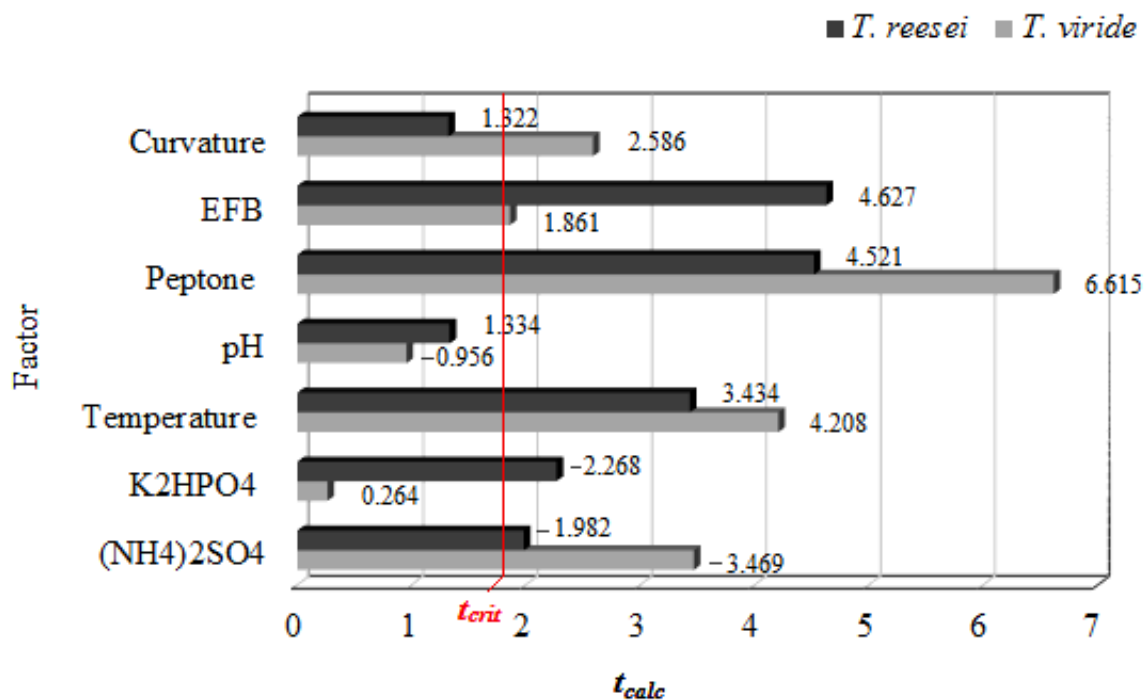
Figure 2. Pareto chart of the standardized effects of independent variables. Response HA mg/L and alpha = 0.10.

difficult when the fungal hyphae penetrates into and binds tightly to the solid substrate particles (Abd-Aziz et al., 2008).

The present study adopted two methods for biomass quantification, one direct and one indirect, consisting of dry biomass and cellular proteins quantification, respectively. Christias et al. (1974) determined in their work the amount of protein present in the biomass of five different genera of fungi, and they obtained a range of 30 to 40%. By applying these results to the present study, it is possible to observe that the ratio between protein and biomass concentration is within the range obtained by Christias et al. (1974).

According to the optical Microscopic image analysis, after 120 h of fermentation, the sporulation phase is complete, and only spores are present in the culture medium. Therefore, the spore counts were performed at 0 and 120 h of fermentation to determine the increase in the fungal population using oat medium for submerged fermentation. The comparison between the initial concentration of spores in the culture and after 120 h of fermentation indicated that there were two phases of log growth during the 5 days of oatmeal-based fermentation.

Watanabe et al. (2006) used a culture medium containing soluble starch and soybean meal supplemented with KH_2PO_4, KCl, $MgSO_4 \cdot 7H_2O$ and $FeSO_4 \cdot 7H_2O$ to produce *T. asperellum* spores by submerged fermentation. They obtained 7.8 x 10^8 spores/mL after 7 days of fermentation from a starting inoculum of 1.0 x 10^6 spores/mL. Jakubíková et al. (2006) optimized *T. atroviride* sporulation in submerged fermentation on cellobiose supplemented with $NaNO_3$, K_2HPO_4, KCl, $MgSO_4$, and $FeSO_4$. The culture medium was inoculated with 1 x 10^6 spores/mL, and the spore concentration reached a maximum level of 2.68 x 10^8 spores/mL after 4 days of fermentation. Comparing the results obtained in the present work to past results indicate that the submerged fermentation of oatmeal is a good alternative for the production of *T. reesei* spores. Moreover, the culture medium used in this study for spore production was supplemented only with peptone, a safe supplement for culture media that is animal-and endotoxin-free. These compounds can cause illness in humans and are considered as contaminants that must be avoided or minimized in the preparation of pharmaceutical products. Because both species were grown under identical conditions and are completely transformed into spores after 120 h of fermentation, it is possible to make a comparison between the two species regarding growth in oat medium. For *T. viride*, the initial concentration of spores (0 h) was 4.20 x 10^4 spores/mL, reaching 3.52 x 10^6 spores/mL at the end of the fermentation (120 h) (Motta and Santana, 2012). With respect to the *T. reesei* spore concentration, the initial and final values were 4.54 x 10^4 and 6.36 x 10^6, respectively. Therefore, it is possible to observe that

there was an 84-fold increase in the population of *T. viride* and a 140-fold increase in the population of *T. reesei* after 120 h of cultivation under the same conditions. The highest growth potential for *T. reesei* with respect to *T. viride* can also be observed by comparing the biomass concentration (g/L) throughout the fermentation for both species (Figure 1). *Trichoderma* species produces large quantities of hydrolytic enzymes (Papavizas, 1985) including chitinases, β-1,3-glucanases (Howell et al., 2000), cellulases, amylases and proteases (Bastos, 1996), which explains the high growth capability of each species. However, *T. reesei* is regarded in the literature as one of the main producers of endoglucanases, secreting at least five types of these enzymes and justifying the increased growth in culture compared to *T. viride*, because the oat media is comprised of β-glucans that can be hydrolyzed to glucose, providing the fungus with a readily usable carbon source for growth (Herpoël-Gimbert et al., 2008).

The *T. reesei* and *T. viride* spores were then used in the submerged fermentation of EFB for HA production. By performing the PB trials, the species were compared in terms of the HA produced as well as analyzed for the effect of fermentation conditions on the production of HA. By comparing the central points of the PB for both species, it is possible to observe that *T. reesei* HA production was 2.4-fold greater than *T. viride*. With respect to the highest HA production for each species (Trial 2 for *T. reesei* and Trial 5 for *T. viride*), this value increases to 3-fold. Although many species of *Trichoderma* have previously been used for industrial enzyme production and lignocellulosic degradation (Nevalainen et al., 1994), *T. reesei* is known to be the main producer of cellulases and hemicellulases acting in synergy to degrade lignocellulosic materials (Herpoël-Gimbert et al., 2008), which explains its increased production of HA. To understand the effect of the variables involved in the proposed process, PB statistical analysis was used, in which the response variable (HA concentration) was examined at 120 h, because HA accumulated in the culture medium, due to the resistance of HA to microbial degradation compared to other organic materials (Aiken, 1985). This idea was confirmed by the work of Motta and Santana (2013), in which the production of HA by submerged EFB fermentation was studied. The profile obtained for HA concentration increased as fermentation time increased, demonstrating accumulation in the media. The Pareto chart (Figure 2) distinguishes the statistically significant variables from the statistically insignificant among the factors studied, as previously discussed, by comparing the value of the statistic (t_{cal}) for each variable to the critical value (t_{crit} = 1.860). Center points were added in the statistical analysis, allowing the performance of explicit statistical significance tests of curvature. These values were not statistically significant for *T. reesei*, indicating that there is

a linear relationship between the factors and the dependent variable. For *T. viride*, the curvature was statistically significant, meaning that at least one variable is involved at an order higher than one. Among the analyzed variables, only pH was considered to have no effect on HA production at the confidence level studied (90%) for both *Trichoderma* species. According to Bhattiprolu (2008), *T. viride* is able to grow in wide range of pH (4.0 to 9.0), which may explain the fact that this factor was not been statistically significant for either species in this work. Regarding the nitrogen sources, peptone (L) and $(NH_4)_2SO_4$ (L) concentration had a significant effect on HA production by both species, although the first had a large positive effect and the second a negative effect. Juwon and Emmanuel (2012) tested the influence of nitrogen sources on growth and enzyme production in *T. viride* and observed that organic nitrogen substrates, like peptone, supported increased biomass yield and enzyme activity of the fungus compared to the inorganic nitrogen substrates tested.

The variable that affected HA production is the least in *T. reesei* and K_2HPO_4 concentration, and its effect was not significant in *T. viride*. According to the average chemical formula elucidated by Schnitzer and Khan (1978) (C_{187} H_{186} O_{89} N_9 S_2), HA does not contain phosphorus and potassium, explaining the minimal influence of this compound on the production of HA. Temperature has a large effect on both species, likely because enzyme activity as well as regulation and transport systems are generally largely affected by temperature in microbial systems (Anastassiadis, 2006). The linear effect of EFB concentration was larger in *T. reesei* than in *T. viride* and had the largest effect on this species. Although this component is the main carbon source for HA production in the studied media for both species, its effect was greater for *T. reesei*, possibly because this species is capable of utilizing the best carbon and energy source available when exposed to a mixture of carbon sources and down regulates the expression of genes involved in the degradation of less favorable and complex carbon sources (Seiboth et al., 2011).

Conflict of Interests

The author(s) have not declared any conflict of interests.

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