Experimental design for optimization of *β*-Xylosidase production by A. *fumigatus* isolated from the Atlantic Forest (Brazil).

Abstract

The production of *β*-Xylosidase by a new strain of *Aspergillus fumigatus* (PC-7S-2 M), isolated from the Brazilian Atlantic Forest, was analyzed at 28° C using modified Czapeck media supplemented with different agroindustrial residues at 1% (w/v). Conidia were inoculated for 7 days, and the best activity for *β*- Xylosidase was obtained in the presence of barley brewing residue after 4 days (15 U mL^{-1}). To optimize the production of *β*-Xylosidase, this carbon source was used for a central composite rotational design (CCRD) to obtain a significance level of $p < 0.10$, which predicted an enzyme activity of 245.04 U mL^{-1}. The model validation revealed *β*-Xylosidase activity was optimized at 229.06 U mL^{-1}. Furthermore, the production of intracellular *A. fumigatus β*-Xylosidase increased by 1,500% (15 times) over that initially obtained, achieving 93.47% of the predicted model. This finding emphasizes the availability of *A. fumigatus β*-Xylosidase production with possible applications in biotechnological processes.

Keywords: barley brewing residue; experimental design; agroindustrial residue; hemicellulose; *β*-Xylosidase, *Aspergillus fumigatus*.

Introduction

The xylan molecule is one of the most important hemicellulose components in plant cell walls. It is present in agricultural residues and is degraded by synergistic action of several enzymes, mainly by endo-Xylanases (EC 3.2.1.8) and β-Xylosidases (EC 3.2.1.37) (Liu et al. 2008). The endo-Xylanases cleave the main chain of xylan and release smaller sugars, such as xylobiose, which is degraded to xylose by β-Xylosidases (Teng et al. 2011). These enzymes are well characterized by different biochemical and molecular approaches and can be used in industrial processes to degrade the hemicellulose present in agricultural waste. Therefore, these enzymes have great applicability in different textiles, food industries, such as juices and animal feed, and the production of cellulosic ethanol (Lasrado and Gudipati, 2013).

In view of the fact that the variability in the production of various enzyme systems fungi are usually capable of degrading contaminants from soil and water at low concentrations; they are excellent degraders in the absence of nutrients, such as in low nitrogen conditions found in polluted sites (Oliveira et al. 2011). Additionally, fungi are able to extract energy from organic compounds and carbon found in wastewater and agro-industrial residues, such as corn stover, rice husks, and coconut fiber residues from the brewing industry, which are generated in abundance in Brazil. The global need to decreases agricultural waste contamination to the environment has stimulated investment in innovative processes to obtain these enzymes from waste yeast in recent decades (Benassi et al. 2012).

Aspergillus species have been widely studied in response to its wide distribution in different regions on the planet. The species of this genus are known to be great at degrading soil contaminants by producing higher levels of secreted proteins. They present a viable maintenance cost and their genetic properties are better characterized than several available mutant strains (Wongwisansri et al. 2013). *Aspergillus fumigatus* is specifically used in various biotechnological processes, such as the production of antibiotics and enzymes, and the biodegradation of toxic compounds, such as dyes from textile environments, thereby providing an alternative to the treatment of effluent while producing fewer by-products (Jin et al. 2007). The microorganism presents asexual spores, is saprophyte and mesophilic and is found in different environments, e.g., air, land and marine ecosystems. Furthermore, they can be cultured at temperatures from 28 °C to 40 °C (Saqib et al. 2010).

The application of experimental design techniques is a very useful tool to optimize the process the production of enzymes that are of biotechnological interest, while minimizing costs and maximizing yield, productivity and quality.

This statistical tool is known to determine and quantify the influence of variables on the desired responses (Sena et al. 2011).

In the present work, we analyzed and optimized the production of intracellular β-Xylosidase from a new strain of *A. fumigatus* (PC-7S-2 M), isolated specifically from the Atlantic Forest of West Paraná State, Brazil, in an area under environmental protection named Dog Head State Park, which presently features a great biodiversity of microorganisms that have been little explored. The production of β-Xylosidase was analyzed using different agro-industrial residues as carbon sources for growth of the fungus, and the best conditions for inducing enzyme activity were analyzed by applying experimental design techniques and statistical approaches to maximize optimal production and induction of fungal β-Xylosidase activity using different combinations of variables.

Materials and Methods

Description of the collection site of the fungus

A. fumigatus (PC-7S-2 M) was isolated during collections in the Dog Head State Park, Paraná, Brazil. The park has a total area of 60.98 hectares and is located in the south central portion of the municipality of São Pedro do Iguaçu, under the coordinates 24 54 '47' latitude (south) and 53 54 '35' longitude (west). The unit of conservation receives this name because the area is shaped similar to a dog's head from aerial views of the area by camera and satellite. The park also has a subtropical climate with hot summers, infrequent frosts and concentrated rainfall trends in the summer months. The formation of the vegetation is semideciduous forest submontane, and its surroundings are predominantly composed of forest and agricultural areas, which are considered important remnants from the development for the entire western region of Paraná.

Identification and maintenance of the fungal species

A. fumigatus (PC-7S-2 M) was first identified by morphological taxonomists by the Federal University of Pernambuco, Brazil. However, identification of the isolate at genus and species levels was confirmed by nucleotide sequence analysis of the ITS region corresponding to the rRNA of the microorganism in the Laboratory of Molecular Biochemistry of UNIOESTE by extracting the total DNA from the fungus followed by DNA replication using an amplicon target for sequencing (HELIXXA-DNA Sequencing Service, Brazil). The obtained sequence was analyzed by the algorithm Blast-X from the National Center for Biotechnology Information (NCBI). The sequence was deposited in GenBank under accession number *KM 349230.*

A. fumigatus (PC-7S-2M) conidia were maintained in test tubes containing 5 mL of solid medium potato dextrose agar (PDA), composed of 1.5% (w/v) glucose, 2.0% (w/v) agar and 20% (v/v) potato juice. Thse samples were cultured and grown at 28 °C for 7 days, and after its growth, the microorganism was kept and stored at 4 °C for 30 days. The isolate was deposited in the fungal collection of the Laboratory of Biochemistry (UNIOESTE, Cascavel).

Preparation of by-products and residues from agriculture

The carbon sources tested in these trials for fungal growth and induction of enzyme activity were randomly selected: barley brewing residue, wheat bran, passion fruit peel, pokan peel and ripe banana peel. The residues used in the tests were prepared starting with oven drying at 70 °C for 24 hours followed by crushing using a mill (SL30 slicer - SOLOLAB) and a sieve. The shredded waste was sieved using four screens ranging from 12 to 48 mesh and subsequently stored in clear glasses at room temperature.

Culture conditions of A. fumigatus

Conidia of the fungus *A. fumigatus* (PC-7S-2M) were maintained on PDA medium at 4 °C and, after the fresh state, were used for the preparation of 1 mL suspensions (1 x 10^5 conidia mL^{-1}) using sterile distilled water and were inoculated in 25 mL of modified mineral Czapeck medium (0.3 g $NaNO_3$, KH_2PO_4 0.1 g; $MgSO_4.7H_20$ 0.05 g, KCl 0.05 g, 0.001 g FeSO4.$7H_20$; 0.1 mg casein at pH 6.0), separately supplemented with 1% (w/v) of the different agricultural residues, such as passion fruit peel, pokan peel, barley brewing residue, soya flakes and ripe banana peel. The cultivations were carried out in two ways: stationary liquid and agitated liquid, maintained at 28 °C for 10 days in duplicates. After this period, cultures were vacuum filtered on sterile Whatman paper, and the frozen mycelium was macerated using glass beads in 5 mL of distilled water and centrifuged at 4 °C 8,000 x g for 5 minutes. The supernatant was used to determine the amount of β-Xylosidase and the optimum pH and temperature.

Activity assay for β-Xylosidase

Fifty µl of the enzyme extract was added to 250 µL of the synthetic substrate *p*-nitrophenyl β-D-xylopyranoside (pNPX) (Sigma-Aldrich\) and incubated in a water bath at 50 °C for 10 minutes. The reaction was stopped by adding 1 mL of saturated solution of sodium tetraborate, and the product of the reaction was read by a spectrophotometer at 410 nm. Enzyme activity values were calculated using the formula obtained from the standard calibration curve using *p*-nitrophenol, 1 mg mL^{-1}, and the results were expressed in mmol mL^{-1}. One unit of enzyme activity was defined as being able to release 1 micromole of *p*-nitrophenol per milliliter of reaction (Simão et al. 1997).

Influence of time on A. fumigatus β-Xylosidase production

A. fumigatus (PC-7S-2 M) was grown in culture medium containing modified Czapeck medium with the different tested residues. We selected the best carbon source for the production of β-Xylosidase. Furthermore, standardized stationary liquid culture was the most efficient for production of the enzyme. Therefore, the fungus was inoculated in liquid phase in the presence of the agroindustrial residue that operated as the best inducer of β-Xylosidase activity for a period of 7 days, and every day, two vials were withdrawn. The samples were taken, filtered and submitted

to enzymatic assays. We measured the intracellular β-Xylosidase and determined the best day of production of the protein. All assays were conducted in duplicate, and measurements were performed in triplicate.

Influence of pH and temperature on the activity of A. fumigatus β-Xylosidase.

β-Xylosidase was also measured under different values of pH (5 to 7.5) using McIlvaine solution buffer (McIlvaine, 1921). To analyze the effect of temperature on the enzymes activity in crude extracts, samples were incubated at temperatures from 35 °C to 60 °C, with 5 degree increments. The enzymatic reaction was performed according to the optimal assay conditions. The experiments were performed in duplicate and measurements in triplicate.

Experimental design

From the experiments highlighted above, it was possible to obtain the optimal conditions for the production of *A. fumigatus* intracellular β-Xylosidase (PC-7S-2 M). Therefore, we determined the variables that had the greatest influence on the performance of the fungus to produce β-Xylosidase, enabling a reduction in process variation and obtaining better agreement between the nominal values and the desired values, which reduces the process time, reduces operating costs, improves process yield and utilizes industrial waste, which is widely produced in large quantities in Brazil by the brewing industry.

The factorial experimental design CCRD was conducted to determine the optimal conditions for β-Xylosidase production from the fungal isolated from the selected residue, barley brewing residue (Saini et al. 2013). The combination of the variables was determined using two levels, three factors, an additional six axial points and center point repetition, totaling 17 samples. The main effects of variables on enzyme production, their interactions, and analysis of variance (ANOVA) with a significance level of 10% were calculated. The CCRD experimental design and response surface methodology were used for optimizing the conditions and providing a suitable mathematical model specifically for the activity of β-Xylosidase in this process (Sukhbaatar et al. 2014), aiming to maximize the production of the enzyme and progressively facilitate the selection of variables, such as temperature, days of incubation, agitation, and the concentration of the residual, to increase the production of the desired enzyme.

The First Experimental design: Central Composite Design (CCD)

The central composite design was characterized by an experiment with a central point and run in duplicates, giving an estimate for the pure error, and two levels of axial points and factors that determined the quadratic terms (Walia et al. 2013). This design was adjusted to the working conditions of the laboratory to obtain the main effects of the studied factors. The levels used for each factor are presented in Table 1. The coded and actual values of the factors are shown in Table 2, where testing was performed with 11 different combinations. The statistical analysis was performed to obtain linear effects, and a model has been adjusted according to Eq. 1 below to describe the response surface.

$$\hat{y} = b_0 + \sum_{i=1}^{3} b_i X_i + \sum_{i,j=1}^{3} b_{ij} X_i X_j \qquad \text{(Eq. 1)}$$

Path of Maximum Slope

After obtaining the results from the first design, 8 combinations were employed to figure out the greatest points for the factors using the barley brewing residue and nitrogen source yeast extract. Once a mesophilic microorganism was isolated, the temperature of the experiment was set at 28 °C, as shown in Table 3, and the experimental approach was determined based on which strategies would be improving the dependent variables and responses. The test showed that the best response for the production of β-Xylosidase was selected as the central point of the second event, when the CCRD was prepared. The tests with the combinations described were used for establishing the relationships between the response variables and the independent variables because these were not known and needed to be determined to allow analysis of the data if these are far from optimal points. Such tests were performed as punctual goals to enable the alignment of points and finally to select the optimum point (Córdoba et al. 2012).

Second Experimental Design: CCRD

The design was based on three factors: concentration of residue (barley brewing residue) as a carbon source; temperature; and concentration of yeast extract (nitrogen source). The microorganisms were cultivated for 4 days at 28 °C in stationary liquid. The experimental matrix consisted of three central points and six axial points, and the two factors, combined with three levels, totaled 17 trials. We selected the combination of 0.750 g of barley brewing residue with 0.097 g of yeast extract as a central point because the test showed the highest production of β-Xylosidase from the combinations shown in Table 3. Table 4 shows factor levels of the second event. In Table 5, the coded values and the actual values of the planning conducted with the three central points and six axial points are presented, totaling 17 tests.

208 *Optimization Process*

209 Optimization is performed to identify the setting that best determines both sets of response variables (Córdoba et
210 al. 2012). The optimal setting corresponds to the setting that minimizes the multivariate quadratic loss function, i.e., the
211 optimal setting incurs the lowest overall loss of all related factors. Therefore, process validation was performed until the
212 preparation of six samples equaled the optimal conditions defined by the experimental designs carried out according to
213 Table 5.

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215 *Validation of the Model*

216 The statistical analysis was performed to determine which coefficients were significant and adjust a second
217 order model (Eq. 2) to correlate the variables with their responses ($p < 0.1$).

218 $$\hat{y} = b_0 + \sum_{i=1}^{3} b_i X_i + \sum_{i=1}^{3} b_{ii} X_i^2 + \sum_{i,j=1}^{3} b_{ij} X_i X_j \quad \text{(Eq. 2)}$$

219 The significance of the coefficients of the model was evaluated using "t", where b_0, b_i, b_{ii}, b_{ij} are the regression
220 coefficients of the model, and X_i and X_j are the independent variables in the coded values test.

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Results and Discussion

The influence of different concentrations of waste and by-products in the agro-industry on the production of β-Xylosidase by the isolated fungus *A. fumigatus* (PC-7S-2 M) was observed in Fig. 1a, which shows the results of incubating for 10 days under constant agitation or in stationary liquid at 28 °C. The barley brewing residue was the most efficient at producing β-Xylosidase under stirring conditions compared to the other carbon sources; the differences between these residues were very constrained. However, the residue from the brewing industry was very efficient in inducing improved production of β-Xylosidase between different carbon sources tested under the conditions of stationary liquid culture, providing a maximum enzyme activity of 15.45 U mL^{-1}.

Michelin et al. (2012) conducted experiments on the induction of β-Xylosidase activity of the fungi *A. ochraceus* and *A. terricola*, and enzymatic activity levels of 30 U mL^{-1} and 56 U mL^{-1}, respectively, were obtained in the presence of another agroresidue that is very abundant in North America: corn cob. Therefore, induction of the activity of β-Xylosidase by lignocellulosic-rich waste material in species of the genus *Aspergillus* has been reported in the literature. However, to our knowledge, this is the first time that barley brewing residue has been used for induction of intracellular β-Xylosidase from a species of the genus *Aspergillus.*

The levels of β-Xylosidase produced by the fungus *A. fumigatus* (CP-2 M-7S) during incubation in the presence of barley brewing residue1% (w/v) at 28 °C in stationary liquid culture were more than 2.5 times higher (41.20 U mL^{-1}) on the fourth day at a steady temperature of 28 °C, than that previously observed after 10 days in liquid culture, showing a positive influence of lower incubation time on enzyme activity (Fig. 1b).

According to Benassi et al. (2012), incubation of *A. phoenics* with different wastes, such as corn flakes, cassava flour and rye flake, resulted in β-Xylosidase production values of 0.36 U ml^{-1}, 0.66 U mL^{-1} and 7.60 U mL^{-1}, respectively. These values are bellow the values obtained by the non-optimized experiments that we present in Fig. 1a and b in the present report. To our knowledge, this work is also the first to use statistical approaches to optimize the production of β-Xylosidase by varying barley brewing residue conditions in a species of *Aspergillus*.

The effects of pH and temperature on enzymatic activity were also analyzed for intracellular β-Xylosidase using the crude enzyme extract obtained after culturing for 4 days in stationary and liquid cultures in the presence of barley brewing residue (1% w/v) at 28 °C. In these experiments, changes in enzyme activity with pH values were analyzed using McIlvaine (1921) buffer in a pH range from 5 to 7.5, and the samples were incubated in a temperature range from 35 °C to 60 °C (5 degree intervals) with the synthetic substrate pNPX, as described in the Materials and Methods, to analyze the effect of temperature on β-Xylosidase activity. Our data showed that after 4 days of cultivation under the above conditions, β-Xylosidase and *A. fumigatus* showed optimal activity at pH 5.5 and 45 °C.

Omardien (2013) conducted experiments to analyze the activity of β-Xylosidase with *Aerobasidium pullulans* (isolate 23B25) by testing several residues, such as wheat bran and wheat bran mixed with rye, and the enzyme activity was 0.22 U mL^{-1} after 5 days of growth for the mixture with rye bran, showing an optimum pH at 3 and an optimum temperature range of 80 to 90 °C. However, comparing the data obtained in the present work with that carried out by Omardien, we obtained higher production of β-Xylosidase (41.20 U mL^{-1}) using barley brewing residue in just 4 days and an optimum pH and temperature of 5.5 and 45 °C, respectively (data not shown). Knob and Carmona (2009) studied the induction of β-Xylosidase by the fungus *Penicillium sclerotiorum* cultured in medium supplemented with waste; after five days of cultivation at 28 °C under agitation, enzyme production was 1.47 U mL^{-1}, 0.24 U mL^{-1} and 0.05 U mL^{-1}, in the presence of bran, oat bran and corn cobs, respectively, applying 0.01 citrus pectin as the carbon source. In these assays, the optimal temperature for activity was 60 °C and the optimum pH equal to 2.5.

The coefficient of linear correlation is observed between -1 and +1 levels, allowing quantification and determination of the direction of the relationship, whereas the extreme values -1 and +1 refer to perfect, negative and positive linear correlations, respectively. Intermediate coefficient values indicate weak relationships, coefficient close to the extreme values represents a strong relationship, and coefficient near or equal to zero indicates the absence of a linear (Rodrigues and Iemma, 2014) correlation. Therefore, the best culture medium was investigated by means of DCC linear effects. The temperature had a negative effect, indicating that increasing cultivation from temperatures of 28 °C (-1) to 42 °C (+1) reduced the production of β-Xylosidase by the fungus *A. fumigatus* by 28 U mL^{-1} (Fig. 2). In contrast barley brewing residue showed a positive effect of 21 U mL^{-1} when larger quantities were added, changing from 0.125 g (-1) to 0.375 g (+1). There was an increase of 9.9 U mL^{-1} in the production of β-Xylosidase when the concentration of yeast extract was increased from 0.025 g (-1) to 0.080 g (+1). We observed that the addition of yeast extract influenced the production of the enzyme, which is an important source of nitrogen and other nutrients added to the culture medium; however, when yeast extract is added in large quantities, it is possible to statistically observe inhibition in the production

of β-Xylosidase. Therefore, it is necessary to establish optimal quantities for optimal enzyme levels when combined with crushed barley.

Using yeast extract as a nitrogen source, Vaithanomsat and colleagues (Vaithanomsatet al. 2011) optimized the fermentation conditions using *A. niger* for the production of β-glucosidase using the response surface methodology in medium consisting of 0.275% (w/v) yeast extract, 1.125% (w/v) cellobiose and 2.6% (w/v) ammonium sulfate, with a pH of 3, resulting in a specific activity of 8.99 U mg^{-1}.

In the present work, analysis of variables has produced a first order mathematical model, and the summary from ANOVA for the enzymatic activity with significant terms at 10% probability ($p < 0.10$) is presented in Table 6. The coefficient of determination (R2) is equal to 0.68, and the F test showed that the model is adequate at predicting the results using a response surface. The model is presented in Eq. 3 and *B* represents barley brewing residue, *T is* temperature and *E* is yeast extract:

$$\hat{\beta} = 17{,}96 + 10{,}53B - 13{,}87T + 4{,}95E \quad \text{(Eq. 3)}$$

The surfaces from the response model (Eq. 3), shown in Fig. 3, show the relationship between temperature, yeast extract and barley brewing residue. It was observed that the lower the temperature used in combination with a greater amount of crushed barley, the greater was the activity of β-Xylosidase from *A. fumigatus* (40 U mL^{-1}) (Fig. 3a). However, when the two factors were provided at steady increases, activities above 40 U mL^{-1} were observed for β-Xylosidase (Fig. 3b).

From the standpoint of utilizing agro-industrial waste, which may be cumulative in nature, it is advantageous to optimize a process in which a larger amount of residue and less yeast extract are used for the industrial process of enzymatic synthesis because it is less costly and beneficial to the environment. Lastly, when comparing yeast extract with temperature, gradually increasing the amount of yeast extract combined with a lower temperature, approximately 28 ° C, led to larger quantities of β-Xylosidase 40 U mL^{-1} (Fig. 3c).

The production of β-Xylosidase by *Colletotrichum graminicola* when in contact with wheat bran was 57.9 U ml^{-1} when grown at 65 °C for 72 hours with an optimum pH of 5 (Zimbardi et al. 2013). Supplementing the medium with 1% peanut shell led to an increase in enzyme production, up to 79 U mL^{-1}. With the application of experimental design, the fungus grown in medium containing the residue peanut hulls optimized production to 126 U mL^{-1}, whereas temperature had no significant effect when compared with other independent variables, such as the concentration of the residual, time for cultivation and initial moisture content.

Compared to classical studies (not CCRD) performed with xylan-degrading enzymes and cellulolytic fungus with *A. fumigatus* by Sherief and colleagues (Sherief et al. 2010) in solid state fermentation using mixed substrates containing rice straw with wheat bran in different proportions, high enzyme production was observed after 4 days of incubation in 75% humidity, an initial pH of 5-6, at 40 °C, and in the presence of $NaNO_3$ as the nitrogen source, yielding activities of 8.51 and 42.7 IU g^{-1} for -Xylanase and β-glycosidase, respectively.

Regarding the data presented here, given that the optimum point could not be found for the enzymatic activity of β-Xylosidase from *A. fumigatus* (CP-2 M-7S), additional tests were performed combining the maximum slope of the surfaces, generating new data to set a point for maximum enzyme production. The results of the tests from the parameters in Table 3 are shown in Fig. 4. The combinations in test 4 showed the best results, with 0.750 g of barley brewing residue and 0.097 g of yeast extract in 25 mL of modified Czapeck medium, generating enzymatic activity equal to 154.01 U mL^{-1}. Therefore, this combination was adopted as a central point for the second experimental design, entitled CCRD.

Table 7 presents the planning array for 2^3 levels and the values of each variable used at each level with the yield response of β-Xylosidase from *A. fumigatus* (PC-7S-2M), noting that the enzyme production varied from 150.86 to 252.26 U mL^{-1}. According to Abdeshahian and colleagues (Abdeshahian et al. 2010), the maximum production of β-Xylosidase was 6.13 U mL^{-1} when applied to an experimental design using *A. niger* grown on palm kernel cake as the substrate. Lenartovicz and co-authors (Lenartovicz et al. 2003) used corn cob powder as the carbon source for culturing another strain of *A. fumigatus* and obtained 45 U mL^{-1} extracellular β-Xylosidase at a temperature of 42 °C for 72 hours.

Interesting data were shown by Gottschalk et al. (2013) evaluating the production of β-Xylosidase by the fungus *A. awamori* in submerged culture at 30 °C with agitation at 200 rpm in medium supplemented with 30 g mL^{-1} of wheat bran in a pH range between 5.5 to 6.5. They used different nitrogen sources, such as yeast extract, sodium nitrate, ammonium sulfate and urea, to give a high yield of the enzyme in media containing 685 U mL^{-1} of urea at 144 h of culture, and used less yeast extract in 210 U mL^{-1} at 168 h.

In addition to the above analyses, there were no CCRD studies that showed the production of β-Xylosidase by *A. fumigatus* using barley brewing residue. Additionally, these variables are differentiated, the enzyme production was also optimized by applying a CCRD to obtain high levels of the enzyme for preliminary statistical tests and experimental activity. Table 8 shows the ANOVA (analysis of variance) model that describes the variables studied, i.e., the production of β-Xylosidase as a function of barley brewing residue, temperature and yeast extract, with a significance level of $p < 0.10$, indicating that it was possible to obtain a predictive model.

$$\hat{\beta} = 242{,}88 - 14{,}07B^2 - 7{,}61T - 13{,}69T^2 - 6{,}24E - 25{,}02E^2 - 10{,}37BE \quad \text{(Eq. 4)}$$

ANOVA was used for assessing the significance of the model fit. The coefficient of determination (R2) was 0.74, which means that the biological process studied was considered predictive because predicted variables from barley brewing residue, temperature and yeast extract corresponded to the production of β-Xylosidase. The model with significant terms is shown in Eq. 4; the F calculated from the regression (4.90) was greater than the tabulated F (2.46), which proved that the quadratic model was significant for the selected experiment. The lack of fit of the model was determined from the calculated F (8.26) and tabulated F (9.37); the calculated F value is less than the tabulated F value, which represents that there is no lack of fit in the mathematical model.

The surfaces represent the responses generated by the mathematical model, which enables verification of the combinations of the three factors analyzed in the experiment, displaying the influence of each and the maximal activity of the β-Xylosidase enzyme (Fig. 5). The conditions for increased production of β-Xylosidase are obtained at concentrations that are close to the central point condition for the three factors; however, the surfaces have regions of contour curves, which indicate that there are slightly lower values for the center point and that the three variables lead to the same responses.

It is therefore an important step to validate the data to confirm that they were obtained in this graphical representation. This process assists with obtaining the ideal conditions, enabling the formulation of the composition of the medium that leads to the maximum production of β-Xylosidase by the fungus while minimizing the final cost of the process because it uses a small number of trials without losing quality information about the results. Fig. 6 shows the regression model that illustrates the independent variables and the interactive effects from the graphical response surfaces.

From Fig. 6 (a), barley brewing residue shows better interaction with temperature, with the optimal point located in the temperature range from 24 °C to 28 °C, combined with 0.725 g of barley brewing residue, and the desirability of production of β-Xylosidase is greater than 240 U mL^{-1}. As shown in Fig. 6 (b), the optimal conditions of yeast extract and barley brewing residue would be 0.097 g and 0.725 g, respectively, to obtain values greater than 200 U mL^{-1} of enzyme. From Fig. 6 (c), the optimum temperature range is from 24 °C to 28 °C with 0.097 g of yeast extract, enabling enzyme production greater than 200 U mL^{-1}.

The trend of the results in this report was proven to be statistically significant because the calculated F values were higher than the tabulated F values (Table 6). It was certainly possible to prove that the proposed model is valid, generating first and second order mathematical models (Eqs. 3 and 4, respectively) that were represented by surface graphs (Fig. 3 and 5).

After determining the conditions that maximize the production of the enzyme of interest, it is also necessary to analyze the behavior of individual answers to ensure that all of them are acceptable in all regions with satisfied constraints. Therefore, it is necessary to perform confirmatory experiments on the selected optimal conditions.

The optimum conditions for the production of β-Xylosidase presented on the desirability chart allow you to select the optimal points for the production of this enzyme, in which the combination between the analyzed factors enables the formulation of medium and cultivation conditions with points greatly under appropriate conditions that lead to maximum production of the protein of interest (Rodrigues and Iemma, 2014). There is an optimum range for barley brewing residue, which is greater than the range of values for yeast extract temperature. The data show that increasing minimal amounts of yeast extract leads to rapid decline in enzyme production. Additionally, the combination of barley brewing residue and temperature are lower in sensitivity, leading to a more slow decrease in the production of β-Xylosidase.

Therefore, adding a greater amount of barley brewing residue (0.730 g) to the medium and growing *A. fumigatus* at the optimal temperature of 27 °C with 0.100 g of yeast extract are the most favorable conditions for obtaining higher amounts of β-Xylosidase. Therefore, changing the amount of yeast extract from 0.100 g to 0.125 g led to a sharp decrease in enzyme production.

The conditions set in the experiment were validated, with up to 6 replications of dosages and performing duplicates of the following conditions: barley brewing residue 0.730 g, 27 °C and 0.100 g of yeast extract. The cultures were incubated for 4 days in liquid medium without agitation, as proposed initially, and then doses of β-Xylosidase were added according to established protocols in the Materials and Methods.

The validation data showed an experimental result with average production of β-Xylosidase of 229.06 U mL^{-1}, very close to the result predicted by the model (245.04 U mL^{-1}). Therefore, the experimental design was extremely important for improving the enzymatic activity of the isolated fungal *A. fumigatus* (PC-7S-2M) in the presence of barley brewing residue. Initially, classic growth and cultivation without application of experimental design obtained activity of 15 U mL^{-1} for β-Xylosidase from this microorganism. After applying the experimental design and the variable conditions discussed previously, it was possible to reduce spending on materials, reagents, and time of experiment, while obtaining an average increase in the activity of β-Xylosidase, up to 229.06 U mL^{-1}. The production of the enzyme of interest has been improved by 1,500% (15 times). To our knowledge, this is the first record of such significant enzyme activities for β-Xylosidase from *A. fumigatus* using barley brewing residue as a carbon source.

The discovery of new fungal strains capable of producing enzymes can add scientific knowledge to the development of sustainable and environmental technologies. Here, we have shown that the A. fumigatus strain (PC-7S-

2M) was new and isolated from an environment whose biodiversity is unexplored, which comprises western Paraná. The results presented here provide a positive contribution to the survey of microorganism biodiversity. Moreover, we have indicated that the produced enzyme could be tested for biotechnological purposes because it is abundantly produced at high levels from the residue of a brewery in Brazil, which decreases its accumulation in the environment.

Conclusions

In this study, the best substrate for high β-Xylosidase activity among the tests was barley brewing residue, which has not been previously used for this enzyme in the *Aspergillus* genus. This crude enzyme extract showed an optimum temperature of 45 °C and optimum pH of 5.5. The application of experimental design was effective for optimizing the production of β-Xylosidase from a new strain of *A. fumigatus*, leading to production of high enzyme levels under the optimum conditions, increasing the initial production of the enzyme from 15 U mL^{-1} to 229.06 U mL^{-1}. This result corresponds to a 1,500% (15 times) improvement in the efficiency of enzymatic activity. The application allowed for the CCRD to achieve 93.47% of the predicted model. Therefore, β-Xylosidase from *A. fumigatus* (PC-7S-M 2) is an interesting enzyme to test for application in various industrial and biotechnological processes.

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447 **Reference**

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Figure legends

Fig. 1 (a) Intracellular activity of β-Xylosidase from *A. fumigatus* (strain PC-7S-2) was measured after culturing for 10 days at 28°C in 25 ml of modified Czapeck liquid medium under shaking at 120 rpm (black columns) or under steady state (white columns) using 1% (w/v) of the different agroindustrial (PFP: Passion fruit peel; PP: Pokan Peel; BBR: Barley brewing residue; SF: soy flakes and RBP: Ripe banana peel) residues as carbon sources. (b) Measurement of intracellular β-Xylosidase mycelia of *A. fumigatus* (strain PC-7S-2) were grown in stationary liquid culture for 7 days using 1% (w/v) barley brewing residue as the carbon source. The experiments were performed in duplicate, and measurements were taken in triplicate. The error bars represent the means of independent results.

Factors	-1	0	+1
Barley brewing residue (g)	0.125	0.250	0.375
Temperature (°C)	28	35	42

Fig. 2 Comparison of linear effects for the production of β-Xylosidase (absolute values).

Fig. 3 (a) Response surfaces of the production of β-Xylosidase as functions of barley brewing residue and temperature, (b) yeast extract and barley brewing residue and (c) temperature and yeast extract, without the axial points.

Fig. 4 β-Xylosidase activity (U mL^{-1}) in eight different concentrations (g) of the barley brewing residue (BBR) and yeast extract (YE).

Fig. 5 Response surfaces of the production of β-Xylosidase as functions of barley brewing residue and temperature (a); yeast extract and barley brewing residue (b); and, temperature and yeast extract (c).

Fig. 6 Desirable optimal conditions for the production of β-Xylosidase.

Yeast extract (g)	0.025	0.052	0.080

Table 1 Levels used in CCD for the three factors studied.

Table 2 Central composite design (CCD) with the coded and real values for the factors in the study: residue concentration, temperature and concentration of yeast extract

Runs	**Coded values**			**Real values**				
	BBR (g)	**ºC**	**YE (g)**	**BBR (g)**	**%**	**ºC**	**YE (g)**	**%**
1	-1	-1	-1	0.125	0.5	28	0.025	0.10
2	1	-1	-1	0.375	1.5	28	0.025	0.10
3	-1	1	-1	0.125	0.5	42	0.025	0.10
4	1	1	-1	0.375	1.5	42	0.025	0.10
5	-1	-1	1	0.125	0.5	28	0.080	0.32
6	1	-1	1	0.375	1.5	28	0.080	0.32
7	-1	1	1	0.125	0.5	42	0.080	0.32
8	1	1	1	0.375	1.5	42	0.080	0.32
9	0	0	0	0.250	1.0	35	0.052	0.20
10	0	0	0	0.250	1.0	35	0.052	0.20
11	0	0	0	0.250	1.0	35	0.052	0.20

BBR: Barley brewing residue; YE: Yeast extract

576 **Table 3**Maximum inclination
577

Runs	BBR (g)	%	YE (g)	%
1	0.375	1.5	0.064	0.25
2	0.500	2.0	0.075	0.30
3	0.625	2.5	0.086	0.34
4	0.750	3.0	0.097	0.38
5	0.825	3.5	0.108	0.43
6	1.000	4.0	0.119	0.47
7	1.120	4.5	0.130	0.52
8	1.250	5.0	0.141	0.56

578 BBR: Barley brewing residue; YE: Yeast extract
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584

585 **Table 4**- Levels for the factors used in the second design, CCRD.
586

Factors	-1.68	-1	0	1	+1.68
Barley brewing residue (g)	0.515	0.600	0.725	0.850	0.935
Temperature (°C)	22	24	28	32	35
Yeast extract (g)	0.074	0.085	0.097	0.115	0.125

587
588
589

590 **Table 5** - CCRD with the coded and real values given the factors in the study: temperature,barley brewing
591 residue and yeast extract concentration.
592

Runs	Coded values			Real values				
	BBR (g)	°C	YE (g)	BBR (g)%		°C	YE (g) %	
1	-1	-1	-1	0.600	2.4	24	0.085	0.3
2	1	-1	-1	0.850	3.4	24	0.085	0.3
3	-1	1	-1	0.600	2.4	32	0.085	0.3
4	1	1	-1	0.850	3.4	32	0.085	0.3
5	-1	-1	1	0.600	2.4	24	0.115	0.5
6	1	-1	1	0.850	3.4	24	0.115	0.5
7	-1	1	1	0.600	2.4	32	0.115	0.5
8	1	1	1	0.850	3.4	32	0.115	0.5
9	0	0	0	0.725	2.9	28	0.097	0.4
10	0	0	0	0.725	2.9	28	0.097	0.4
11	0	0	0	0.725	2.9	28	0.097	0.4
12	-1.68	0	0	0.515	2.1	28	0.097	0.4

13	+1.68	0	0	0.935	3.7	28	0.097	0.4
14	0	-1.68	0	0.725	2.9	22	0.097	0.4
15	0	+1.68	0	0.725	2.9	35	0.097	0.4
16	0	0	-1.68	0.725	2.9	28	0.074	0.3
17	0	0	+1.68	0.725	2.9	28	0.125	0.5

593 BBR: Barley brewing residue;YE: Yeast extract

594

595 **Table 6 -**Summary of ANOVA of the first order mathematical model for *β*-xylosidase
596

Variation source	SS	DF	MS	*F* Value	*F*Tab
Regression	2,624.442	3	874.81	5.13	3.07
Residual	1,192.580	7	170.37		
Total	**3,817.022**	**10**			

597 SS: sum of squares; DF: degree of freedom, MS: mean square, R^2 =0.68; p-value < 0.10
598

599

600

601 **Table 7** - Planning with 2^3 levels for the factors and the production of *β*-Xylosidase
602

	Levels for the factors					
Runs	**BBR (g)**	**°C**	**%**	**YE (g)**	**%**	***β*-Xylosidase(U ml^{-1})**
1	0.600	2.4	24	0.085	0.3	205.09
2	0.850	3.4	24	0.085	0.3	231.89
3	0.600	2.4	32	0.085	0.3	197.82
4	0.850	3.4	32	0.085	0.3	220.04
5	0.600	2.4	24	0.115	0.5	220.95
6	0.850	3.4	24	0.115	0.5	184.81
7	0.600	2.4	32	0.115	0.5	169.9
8	0.850	3.4	32	0.115	0.5	174.18
9	0.725	2.9	28	0.097	0.4	252.26
10	0.725	2.9	28	0.097	0.4	237.73
11	0.725	2.9	28	0.097	0.4	244.43
12	0.515	2.1	28	0.097	0.4	184.23
13	0.935	3.7	28	0.097	0.4	192.38
14	0.725	2.9	22	0.097	0.4	209.88
15	0.725	2.9	35	0.097	0.4	183.14
16	0.725	2.9	28	0.074	0.3	150.86
17	0.725	2.9	28	0.125	0.5	163.94

603 BBR: Barley brewing residue; YE: Yeast extract

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608 **Table 8 -** Summary of ANOVA of the 2nd order mathematical model for the production of *β*-
609 xylosidase
610

Variation source	SS	DF	MS	*F*Value	*F*Tab
Regression	10,589.578	6	1,764.930	4.900	2.46
Residual	3,601.789	10	360,179		
Lack of fit	3,496.015	8	437,002	8.263	9.37
Pure error	105,773	2	52,887		
Total	**14,191.367**	**16**			

611 SS: sum of squares; DF: degree of freedom, MS: mean square; R^2=0.74; p-value < 0.10

612
613

Fig. 1

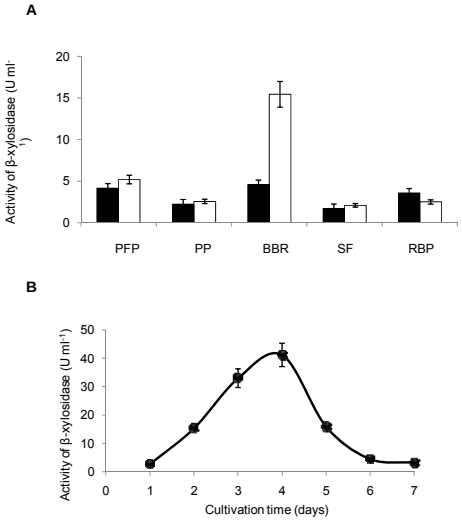

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Fig. 2

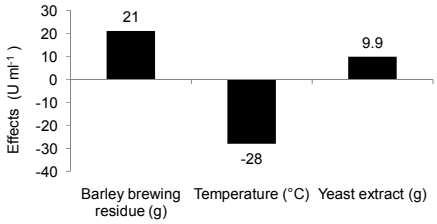

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Fig. 3 - print version

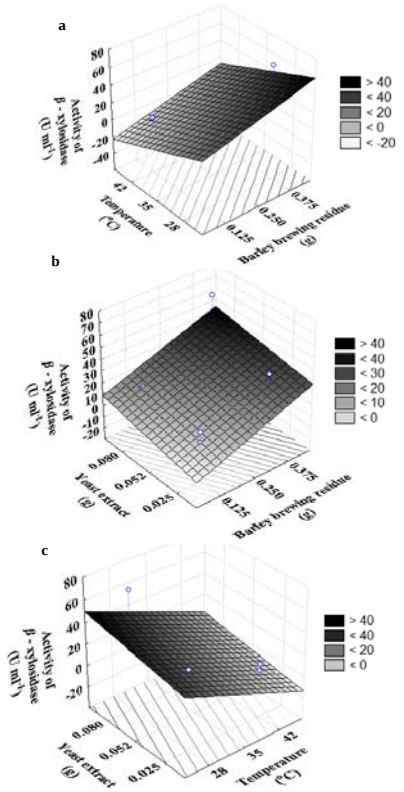

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Fig. 3 – web version

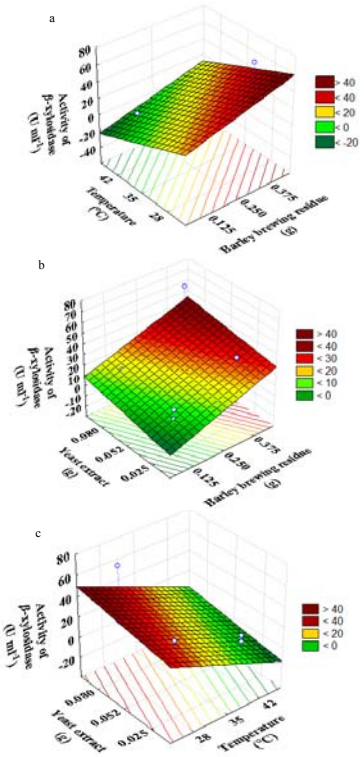

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Fig. 4

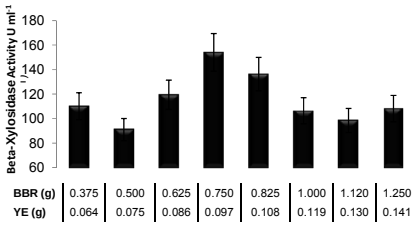

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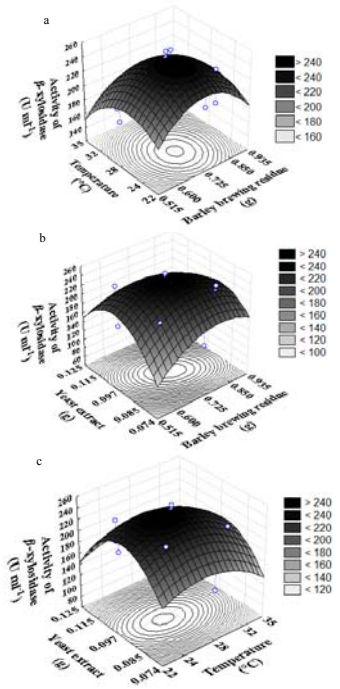
Fig. 5 print version



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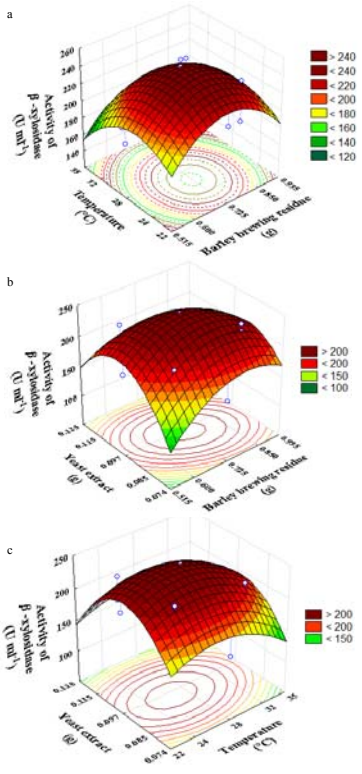

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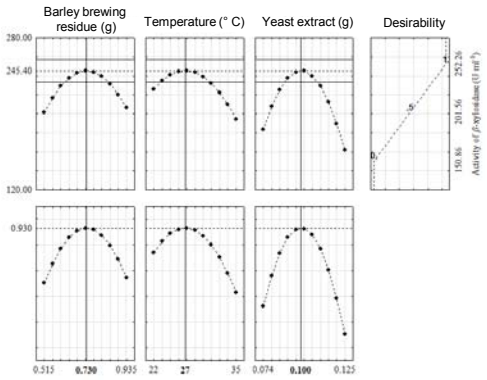
Fig. 6 – Print version



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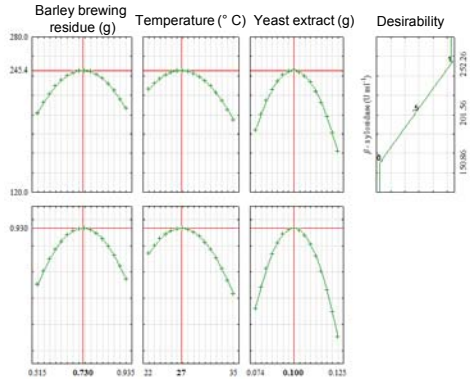

623

$$\hat{y} = b_0 + \sum_{i=1}^{3} b_i X_i + \sum_{i,j=1}^{3} b_{ij} X_i X_j \quad \text{(Eq. 1)}$$

$$\hat{y} = b_0 + \sum_{i=1}^{3} b_i X_i + \sum_{i=1}^{3} b_{ii} X_i^2 + \sum_{i,j=1}^{3} b_{ij} X_i X_j \quad \text{(Eq. 2)}$$

$$\hat{\beta} = 17{,}96 + 10{,}53B - 13{,}87T + 4{,}95E \quad \text{(Eq. 3)}$$

$$\hat{\beta} = 242{,}88 - 14{,}07B^2 - 7{,}61T - 13{,}69T^2 - 6{,}24E - 25{,}02E^2 - 10{,}37BE \quad \text{(Eq. 4)}$$

624