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*Full Length Research Paper*

# The use of Amazon fungus (*Trametes* sp.) in the production of cellulase and xylanase

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**In order to use the residue from the beneficiation of Brazil nuts (Bertholletia excelsa HBK) as substrate in solid-sate fermentation (SSF), in this work, the production of cellulase and xylanase by the fungus *Trametes* sp. was investigated, using the residue as a carbon source. Employing a $2^{4-1}$ fractional experimental design, the influence of substrate moisture, nutrient addition and inoculum quantity on enzymatic activities was verified. Moisture was detected to be statistically significant for the production of both enzymes, and increasing the moisture leads to the improvement of cellulase and xylanase activities. Nitrogen and phosphate were also important for enzymes production by the Amazon *Trametes* sp. The use of this Amazon strain to obtain cellulase and xylanase via SSF of Brazil nut residue appears to be feasible when maintaining substrate moisture at 80%, nitrogen source at 0.9% and low inoculum concentrations.**

**Key words:** Cellulolytic activity, xylanolytic activity, *Trametes* sp., experimental design.

## INTRODUCTION

Among the most important microbial metabolites obtained through biotechnological processes are the enzymes, which have massive industrial applications in several processes and products (Bajpai, 1999; Souza and Magalhães, 2010). Cellulase is a complex mixture of enzyme proteins with different specificities, which act synergistically to hydrolyze glycosidic bonds. The three major cellulase enzyme activities are: endocellulase or1,4-β-D-glucan glucanohydrolases (EC 3.2.1.4.), exocellulase or 1,4-β-D-glucan cellobiohydrolase (EC

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3.2.1.91) and beta-glucosidase or β-D-glucoside glucohydrolases (EC 3.2.1.21) (Zhou et al., 2004; Martins et al., 2008; Farinas et al., 2011). Cellulases are used in food and animal feed industries, pulp and paper processing, as well as in the textile industry (Bajpai, 1999; Ögel et al., 2001). Degradation of lignocellulosic materials to monomeric sugars through the concerted action of cellulolytic enzymes has great importance, since sugars can be converted to ethanol, lactic acid and hydrogen (Juhász et al., 2005).

The best known xylanolytic enzymes are endo-β-1,4-xylanases (EC 3.2.1.8), which act on the chain of xylan, cleaving the internal glycosidic linkages of the backbone that results in chains with lower polymerization degrees and β-xylosidase (EC 3.2.1.37), which hydrolyses xylobiose and short xylooligosaccharides to xylose (Silva and Carmona, 2008). Xylanases show great potential for industrial applications mainly for the bioconversion of lignocelluloses to sugar, ethanol, and other useful substances; clarification of juices and wines; improving the nutritional quality of silage and green feed; the de-inking processes of waste papers, and for the treatment of agro industrial residues (Bajpai, 1999; Viikari et al., 1994; Kheng and Omar, 2005).

The use of purified substrates for bioconversion into hydrolytic enzymes, such as cellulase and xylanase, increases the cost of enzyme production. Consequently, for commercial applications, there have been attempts to develop bioprocesses to produce these enzymes in high quantities from simple and inexpensive substrates. Abundantly available residues are an interesting choice as a substrate (Bakri et al., 2008), and Brazil has an agricultural-based economy, producing large amounts of agro-industrial byproducts every year (Soccol and Vandenberghe, 2003). Amazonas State stands out as the largest Brazil nut (*Bertholletia excelsa* HBK) producer in Brazil. The beneficiation of Brazil nut for exportation generates significant amounts of residues, which consist mainly of cellulose, lipids, starch, ash and sugars, therefore presenting great potential for use as substrate in solid state fermentation (Pacheco and Scussel, 2006; Martins et al., 2012).

Solid-sate fermentation (SSF) has been used to obtain microbial metabolites of commercial interest, such as hydrolytic enzymes (Bakri et al., 2008; Mrudula and Murugammal, 2011), organic acids (Prado et al., 2005; Torrado et al., 2011), biopesticides (Balakrishnan and Pandey, 1996), biosurfactants (Castiglioni et al., 2009), single cell proteins (Alberton, 2004; Albuquerque et al., 2006), and many other compounds. Solid-sate fermentation is characterized by the development of microorganism in a low aqueous content on an insoluble material that can act as both a physical support and as a nutrient source (Auria et al., 1995; Pandey et al., 2000; Vandenberghe et al., 2000). This low water and energy consumption bioprocess offers the possibility to employ agro industrial residues, which usually consist of heterogeneous materials containing complex polymers like lignin, pectin and lignocellulose, as an alternative for conventional submerged fermentations (Raimbault, 1998).

Lignocellulose comprises the world's most abundant polymeric biomass and appears in a significant portion of solid residues. These residues are formed mainly by cellulose (32 to 50%), hemicellulose (19 to 25%) and lignin (23 to 32%), as well as minerals, salts and organic acids (Castro and Pereira Jr., 2010), therefore being defined as lignocellulosic residues.

Environmental factors such as temperature, pH, water activity, oxygen levels and concentrations of nutrients and products significantly affect microbial growth and product formation during SSF. The control of these parameters is difficult and often inaccurate, thereby limiting the industrial potential of this technology. Due to these problems, the microorganisms that have been selected for SSF are the most tolerant to a wide range of cultivation conditions (Raimbault, 1998).

Cellulase and xylanase can be produced by a number of microorganisms including bacteria, yeasts and fungi (Kheng and Omar, 2005). However, filamentous fungi are preferred for commercial enzyme production, because of the level of the enzymes produced by these cultures (Mrudula and Murugammal, 2011). Fungi of the *Trametes* genus are white-rot basidiomycete, agents of wood degrading processes (Archibald et al., 1997). These fungi promote the simultaneous degradation of lignin, cellulose and hemicellulose (Tanaka et al., 1999), which confer to these organisms a great potential for the production of lignocellulolytic enzymes.

Several studies have demonstrated the effectiveness of statistical experimental designs and surface response methodology for the optimization of microbial enzyme production (Albuquerque et al., 2006; Singh et al., 2009; Rajendran and Thangavelu, 2009; Chicatto et al., 2014). The fractional factorial experimental design is useful as a first effort to evaluate the impact of independent variables (factors) on the dependent variables (response), allowing the selection of statistically significant parameters (Montgomery, 1997; Rodrigues and Iemma, 2005). Hence, the present work aimed to use a statistical experimental design to study the influence of substrate moisture content, nutrient addition and inoculum quantity on the cellulase and xylanase produced by an Amazon *Trametes* sp., cultivated through solid state fermentation on the residue of Brazil nuts.

## MATERIALS AND METHODS

### Chemical

Potato dextrose agar (PDA) medium was purchased from Himedia Laboratories (Mumbai, India). Birch wood xylan, carboxymethylcellulose (CMC) and 3,5-dinitrosalicylic acid (DNS) were purchased from Sigma Chemical Co. (St. Louis, MO). Other chemicals were of analytical grade and were not previously purified.

**Table 1.** Cultivation conditions and experimental results for cellulase and xylanase production using Brazil nut residue as substrate.

| Experiment | Variables (Coded variables) | | | | Enzymatic activity | |
|---|---|---|---|---|---|---|
| | Inoculum (disks) | Moisture[a] (%) | $KH_2PO_4$ (%) | $NH_4NO_3$ (%) | Cellulase (IU/g) | Xylanase (IU/g) |
| 1 | 1 (-1) | 60 (-1) | 0.1 (-1) | 0.1 (-1) | 0.18 | 0.33 |
| 2 | 5 (+1) | 60 (-1) | 0.1 (-1) | 0.9 (+1) | 0.23 | 0.00 |
| 3 | 1 (-1) | 80 (+1) | 0.1 (-1) | 0.9 (+1) | 2.97 | 2.37 |
| 4 | 5 (+1) | 80 (+1) | 0.1 (-1) | 0.1 (-1) | 1.20 | 2.43 |
| 5 | 1 (-1) | 60 (-1) | 0.3 (+1) | 0.9 (+1) | 0.65 | 1.15 |
| 6 | 5 (+1) | 60 (-1) | 0.3 (+1) | 0.1 (-1) | 0.29 | 0.18 |
| 7 | 1 (-1) | 80 (+1) | 0.3 (+1) | 0.1 (-1) | 0.00 | 2.02 |
| 8 | 5 (+1) | 80 (+1) | 0.3 (+1) | 0.9 (+1) | 3.12 | 3.84 |
| 9 | 3 (0) | 70 (0) | 0.2 (0) | 0.5 (0) | 1.30 | 1.22 |
| 10 | 3 (0) | 70 (0) | 0.2 (0) | 0.5 (0) | 1.15 | 1.42 |
| 11 | 3 (0) | 70 (0) | 0.2 (0) | 0.5 (0) | 1.31 | 1.13 |

[a]60% Moisture content was adjusted by adding 2.76 mL of nutrient solution; 80% moisture content was adjusted by adding 3.76 mL of nutrient solution; 70% moisture content was adjusted by adding 3.26 mL of nutrient solution.

**Microorganism**

*Trametes* sp. used in this investigation was isolated from the bark of a decomposing tree at km 8 of BR-174 road (Manaus-Presidente Figueiredo, Amazonas State, Brazil) in February 2010, according to the methodology described by Araújo et al. (2002). Stock cultures were maintained on potato dextrose agar (PDA) slants at 5°C, at the Collection of the Laboratory of the School of Health Sciences, State University of Amazonas (ESA/UEA). The fungus was identified by classic taxonomy, according to its macro and micromorphological characteristics. Macroscopic vegetative characteristics (color, texture, topography, diffuse pigmentation, color and topography of the back of the colony), as well as microscopic reproductive structures (through microculture technique), were analyzed and compared with taxonomic keys (Barnett et al., 1972; Hanlin, 1996).

**Solid substrate**

Brazil nut (*Bertholletia excels* HBK) residue, consisting of nut, shell and pellicle was kindly provided by Usina CIEX (Manaus, Amazonas), a Brazil nut exporter. After collecting it at the factory, the residue was milled and stored in plastic bags at -18°C until used. The substrate was characterized at the Laboratory of Fish Nutrition from National Institute of Amazon Research (INPA). The residue had on dry basis, 23.5% lipid, 8.9% protein and 1.8% ash. It contained 4.8% moisture, determined by desiccation method at 50°C, until constant weight.

**Inoculum**

*Trametes* sp. was activated by growing on PDA dishes at 30°C for 7 days. Disks (0.9 cm diameter) of solid media containing active growing mycelium were removed from the dishes and inoculated on 5 g of solid substrate. The number of disks used varied according to the experimental design matrix (Table 1).

**Enzymatic qualitative assay**

*Trametes* sp. was tested on solid media for the production of cellulase and xylanase. To assess the cellulase production, the solid media was prepared with 1.8% (v/w) agar, 1.0% (v/w) CMC and 0.1 M sodium acetate buffer solution pH 5.0 (Teixeira, 1994). Xylanase activity was evaluated on a solid media prepared with 1.8% (v/w) agar, 1.0% (v/w) birch wood xylan, and 0.1 M sodium acetate buffer solution pH 6.0 (adapted from Silva et al., 2005). In order to verify the halo production, a 0.1% (v/w) Congo red solution was used. Tests were performed in triplicate.

**Solid state fermentation**

Solid state fermentation was conducted in 250 mL Erlenmeyer flasks without agitation. Each flask was filled with 5 g of Brazil nut residue and nitrogen (ammonium nitrate, $NH_4NO_3$) and phosphate (potassium dihydrogen phosphate, $KH_2PO_4$) sources were added. Solutions containing nitrogen and phosphate sources were added in order to adjust moisture content, according to the experimental design matrix (Table 1). Flasks containing the supplemented substrate were sterilized (121°C, 15 min) prior to the fungus inoculation. The flasks were incubated at 30°C for seven days.

The cultivated media was suspended in 20 mL of sterile distilled water. This was kept in an ultrasound bath for 1 min for enzymes extraction, filtered and used for enzyme activities analysis.

**Enzymatic activities assays**

The cellulolytic and xylanolytic activities were determined in the enzymatic extract according to the methodology described by Silva and Carmona (2008). The cellulolytic activity was evaluated as endoglucanase activity. Carboxymethylcellulase (CMCase) activity was determined using carboxymethylcellulose (CMC) 1.0% (w/v) as substrate. Standard cellulolytic assay conditions were 45°C and pH 5.0 buffered with 50 mM sodium acetate. The reducing sugars released were determined by the dinitrosalicylic acid method (Miller et al., 1959). One unit of cellulase activity was defined as the amount of enzyme that releases 1.0 µmol of reducing sugar equivalent to glucose per minute.

Xylanase activity was determined at 50°C using 1.0% (w/v) birchwood xylan in 50 mM sodium phosphate buffer pH 6.0. One unit of xylanase activity was defined as the amount of enzyme which releases 1.0 µmol of reducing sugar equivalent to xylose per

minute.

**Experimental design**

A $2^{4-1}$ fractional factorial planning added to three repetitions in the central point was used to determine the effects of nutrients (nitrogen and phosphate) supplementation, substrate moisture content and inoculum quantity on enzymatic activities. The experimental conditions of each experiment are shown in Table 1. Two analytical steps-analysis of variance (ANOVA), and plotting of response surface were performed to select the most significant variables. The results obtained from experiments were submitted to ANOVA variance analysis, and effects were considered significant at $p \leq 0.05$. Statistical analysis was done using the software Statistica 6.0 (StatSoft). Experimental error and standard deviation were assessed through the triplicate experiment performed at the central point (Montgomery, 1997; Rodrigues and Iemma, 2005).

## RESULTS AND DISCUSSION

*Trametes* sp. presented positive results in the qualitative assay for both enzymes, indicating the production of cellulase and xylanase. Therefore, the isolate was used for the production of these enzymes in SSF experiments. It was observed that after seven days of solid state fermentation on the Brazil nut residue, the fungus *Trametes* sp. showed the ability to grow on the substrate, colonizing the entire Erlenmeyer flasks.

Fungi of the genus *Trametes* have been cited as cellulase and xylanase producers (Mswaka and Magan, 1998; Valášková and Baldrian, 2006; Levin et al., 2008; Jeya et al., 2009). Márquez-Araque and colleagues (2007) evaluated the cellulase and xylanase production by a *Trametes* sp. grown on sugar cane bagasse, through solid state fermentation. After comparing the results with *Pleurotus ostreatus* and *Aspergillus niger* strains, the authors verified that *Trametes* sp. strain presented the highest enzymatic activities (141.77 IU/g for xylanase and 9.04 IU/g for cellulase). In another study, Jeya and colleagues (2009) identified *T. hirsute* as a remarkable producer of different cellulolytic enzymes (endoglucanase, cellobiohydrolase and β-glucosidase), which was used on rice straw hydrolysis. Mswaka and Magan (1998) verified outstanding values for specific cellulase activities in *Thalassoplanes modesta* (303 IU/mg) and *T. pocas* (293 IU/mg), among 14 basidiomycete species grown on Mandels cellulose medium. These findings reported in literature justify the attempt to use *Trametes* sp. for the production of cellulase and xylanase using the Brazil nut residue as substrate, since this wood-degrading fungus is abundant in the Amazon Region, where large amounts of this residue are produced (Martins et al., 2012).

The cellulase and xylanase activities obtained through the fractional $2^{4-1}$ experimental design are presented in Table 1. It should be noted that culture conditions used for experiment 8 promoted the highest cellulase (3.12 IU/g) and xylanase (3.84 IU/g) activities. Standard deviation obtained for cellulase activity was 0.0902 IU/g and for xylanase activity SD = 0.1191 IU/g (calculated from the central point experiments).

The highest enzyme production by Amazon *Trametes* sp. was observed when maximum levels of substrate moisture (80%), phosphate concentration (0.3% $KH_2PO_4$), nitrogen concentration (0.9% $NH_4NO_3$) and inoculum quantity (5 disks) were used for solid state fermentation. The Pareto chart presents the effects that are statistically significant for a process response. It can be noted in Figure 1 that the factors which statistically influenced the cellulase activity were substrate moisture and the addition of nitrogen source ($p \leq 0.05$).

According to Raimbault, (1998), the moisture content in SSF processes has a marked effect on growth kinetics, and consequently on enzyme production, which was corroborated by Mrudula and Murugammal (2011). Santos et al. (2011) mentioned that the available water in SSF is a key factor that directly interferes in the microorganisms' enzyme excretion, since water promotes the fungus growth through oxygen transfer, nutrients diffusion, as well as temperature control.

Agro-industrial residues have been used for the production of cellulase. Using an *A. niger* strain grown on mango residue for the production of endoglucanase, Santos et al. (2011) verified 7.26 IU/g after 75 h of cultivation. In the present work, cellulase activity presented by the Amazon *Trametes* sp. grown on nut residue was 3.12 IU/g after 168 h of cultivation, indicating the need for further optimization of the culture media in order to reach higher endoglucanase activities. It was observed that a nitrogen source presented a positive effect on cellulase activity. This nutrient, in general, represents one of the main factors that affect the cellulase biosynthesis in fungi (Nascimento et al., 2009). Similar to the present study findings, Soni et al. (2010) verified the need for a nutrient supplement of rice straw to be used in SSF. The solid substrate was added with phosphate (0.4% $KH_2PO_4$) and nitrogen (0.45% $CH_3COONH_4$ and 1.3% $(NH_4)_2SO_4$) in order to reach optimal conditions for the production of cellulse by *A. fumigatus*. The same was observed in the work performed by Vu et al. (2011), where the highest cellulase activity (82.50 IU/g) was verified when the solid substrate (wheat bran) was supplemented with 1% urea or $NH_4Cl$, 1% rice starch, 2.5 mM $MgCl_2$ and 0.05% Tween 80, using a mutant *Aspergillus* sp. strain.

The inoculum quantity and the addition of phosphate source were not statistically significant for cellulase production on *Trametes* sp. using the tested experimental conditions. As can be seen in Figure 2, xylanase activity was only significantly affected by the substrate moisture content ($p \leq 0.05$), and this effect was almost three times higher than that observed for cellulase (Figure 1), suggesting that for this Amazon fungus, the water content in the solid substrate is even more important for xylanase than for cellulase production. Pal and Khanum (2010)

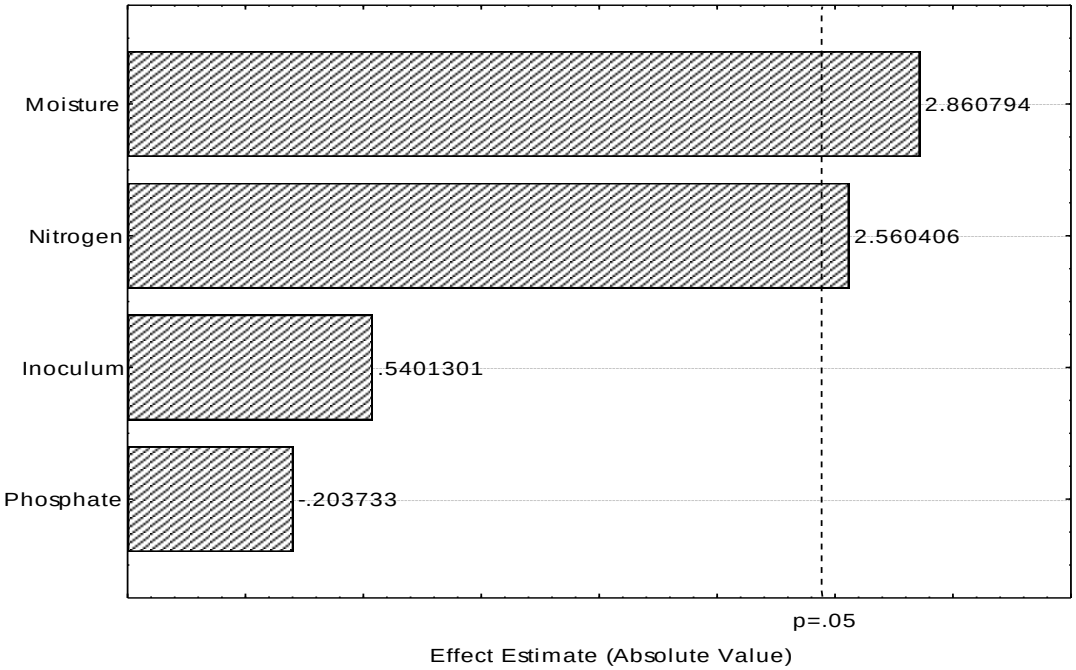


**Figure 1.** Pareto chart for cellulolytic activity of Trametes sp. grown

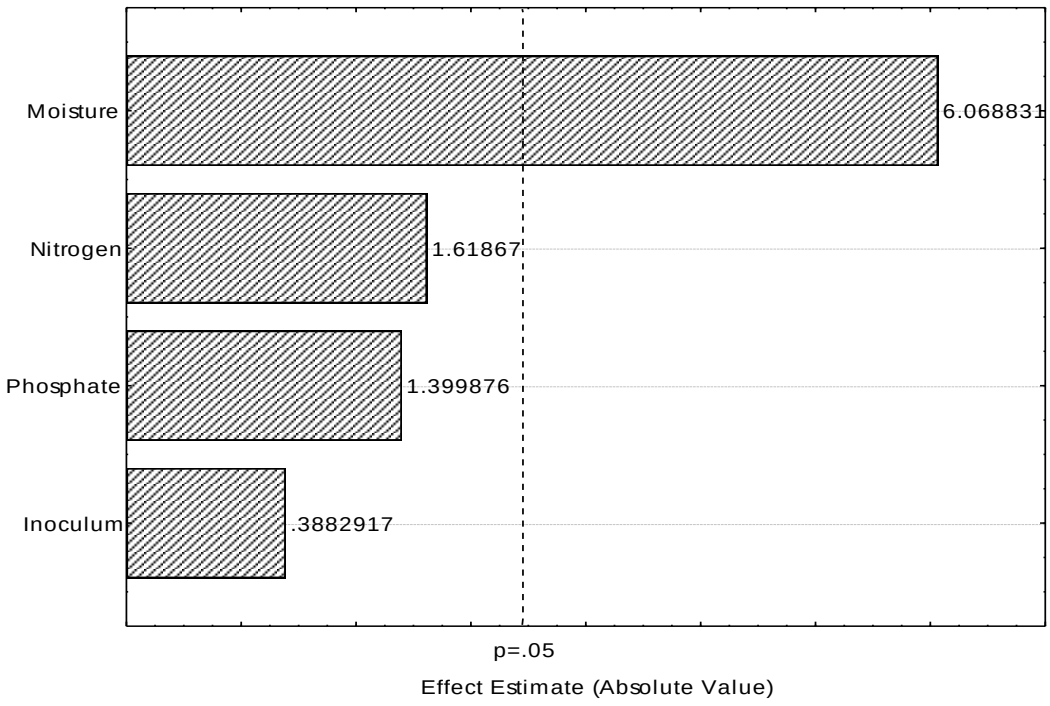


**Figure 2.** Pareto chart for xylanolytic activity of *Trametes* sp. grown on Brazil nut residue.

suggest that for fungal xylanase production, the moisture content should be between 43 and 83%. However, in the present work, the need for higher water quantities (up to 80% initial humidity) was seen for the production of this enzyme. Alberton (2004) also verified that moisture content is an extremely important physical parameter for

**Table 2.** Statistical analysis for cellulolytic activity of *Trametes* sp. grown on Brazil nut residue.

| Factor | Effect | Standard error | t(6) | p |
|---|---|---|---|---|
| Mean | 1.129242 | 0.222361 | 5.078412 | 0.002269 |
| Inoculum | 0.267250 | 0.5211483 | 0.512480 | 0.626628 |
| Moisture | 1.491750 | 0.5211483 | 2.860589 | 0.028779 |
| Phosphate | -0.125250 | 0.5211483 | -0.240180 | 0.818186 |
| Nitrogen | 1.333250 | 0.5211483 | 2.556649 | 0.043103 |

Bold = Statistical significance of the effect (at a 95% confidence range).

the microorganism during the xylanase production in solid state fermentation. The authors observed, as well as in this work, that higher levels of substrate moisture promoted the highest levels of xylanase activity.

Xylanase production using agro-industrial residues through SSF has also been reported. Maciel (2006) observed a 3.09 IU/g of xylanolytic activity using an *A. niger* strain cultivated in a solid substrate composed by sugar cane bagasse and soybean bran. The author used 85% moisture and nutrient supplementation (1.5 g/L $KH_2PO_4$; 0.4 g/L $CuSO_4$ and 0.0012 g/L $CoSO_4$) in a SSF carried out at 30°C and 10 days. Kheng and Omar (2005) used palm kernel cake supplemented with a nitrogen source (0.075%) in SSF with *A. niger* and verified a 33.99 IU/g of xylanolytic activity after 7 days of fungal growth at 28°C.

Different substrates, as well as different fungal species, can justify the differences in the values obtained for cellulolytic and xylanolytic activities, when comparing this work with those reported elsewhere. According to Favela Torres and colleagues (1998), the environmental conditions, such as temperature, pH, water activity, oxygen level and nutrient concentration significantly affect the cellular growth and product formation. It is worthwhile to mention that the Brazil nut residue has not been recorded so far as substrate for cellulase and xylanase production, and further experiments are needed in order to achieve a more significant enzyme production. It is important to mention here that even though the addition of nitrogen and phosphate was not statistically significant on xylanolytic activity (Figure 2), these variables have effects almost 5 times higher than the inoculum quantity, which suggests that these nutrients play an important role in the production of xylanase by the fungus *Trametes* sp.

Table 2 presents the values of variables' effect on the cellulase activity. Moisture content presented a significant and positive effect on cellulase activity (p = 0.028779), and this result indicates that raising the moisture content from 60 to 80% leads to an increase in the cellulase activity. Therefore, the moisture variable should be further evaluated at higher levels, in order to find its optimum value for *Trametes* sp. cellulase production. The addition of $NH_4NO_3$ was also statistically significant for the cellulase production by the fungus *Trametes* sp. grown on Brazil nut residue. Increasing the concentration of nitrogen source from 0.1 to 0.9% leads to an increase in the cellulolytic activity. Mrudula and Murugammal (2011) reported that the supplementation of coir waste with nitrogen sources increased the cellulase production of *A. niger* cultivated in solid state fermentation. Similar to what was observed for moisture content, nitrogen addition on higher levels (up to 0.9%) should be considered in further optimization experiments for cellulase production using the Amazon *Trametes* sp. grown on the Brazil nut residue. Inoculum quantity and phosphate addition were not statistically significant for the production of cellulase. In fact, the phosphate addition presented a negative effect (-0.125250) on cellulase activity, which means that raising the concentration of $KH_2PO_4$ from 0.1 to 0.3% leads to a decrease in enzymatic activity. Since inoculum and phosphate were not significant for the production of cellulase on *Trametes* sp., those variables should be used at their minimum values in further experiments.

Table 3 presents the values of variables' effect on the xylanase activity. It can be noticed that, as observed for cellulase, moisture presented a significant and positive effect (p = 0.000908). The inoculum quantities as well as the addition of nutrients to the Brazil nut residue were not statistically significant for the xilanolytic activity. Contrary to what was observed for cellulase activity, the phosphate supplementation effect on xylanase activity was positive, although not significant. Hence, for xylanase production, the lower levels of these variables should be preferred (1 disk of inoculum, 0.1% $KH_2PO_4$ and 0.1% of $NH_4NO_3$).

Using a statistical experimental design and the analysis of response surfaces, it is possible to investigate the influence of certain variables on a process and the interaction between these variables, as well as obtain the variable values that maximize the results (Montgomery, 1997; Rodrigues and Iemma, 2005). In this work, it was possible to graphically evaluate the variables' effect on enzymatic activities, through the analysis of response surface plots. Figure 3 shows the response surface graphics for cellulolytic activity, considering the effect of

**Table 3.** Statistical analysis for xilanolytic activity of *Trametes* sp. grown on Brazil nut residue.

| Factor | Effect | Standard Error | t(6) | p |
|---|---|---|---|---|
| Mean | 0.085659 | 0.011180 | 7.661.726 | 0.000258 |
| Inoculum | -0.009099 | 0.026220 | -0.347028 | 0.740418 |
| Moisture | 0.091495 | 0.026220 | 3.489.560 | 0.012990 |
| Phosphate | 0.038418 | 0.026220 | 1.465.230 | 0.193207 |
| Nitrogen | 0.037913 | 0.026220 | 1.445.950 | 0.198328 |

Bold = Statistical significance of the effect (at a 95% confidence range).

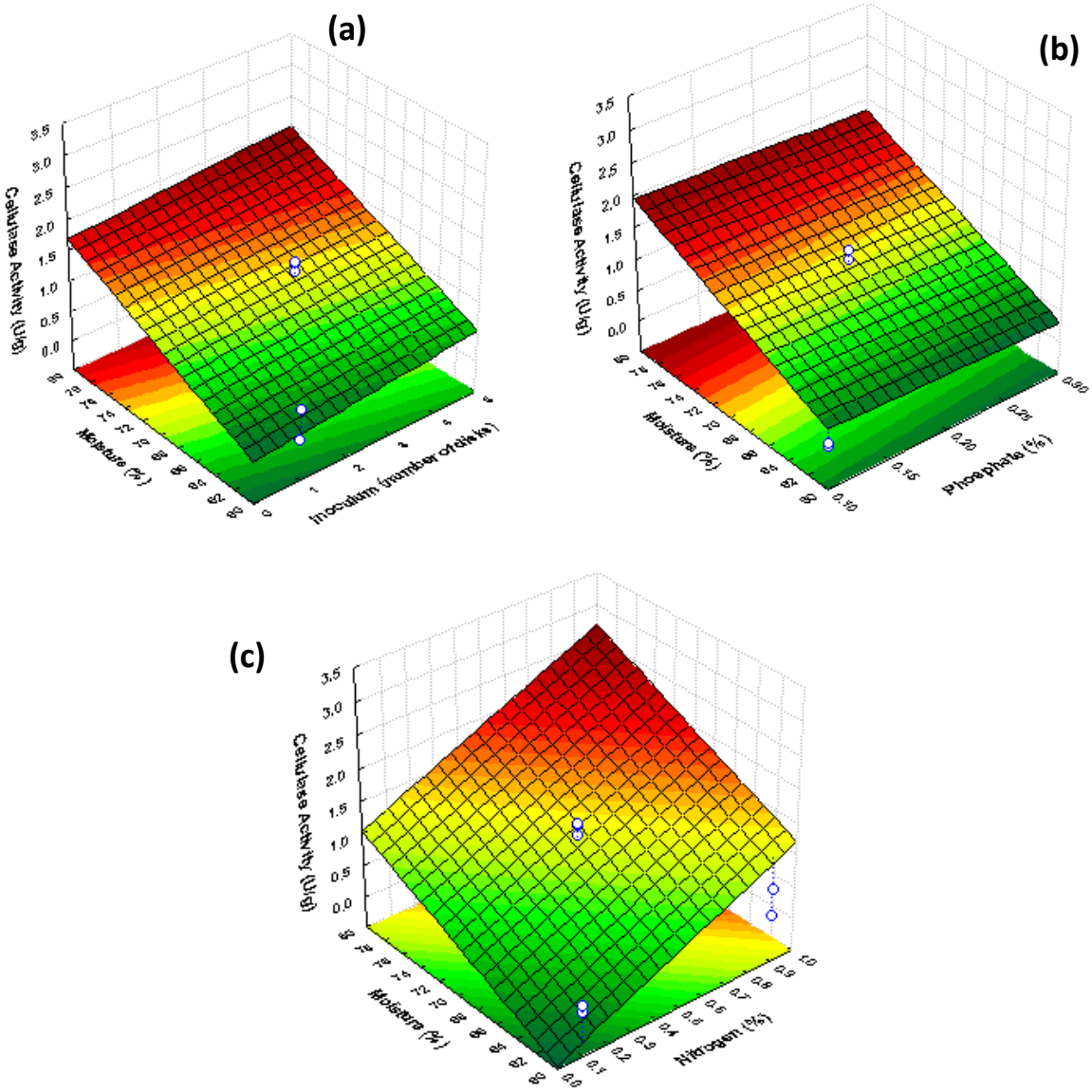


**Figure 3.** Response surface plot for cellulolytic activity as a function of moisture and inoculum quantity (a), $KH_2PO_4$ concentration (b) and $NH_4NO_3$ concentration (c).

interactions between moisture and the other variables studied (inoculum quantity, phosphate and nitrogen addition). Analyzing the response surface shown in Figure 3a, it can be noted that when the inoculum quantity varies, the enzymatic activity does not suffer practically any value change, which is true for any

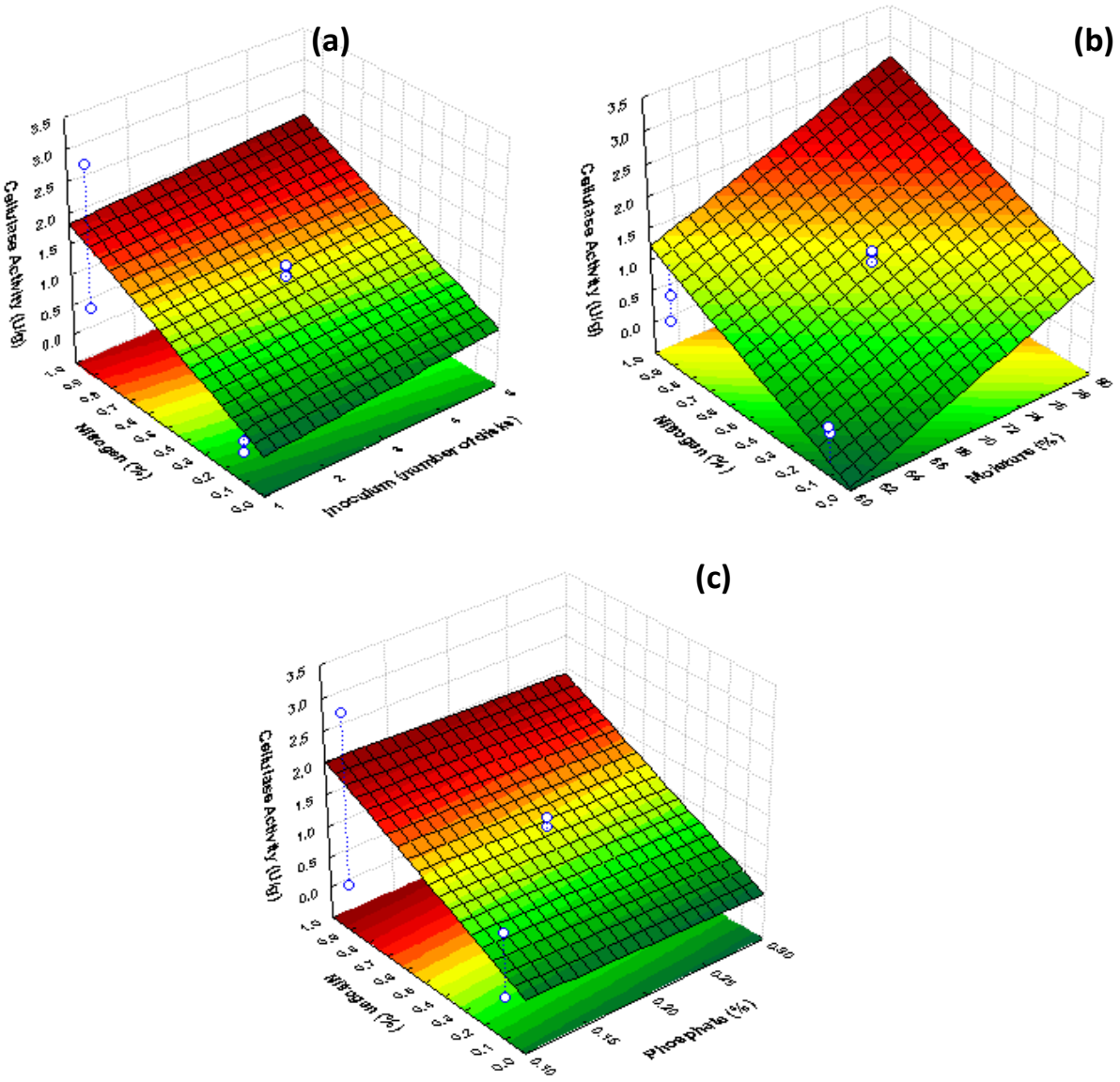


**Figure 4.** Response surface plots for cellulolytic activity as a function of $NH_4NO_3$ concentration and inoculum quantity (a), moisture content (b) and $KH_2PO_4$ concentration (c).

moisture value. A similar behavior can be observed in Figure 3b, since the increase in $KH_2PO_4$ concentration does not cause appreciable changes in cellulolytic activity. A different behavior, however, can be seen Figure 3c, where it shown that the increase of $NH_4NO_3$ concentration leads to an increase in the enzymatic activity, and this effect is even more pronounced at higher levels of moisture. Figure 4 shows the response surface plots correspondent to the interactions between nitrogen concentration and the other variables (inoculum quantity, moisture content and phosphate concentration). Figure 4a shows that, the use of larger amounts of inoculum disks does not have a significant effect on cellulolytic activity as also observed in Figure 3a. However, at higher concentrations of nitrogen source, the enzymatic activity reaches higher values. In Figure 4b, it can be noticed that
the moisture content has a pronounced effect on the cellulolytic activity, which is raised when higher $NH_4NO_3$ concentrations are used.

Figure 4c is similar to Figure 4a, since the addition of higher concentrations of $KH_2PO_4$ does not cause practically any change in the enzymatic activity, unlike the effect observed when nitrogen source concentration is increased.

Response surface plots presented in Figure 5 allow evaluation of the interaction of moisture content and the other variables studied (inoculum quantity, addition of

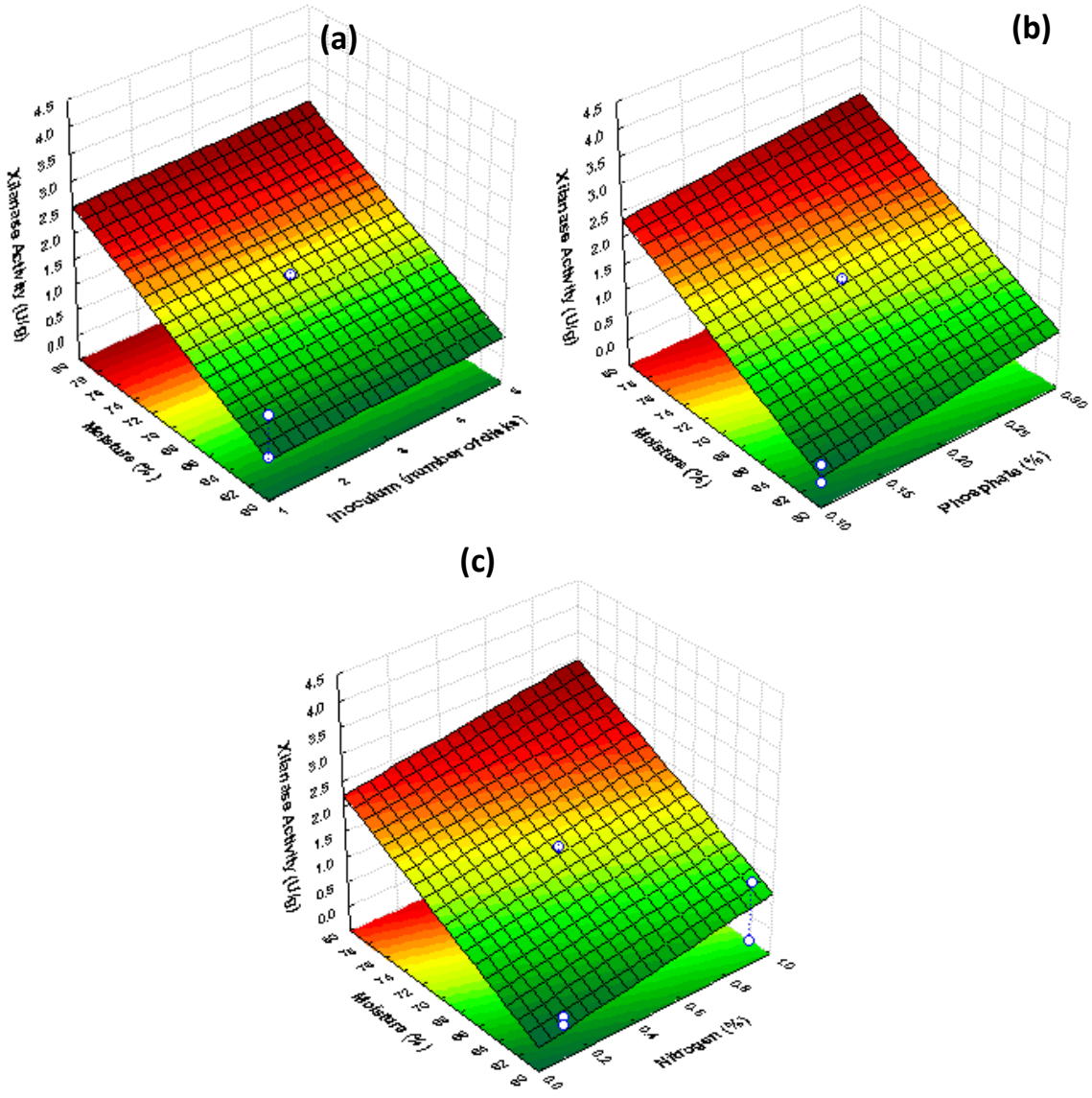


**Figure 5.** Response surfaces for xylanolytic activity as a function of moisture content and inoculum quantity (a), $KH_2PO_4$ concentration (b) and $NH_4NO_3$ concentration (c).

phosphate and nitrogen) for the xylanase production. It can be noticed in Figure 5a that varying the quantity of inoculum does not cause appreciable changes on xylanase activity. The same result was observed for cellulase activity (Figures 3a and 4a), regardless of moisture content. However, when the moisture content increased, a pronounced increase in xylanase activity was observed. In Figure 5b, it can be seen that the increment in $KH_2PO_4$ concentration leads to a slight increase on xilanolytic activity, and that effect is enhanced at higher levels of moisture. Similar behavior is shown in Figure 5c, where the nitrogen concentration, when raised, caused a small increase in enzymatic activity, which is enhanced when high percentages of substrate moisture are used for the fungus growth.

## Conclusion

It is possible to employ the Brazil nut residue as substrate for the production of cellulase and xylanase using an Amazon *Trametes* sp. strain. Using the $2^{4-1}$ fractional experimental design allowed the selection of the most relevant variables for the production of cellulase and xylanase via solid state fermentation. It was shown that moisture content had a significant effect on both cellulase and xylanase production, whereas the inoculum quantity and the addition of phosphate source did not influence the enzymatic activities. Nitrogen addition was important for cellulase production, and this supplementation should be used at a concentration above 0.9%, combined with moisture content higher than 80%. SSF is a technology

that can propose alternative paths for the reuse of agro-industrial wastes, therefore decreasing possible environmental problems, as well as adding economic value to these co-products.

The Brazil nut residue can be used for the production of cellulase and xylanase, which means a low cost substrate, abundant in Northern Brazil. Finally, it was shown that further optimization studies need to be carried out, in order to obtain higher enzymatic activities.

## Conflict of interests

The authors hereby declare that no conflict of interest exists among them

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### REFERENCES

Alberton LR (2004). Produção de xilanase em resíduos agroindustriais por *Streptomyces viridosporus* T7a e aplicação do extrato bruto em veterinária. Ph.D. Thesis, Universidade Federal do Paraná, Curitiba.

Albuquerque PM, Koch F, Trossini TG, Esposito E, Ninow JL (2006). Production of *Rhizopus oligosporus* protein by solid state fermentation of apple pomace. Braz. Arch. Biol. Technol. 49:91-100.

Araújo WL, Lima AOS, Azevedo JL, Marcon J, Kuklinskysobral J, Lacava PT (2002). Manual: Isolamento de Microrganismos Endofíticos. CALQ, Piracicaba.

Archibald FS, Bourbonnais R, Jurasek L, Paice MG, Reid ID (1997). Kraft pulp bleaching and delignification by *Trametes versicolor*. J. Biotechnol. 53:215-236.

Auria R, Ortiz I, Villegas E, Revah S (1995). Influence of growth and high mould concentration on the pressure drop in solid state fermentations. Process Biochem. 30:751-756.

Bajpai P (1999). Application of enzymes in the pulp and paper industry. Biotechnol. Prog. 15:147-157.

Bakri Y, Jawhar M, Imad M, Arabi E (2008). Improvement of xylanase production by *Cochliobolus sativus* in solid state fermentation. Braz. J. Microbiol. 39:602-604.

Balakrishnan K, Pandey A (1996). Production of biologically active secondary metabolites in solid state fermentation, J. Sci. Ind. Res. 55:365-372.

Barnett HL, Hunter BB (1972). Ilustrated Genera of Imperfect Fungi, Burgess, Minneapolis.

Castiglioni GL, Bertolin TE, Costa JAV (2009). Solid-state biosurfactant production by *Aspergillus fumigatus* using agricultural residues as substrate. Quim. Nova. 32:292-295.

Castro AM, Pereira Jr. N (2010). Production, properties and application of cellulases in the hydrolysis of agroindustrial residues. Quim. Nova. 33:181-188.

Chicatto JA, Castamann VA, Helm CV, Avares LBB (2014). Optimization of the production process of enzymatic activity of *Lentinula edodes* (Berk.) Pegler in holocelulases. Nat. Resour. 5:241-255.

Farinas CS, Scarpelini LM, Miranda EA, Bertucci Neto V (2011). Evaluation of operational parameters on the precipitation of endoglucanase and xylanase produced by solid state fermentation of *Aspergillus niger*. Braz. J. Chem. Eng. 28:17-26.

Favela-Torres E, Córdova-Lopes J, García-Rivero M, Gutierrez-Rojas M (1998). Kinetics of growth of *Aspergillus niger* during submerged, agar surface and solid state fermentation. Process Biochem. 33:103-107.

Hanlin RT (1996). Gêneros Ilustrados de Ascomicetos. Imprensa da Universidade Federal Rural de Pernambuco, Recife.

Jeya M, Zhang YW, Kim IW, Lee JK (2009). Enhanced saccharification of alkali-treated rice straw by cellulase from trametes hirsuta and statistical optimization of hydrolysis conditions by RSM. Bioresour. Technol. 100:5155-5161.

Juhász T, Szengyel Z, Réczey K, Siika-Aho M, Viikari L (2005). Characterization of cellulases and hemicellulases produced by *Trichoderma reesei* on various carbon sources. Process Biochem. 40:3519-3525.

Kheng PP, Omar IC (2005). Xylanase production by a local fungal isolate, *Aspergillus niger* USM AI 1 via solid state fermentation using palm kernel cake (PKC) as substrate. Songklanakarin J. Sci. Technol. 27:325-336.

Levin L, Herrmann C, Papinutti VL (2008). Optimization of lignocellulolytic enzyme production by the white-rot fungus *Trametes trogii* in solid-state fermentation using response surface methodology. Biochem. Eng. J. 39:207-214.

Maciel GM (2006). Desenvolvimento de bioprocesso para produção de xilanases por fermentação no estado sólido utilizando bagaço de cana de açúcar e farelo de soja. Master of Science Thesis, Universidade Federal do Paraná, Curitiba.

Márquez-Araque AT, Martínez GDM, Muñoz SSG, Dios SEB, Corral OL (2007). Actividad fribolítica de enzimas producidas por *Trametes* sp. EUM1, *Pleurotus ostreatus* IE8 y *Aspergillus niger* AD96.4 en fermentación sólida. Interciencia 32:780-785.

Martins LF, Kolling D, Camassola M, Dillon AJP, Ramos LP (2008). Comparison of *Penicillium echinulatum* and *Trichoderma reesei* cellulases in relation to their activity against various cellulosic substrates. Bioresour. Technol. 99:1417-1424.

Martins M, Pacheco AM, Lucas ANS, Andrello AC, Appoloni CR, Xavier JJM (2012). Brazil nuts: determination of natural elements and aflatoxin. Acta Amaz. 42:157-164.

Miller GL (1959) Use of dinitrosalicilic acid reagent for determination of reducing sugar. Anal. Chem. 31:426-428.

Montgomery DC (1997). Design and Analysis of Experiments. John Wiley, New York.

Mrudula S, Murugammal R (2011). Production of cellulase by *Aspergillus niger* under submerged and solid state fermentation using coir waste as a substrate. Braz. J. Microbiol. 42:1119-1127.

Mswaka AY, Magan N (1998). Wood degradation, and cellulase and ligninase production, by *Trametes* and other wood-inhabiting Basidiomycetes from indigenous forests of Zimbabwe. Mycol. Res. 102:1399-1404.

Nascimento RP, Junior NA, Pereira Junior N, Bon EPS, Coelho RRR (2009). Brewer's spent grain and steep liquor as substrates for cellulolytic enzymes production by *Streptomyces malaysiensis*. Lett. Appl. Microbiol. 48:529-535.

Ögel ZB, Yarangümeli K, Dü H, Ifrij I (2001). Submerged cultivation of *Scytalidium thermophilum* on complex lignocellulosic biomass for endoglucanase production. Enzyme Microb. Tech. 28(7):689-695.

Pacheco MA, Scussel MV (2006). Castanha-do-Brasil, da Floresta Tropical ao Consumidor. Editorgraf, Florianópolis.

Pandey A, Soccol CR, Mitchell D (2000). New developments in solid state fermentation: I-bioprocesses and products. Process Biochem. 35:1153-1169.

Pal A, Khanum F (2010). Production and extraction optimization of xylanase from *Aspergillus niger* DFR-5 through solid-state-fermentation. Bioresour. Technol. 101:7563-7569.

Prado FC, Vandenberghe LPS, Woiciechowski AL, Rodrígues-León JA, Soccol CR (2005). Citric acid production by solid-state fermentation on a semi-pilot scale using different percentages of treated cassava bagasse. Braz. J. Chem. Eng. 22:547-555.

Raimbault M (1998). General and microbiological aspects of solid

substrate fermentation. Electron. J. Biotechnol. 1(3):26-27.
Rajendran A, Thangavelu V (2009). Statistical experimental design for evaluation of medium components for lipase production by *Rhizopus arrhizus* MTCC 2233. Food Sci. Technol. 42:985-992.
Rodrigues IM, Iemma AF (2005). Planejamento de experimentos e otimização de processos. Casa do Pão, Campinas.
Santos TC, Cavalcanti IS, Bonomo RCF, Santana NB, Franco M (2011). Optimization of productions of cellulolytic enzymes by *Aspergillus niger* using residue of mango a substrate. Cien. Rural. 41:2210-2216.
Silva AO, Carmona CE (2008) Production and characterization of cellulase-free xylanase from *Trichoderma inhamatum*. Appl. Biochem. Biotechnol. 150:117-125.
Silva R, Lago ES, Merheb CW, Macchione MM, Park YK, Gomes E (2005). Production of xylanase and CMCase on solid state fermentation in different residues by *Thermoascus aurantiacus miehe*. Braz. J. Microbiol. 36:235-241.
Singh R, Kumar R, Bishnoi K, Bishnoi NR (2009). Optimization of synergistic parameters for thermostable cellulase activity of *Aspergillus heteromorphus* using response surface methodology. Biochem. Eng. J. 48:28-35.
Soccol RS, Vandenberghe LPS (2003). Overview of applied solid-state fermentation in Brazil. Biochem. Eng. J. 13:205-218.
Soni R, Nazir A, Chadha BS (2010). Optimization of cellulase production by a versatile *Aspergillus fumigatus* Fresenius strain (AMA) capable of efficient deinking and enzymatic hydrolysis of Solka floc and bagasse. Ind. Crop. Prod. 31:277-283.
Souza PM, Magalhães PO (2010). Application of microbial α-amylase in industry - a review. Braz. J. Microbiol. 41:850-861.
Tanaka H, Itakura S, Enoki A (1999). Hydroxyl radical generation by an extracellularlow-molecular-weight substance and phenol oxidase activity during wood degradation by the white-rot Basidiomycete *Trametes versicolor*. J. Biotechnol. 75:57-70.
Teixeira MFS (1994). Obtenção de espécies de *Arpergillus* e *Penicillium* termofílicas e termotolerantes na Amazônia e caracterização de suas enzimas de interesse na indústria de alimentos. Master of Science Thesis, Instituto Nacional de Pesquisas da Amazônia, Manaus, Brazil.
Torrado AM, Cortés S, Salgado JM, Max B, Rodríguez N, Bibbins BP, Converti A, Domínguez JM (2011). Citric acid production from orange peel wastes by solid-state fermentation. Braz. J. Microbiol. 42:394-409.
Valášková V, Baldrian P (2006). Estimation of bound and free fractions of lignocellulose-degrading enzymes of wood-rotting fungi *Pleurotus ostreatus*, *Trametes versicolor* and *Piptoporus betulinus*. Res. Microbiol. 157:119-124.
Vandenberghe LPS, Soccol CR, Pandey A, Lebeault JM (2000). Solid state fermentation for the synthesis of citric acid by *Aspergillus niger*. Biores. Technol. 74:175-178.
Vu VH, Pham TA, Kim K (2011). Improvement of fungal cellulase production by mutation and optimization of solid state fermentation. Mycobiol. 39:20-25.
Viikari L, Kantelinen A, Sundqvist J, Linko M (1994). Xylanases in bleaching: from an idea to the industry, FEMS Microbiol. Rev. 13:335-350.
Zhou X, Chen H, Li Z (2004). CMCase activity assay as a method for cellulase adsorption analysis. Enzyme Microb. Technol. 35:455-459.