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A thermostable xylanase from a new strain of *Aspergillus fumigatus* presents high ability to hydrolyze hemicellulose from corn straw

Uma xilanase termoestável de uma nova estirpe de *Aspergillus fumigatus* que apresenta elevada capacidade de hidrolisar hemicelulose a partir de palha de milho

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ABSTRACT
In order to optimize the production of xylanase from a new thermophilic strain of *Aspergillus fumigatus* (OI-1R-T), Plackett-Burman design (PBD) and central composite rotational design (CCRD) were performed. The response surface plots indicated a trend for increased xylanase biosynthesis with increasing concentrations of corn straw. The optimized xylanase activity was 530 U mL^{-1} in the presence of 6.5% (w/v) of the residual biomass, which was 11 times (1,157%) higher than that obtained with only the PBD (45.8 U mL^{-1}). Interestingly, xylanase thermostability was maintained at 90% at 50 °C for 6 h. Enzymatic hydrolysis assays conducted for 96 h with 2 U mL^{-1} of xylanase and crude corn straw, pre-treated corn straw (hemicellulose) and xylan from beechwood, resulted in the net production of 3.89, 20.96 and 21.64 µmol $mL^{-1}$of reducing sugars, respectively. Thus, *A. fumigatus* xylanase was equally able to hydrolyzes hemicellulose from corn straw and xylan from beechwood. The present data indicate that the xylanase activity of *A. fumigatus* could be applied to the production of low molecular weight sugars for use by pentose-fermenting yeast for the production of fuels and chemicals, among other products.

Keywords: *Aspergillus fumigatus*, corn straw, enzymatic hydrolysis, experimental design, xylanase, agro-industrial residues.

RESUMO
A fim de optimizar a produção de xilanase a partir de uma nova estirpe termofílica de *Aspergillus fumigatus* (OI-1R-T), foram realizados ensaios de otimização experimental pela metodologia de Plackett-Burman (PBD) e pelo desenho rotacional composto central (CCRD). As parcelas de superfície de resposta indicaram uma tendência para um aumento da biossíntese da xilanase com concentrações crescentes de palha de milho. A atividade otimizada da xilanase foi de 530 U mL^{-1} na presença de 6,5% (w/v) da biomassa residual, que foi 11 vezes (1,157%) superior à obtida apenas com o PBD (45,8 U mL-1). Curiosamente, a termoestabilidade da xilanase foi mantida a 90% a 50 °C durante 6 h. Os ensaios de hidrólise enzimática realizados durante 96 h com 2 U mL^{-1} de xilanase e palha de milho em bruto, palha de milho pré-tratada (hemicelulose) e xilana de faia, resultaram na produção líquida de 3,89, 20,96 e 21,64 µmol mL^{-1} de açúcares redutores, respectivamente. Assim, a xilanase do *A. fumigatus* foi igualmente capaz de hidrolisar a hemicelulose a partir da palha de milho e xilano de madeira de faia. Os dados atuais indicam que a atividade da xilanase de *A. fumigatus* poderia ser aplicada à produção de açúcares de baixo peso molecular para utilização por leveduras que fermentam pentoses para a produção de combustíveis e produtos químicos, entre outros produtos.

Palavras-chave: *Aspergillus fumigatus*, palha de milho, hidrólise enzimática, desenho experimental, xilanase, resíduos agro-industriais.

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1 INTRODUCTION

The need for large-scale production of food associated with high global energy consumption and depletion of the planet's energy resources leads to the growing need to implement strategies that optimize the use of agribusiness residues for the production of chemicals and fuels with high added value (Raza et al. 2019).

The large amounts of plant biomass that have accumulated in the world have been identified as having great potential for the production of bioenergy. Several countries have extensive arable areas with suitable climate and soil conditions. This biomass is usually composed of crop residues or the waste from the processing of the agricultural crops. The agro-industrial waste consists largely of lignocellulose, which is the most abundant and renewable biomass available on the planet, consisting of cellulose (45-55%), hemicellulose (25-35%) and lignin (20-30%) (Peng and She, 2014).

Xylan is the major hemicellulose component of the structural unit and contains mainly xylose linked by β-1,4 bonds. Xylan is the second most abundant biopolymer in hemicellulose after cellulose (Berrin and Juge, 2008; Rennie and Scheller, 2014). The hydrolysis of xylan requires the action of different enzymes, most commonly xylanases that hydrolyze the xylan into smaller molecules xylo-oligomers, and β-xylosidases that hydrolyze xylo-oligosaccharides to monosaccharide xylose (Fang et al, 2010; Kiran et al. 2013; Lagaert et al. 2014)

The xylanase enzymes have large industrial applicability in the food industry (Schoenlechner et al. 2013), textile processing (Sanghi et al. 2008), the pulp and paper industries (Sharma et al. 2014), the saccharification of lignocellulosic materials (Machado et al., 2015; Rob et al. 2015) and agricultural silage and poultry feed (Bhattacharya et al. 2015). The use of xylanases and their hydrolysis products is also important for the production of xylooligosaccharide-derived functional foods that assist or modulate the homeostasis of the body to promote health (Ayyappan and Prapulla, 2011). In general, the use of xylanases can improve the processing of lignocellulosic materials for the generation of liquid fuels and chemicals (Bhattacharya et al. 2015).

Among the xylanase-producing microorganisms, filamentous fungi are the best producers from the industrial point of view (Haltrich et al. 1996; Nair et al. 2008) and the members of the genus *Aspergillus* are among the most interesting for the production of xylanases (Botella et al. 2007). The *Aspergillus fumigatus* (OI-1R-1T) strain studied in this work was isolated from an environmental reserve containing a biome fragment of the remaining Atlantic Forest in Brazil. This biome currently has less than 10% of its original area in the country (Sparovek et al. 2012) and is one of the top five biodiversity hotspots of the planet (Myers et al. 2000).

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2 MATERIALS AND METHODS

2.1 CULTURE AND STRAIN

The experiments were carried out using the strain *A. fumigatus* (OI-1R-T) isolated from decomposing plant roots in the *Bela Vista Biological Refuge* (Iguassu Falls, Paraná, Brazil) (latitude 24°55'16"S and longitude 53°54'35"W) and deposited in the library of the Federal University of Pernambuco under number URM 7315. The collection and isolation of the microorganism was authorized by Itaipu Binational, and the microorganism was identified by morphological analysis at the Federal University of Pernambuco. Extraction of genomic DNA, amplification of rDNA ITS1 and 4 regions to molecular identification of fungal isolate OI-1R-T was carried out as described by Corrêa et al. (2019). Preservation and sporulation of the strain were performed on solid potato dextrose agar nutrient (PDA) containing 1.5% glucose (w/v), 2% agar (w/v) and 20% potato broth. The medium was sterilized at 121 °C for 30 min. The inoculated test tubes with slant medium were incubated at 42 °C for seven days and stored at 4 °C for up to 30 days.

2.2 INOCULUM PREPARATION AND XYLANASE PRODUCTION

For the preparation of the inocula, tubes containing fungi suspension in the reproductive stage of conidia in 10 mL of distilled water were prepared followed by homogenization of the solution in a 250 mL conical flask. The inoculum for the experiments was 1 mL (1 x 10^5 conidia mL^{-1}) per flask. The assays for xylanase production were carried out in 125 mL conical flasks containing 25 mL of Czapek mineral medium (modified). The optimization experiments were performed with variations of the medium which has the following standard composition: 0.3% $NaNO_3$, 0.1% KH_2PO_4, 0.05% $MgSO_4.7\ H_2\ O$, 0.5% KCl, 0.00 1% $FeSO_4.\ 7\ H_2O$, 0.1% yeast extract, pH 6.0, 1%, corn straw and distilled water. The cultures were incubated at 42 °C for three days. The corn straw used was obtained from farmers in Western Paraná and prepared as described by Corrêa et al. (2014). The culture media were previously sterilized at 121 °C for 30 min.

2.3 XYLANASE ACTIVITY

After incubation for fungal growth and enzyme production, the cultures were subjected to vacuum filtration using Whatman filter paper. The liquid extracts were frozen for subsequent activity measurements. The xylanase activity was determined using the reducing sugars assay with DNS (3,5-dinitrosalicylic acid) (Sigma) (Miller et al., 1959). One unit (U) of xylanase activity was defined as the amount in μmoles of D-xylose produced per 1 mL of enzyme in 1 minute under the assay conditions (μmol mL^{-1}). The substrate used was xylan from beechwood (Sigma) in sodium

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phosphate buffer at pH 7.0 at 60 °C, as defined in previous experiments as the optimal conditions for xylanase activity from *A. fumigatus* (OI-1R-1T).

3 EXPERIMENTAL DESIGN

3.1 PLACKETT-BURMANN DESIGN (PBD)

Eight independent variables were studied in 16 experiments, including $NaNO_3$, KH_2PO_4, $MgSO_4.7H_2O$, KCl, $FeSO_4.7H_2O$, yeast extract, corn straw and pH. Each variable was analyzed at two levels, indicated by (+1) and (-1) (Tables 1 and 2). Four replicates at the focal points (0) were added to verify the reproducibility of the assay. The results were analyzed using Statistica software, version 8. The variables with $p < 0.05$ in the Pareto chart were considered to influence significantly the production of xylanase.

Table 1 Levels of the four factors used in the CCRD.

Factors	**-2**	**-1**	**0**	**+1**	**+2**
$NaNO_3$ (g)	0	0.150	0.300	0.450	0.600
KH_2PO_4 (g)	0	0.050	0.100	0.150	0.200
$MgSO_4.7H_2O$ (g)	0	0.025	0.050	0.075	0.100
Corn straw (g)	0.125	0.250	0.375	0.500	0.625

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Table 2 Plackett-Burman Design (16 trials) for selecting the variables of the culture medium for the production of xylanase by *A. fumigatus* (OI-1R-T)

			CODED	AND	REAL	VALUES			
	X1	X2	X3	X4	X5	X6	X7	X8	
Treatment	**$NaNO_3$ (g)**	**KH_2PO_4 (g)**	**$MgSO_4.7H_2O$ (g)**	**KCl (g)**	**$Fe_2(SO_4)_3$ (g)**	**YE (g)**	**CS (%)**	**pH**	**Xylanase (U mL^{-1})**
1	1 (0.3)	-1 (0)	1 (0.05)	-1 (0)	-1 (0)	-1 (0.05)	1 (1.5)	1 (8)	45.8
2	1 (0.3)	1 (0.1)	-1 (0)	1 (0.05)	-1 (0)	-1 (0.05)	-1 (0.5)	1 (8)	28.2
3	-1 (0)	1 (0.1)	1 (0.05)	-1 (0)	1 (0.001)	-1 (0.05)	-1 (0.5)	-1 (6)	9.2
4	1 (0.3)	-1 (0)	1 (0.05)	1 (0.05)	-1 (0)	1 (0.15)	-1 (0.5)	-1 (6)	35.7
5	1 (0.3)	1 (0.1)	-1 (0)	1 (0.05)	1 (0.001)	-1 (0.05)	1 (1.5)	-1 (6)	25.2
6	1 (0.3)	1 (0.1)	1 (0.05)	-1 (0)	1 (0.001)	1 (0.15)	-1 (0.5)	1 (8)	39.3
7	-1 (0)	1 (0.1)	1 (0.05)	1 (0.05)	-1 (0)	1 (0.15)	1 (1.5)	-1 (6)	15.3
8	-1 (0)	-1 (0)	1 (0.05)	1 (0.05)	1 (0.001)	-1 (0.05)	1 (1.5)	1 (8)	3.1
9	-1 (0)	-1 (0)	-1 (0)	1 (0.05)	1 (0.001)	1 (0.15)	-1 (0.5)	1 (8)	10.8
10	1 (0.3)	-1 (0)	-1 (0)	-1 (0)	1 (0.001)	1 (0.15)	1 (1.5)	-1 (6)	16.0
11	-1 (0)	1 (0.1)	-1 (0)	-1 (0)	-1 (0)	1 (0.15)	1 (1.5)	1 (8)	7.4
12	-1 (0)	-1 (0)	-1 (0)	-1 (0)	-1 (0)	-1 (0.05)	-1 (0.5)	-1 (6)	2.9
13	0 (0.15)	0 (0.05)	0 (0.025)	0 (0.025)	0 (0.0005)	0 (0.10)	0 (1)	0 (7)	23.3
14	0 (0.15)	0 (0.05)	0 (0.025)	0 (0.025)	0 (0.0005)	0 (0.10)	0 (1)	0 (7)	23.0
15	0 (0.15)	0 (0.05)	0 (0.025)	0 (0.025)	0 (0.0005)	0 (0.10)	0 (1)	0 (7)	23.7
16	0 (0.15)	0 (0.05)	0 (0.025)	0 (0.025)	0 (0.0005)	0 (0.10)	0 (1)	0 (7)	19.7

YE: yeast extract; CS: corn straw.

3.2 CENTRAL COMPOSITE ROTATIONAL DESIGN (CCRD)

CCRD and Response Surface Methodology were employed to optimize the production of xylanase based upon factors previously selected by PBD. The factors selected were $NaNO_3$, KH_2PO_4, $MgSO_4.7H_2O$ and corn straw (Table 2). In addition to the experiments, were included two extrapolated points (-2 and +2) to allow the formation of the surface plot, however this procedure does not pits already described. Thus, the CCRD was constructed using four factors and two levels, including four replicates in the central points and eight replicates in the axial points. Compounds that did not contribute significantly to the production of xylanase were determined and fixed: pH 6.0 and 0.1% of yeast extract (w/v) KCl and $FeSO_4$ were excluded from the culture media. The results were analyzed using Statistica software, version 8. The main effects of the variables and their interactions were analyzed by analysis of variance (ANOVA) with a 5% level of significance.

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3.3 VALIDATION OF THE MODEL TO OPTIMIZE THE XYLANASE PRODUCTION

Once all variables with the greatest influence for the production of xylanase were confirmed, the Statistica program generated a model with a set of tested factors that minimized the multivariate quadratic loss function. Data analysis allowed the determination of significant coefficients and fit the second-order model (Eq. 1).

$$\hat{X} = b_0 + \sum_{i=1}^{4} b_i X_i + \sum_{i=1}^{4} b_{ii} X_i^2 + \sum_{i,j=1}^{4} b_{ij} X_i X_j \qquad \textbf{(Eq. 1)}$$

To confirm the predicted production of xylanase generated by the analysis of the equation, six standardized tests with optimal concentrations defined by the experimental design were conducted. The equation showed the predicted response for the variables, where X is the xylanase activity. The regression coefficients were found at the level of significance using Student's t-test, and experimental data were analyzed by Statistica 8 software. The significant coefficients represented by b_0, b_i, b_{ii}, and b_{ij} are the model of the regression coefficients, and X_i and X_j are the independent variables in coded values.

3.4 VARIATION OF THE CONCENTRATION OF SUBSTRATE

After verifying the optimized data and confirming the statistical model for optimizing the culture conditions for the production of xylanase, an experiment was conducted because the analysis of the response surface generated by the program charts showed a tendency to increase the enzymatic activity with increasing concentration of the carbon source, corn straw. Thus, nine different concentrations of corn straw (2.5 - 7%) were tested in triplicate. The concentration of the residue used for validation of the mathematical model was considered as the initial value.

3.5 ENZYME THERMOSTABILITY ASSAY

The thermostability was analyzed by incubating the crude enzyme extract at different temperatures from 50, 55 e 60 ° C at pH 7 from 10 min to 6 h. After this incubation period, the samples were frozen for later verification of the amount of xylanase activity. The results are expressed as relative activity, considering the highest value of xylanase activity obtained as 100%.

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3.6 ENZYME HYDROLYSIS ASSAY

Enzymatic hydrolysis was performed by incubatingthe crude enzyme extract independently with three different compounds: crude corn straw, hydrolyzed corn straw and xylan from beechwood (Sigma). Duplicate flasks containing 1% (w/v) of each compound in 20 mL of sodium phosphate buffer at pH 7.0 were prepared. The hydrolysis was determined by incubating these different flasks with 2 U mL^{-1} of crude extract of xylanase for 1 up to 96 h at 50ºC (temperature better thermostability). Aliquots of 1 mL of the incubation mixtures were collected at fixed time intervals and frozen for subsequent measurements, including analysis of the amount of total reducing sugars produced. Corn straw was prepared as described by Corrêa et al. (2014). Hydrolyzed corn straw was prepared by self-hydrolysis by incubating 1 g of corn straw with 10 mL water at 200 ºC in a digester tube for 1 h. After this time, the tube was immediately cooled in an ice bath. The liquid phase was filtered through filter paper and the hydrolyzed solution was precipitated by adding 3 volumes of absolute ethanol at 20 °C for 24 h. The liquid fraction obtained was carefully removed by inversion, and the precipitate was dried at 50 °C for 24 h. The culture media containing the various compounds were sterilized at 121 °C for 30 min, and the crude enzymatic extract was sterilized by vacuum filtration using a 0.22 μm membrane (Corning) to avoid the growth of microorganisms during the hydrolysis assay.

4 RESULTS

4.1 PLACKETT-BURMANN DESIGN

Table 2 summarizes the coded and real values of the variables used at each level and the array of planning for the selection of the components of the medium by Plackett-Burmann Design (PBD). The response generated by the fungus *A. fumigatus* (OI-1R-T) with the application of the methodology proposed in PBD allowed the induction of xylanase activity in a range of 2.9 to 45.8 U mL^{-1}. Standard analysis of variance (ANOVA) of the results of the experimental assays for the production of xylanase indicated an R^2 equal to 0.95.

The data for the components that were significant at the level of $p < 0.05$ for enzyme production are presented in the Pareto chart analysis (Fig. 1) showed that, for the production of xylanase of *A. fumigatus* only KCl and pH were not statistically significant, i.e., had high no influence to the improvement of enzyme production, resulting in only one 1.97 and 3.74 U mL^{-1} of enzyme activity, respectively. Thus, these variables were kept at the lowest concentration tested; additional experiments were conducted until the complete removal of KCl (-1: 0 g) while keeping the pH value at 6.0 (-1) was achieved.

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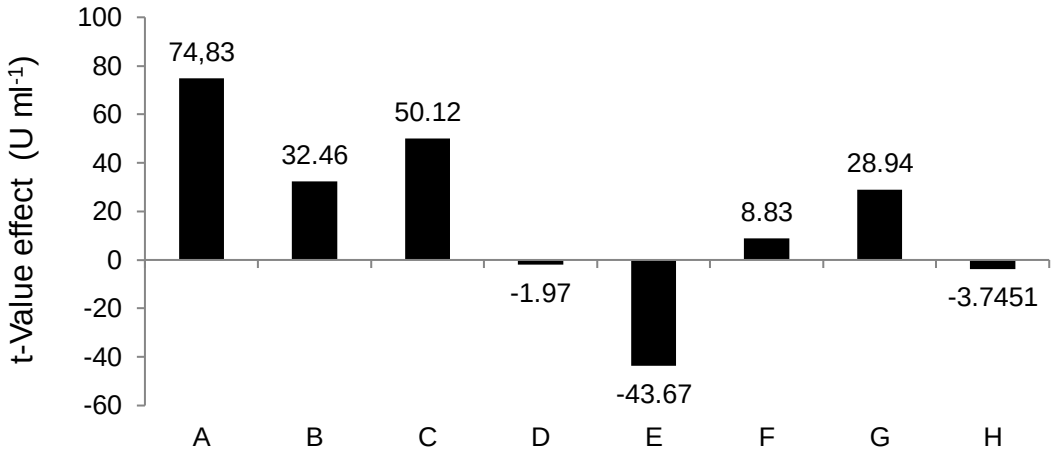
Figure 1 Pareto chart for the effect of each factor on the xylanase synthesis of *A. fumigatus* by Plackett-Burman design. (A) NaNO3, (B) KH2PO4, (C) MgSO4.7 H2O (D) KCl (E) FeSO4. 7 H2O, (F) yeast extract (G) corn straw and (H) pH. Significance level of 5% indicated by the dotted line.



These analyses showed that $NaNO_3$, KH_2PO_4, $MgSO_4.7H_2O$ and corn straw exerted a statistically significant influence on the production of xylanase by the fungus because the higher the concentration used for each component, the higher the enzymatic activity observed. In order of significance, $NaNO_3$ showed a positive effect of 74.83 U mL^{-1} when added in larger amounts, with a shift from 0 g (-1) to 0.3 g (+1). The other components of the medium with a positive influence on the xylanase activity were $MgSO_4.7\ H_2O$, KH_2PO_4 and corn straw, which increased enzyme activity to 50.12, 32.46 and 28.94 U mL^{-1}, respectively.

Based on these results, the experimental design (CCRD) was planned with higher concentrations of factors than those initially tested, and the results are shown in Table 1.

This experience allows verification of variable concentrations of $FeSO_4$ did not contribute for the synthesis of xylanase, since the elevation of its concentration in the culture medium from 0 g (-1) to 0.0005 g (1) resulted a decrease of 43.67 U mL^{-1} (Fig. 1). Thus, this factor was eliminated in later experiments. Finally, the yeast extract factor proved to be significant at the concentrations tested. However, this component had a smaller effect on enzyme production; thus, this variable was set at the value of the tested central point (0.1 g). After verifying the degrees of significance and the most important factors responsible for the increase in production of xylanase by *A. fumigatus* (OI-1R-T), four variables were selected for later experiments: $NaNO_3$, KH_2PO_4, $MgSO_4.7H_2O$ and corn straw.

4.2 CENTRAL COMPOSITE ROTATIONAL DESIGN (CCRD)

The results of the Plackett-Burman Design were used in a 2^4 experimental design to optimize the production of xylanase by *A. fumigatus* (OI-1R-T) using the methodology of Central Composite

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Rotational Design (CCRD). In order to determine the optimum levels for enzyme production, four factors identified as significant were used at different levels as shown in Table 1, namely (-2, 1, 0, 1 and +2). Table 3 shows the experimental design and coded values indicating the actual level used for each of the variables and the experimental response measured for set values of xylanase activity (U mL^{-1}). A variation of the production of this enzyme, from 120 to 430 UmL^{-1}, was observed, thus indicating a significant increase in the enzymatic activity in comparison with the Plackett-Burman Design. Analysis of variance (ANOVA) for the dependent variable is shown in Table 4, where the regression coefficient was found to be significant (5%), as indicated by the calculated F value (8.24), which was higher than the tabulated F (2.66). Therefore, the selected quadratic model was significant for the experiment performed. The R^2 of 0.70 (Table 4) is justified because the experimental design conducted in this work resulted in four-dimensional data. In statistical analysis, an R^2 value is typically used to analyze two-dimensional data, and it is then possible to identify data that do not fit the model. However, statistical relationships are not evaluated only using R^2 values, and it is possible infer the validity of an experiment by observing the trend of the results and demonstrating the significance by examining the calculated F value in relation to the measured F value. Thus, the results presented in the present report were shown to be significant.

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Table 3 Matrix of the 2^4 experimental design for optimizing the production of xylanase by *A. fumigatus* (OI-1R-T).

	CODED AND REAL VARIABLES				
	X1	**X2**	**X3**	**X4**	
Treatments *	**$NaNO_3$ (g)**	**KH_2PO_4 (g)**	**$MgSO_4.7H_2O$ (g)**	**Corn straw (%)**	**Xylanase (U ml^{-1})**
1	-1 (0.15)	-1(0.05)	-1 (0.025)	-1 (1)	270
2	1(0.45)	-1(0.05)	-1 (0.025)	-1 (1)	301
3	-1 (0.15)	1(0,15)	-1 (0.025)	-1 (1)	266
4	1(0.45)	1(0,15)	-1 (0.025)	-1 (1)	307
5	-1 (0.15)	-1(0.05)	1 (0.075)	-1 (1)	120
6	1(0.45)	-1(0.05)	1 (0.075)	-1 (1)	328
7	-1 (0.15)	1(0,15)	1 (0.075)	-1 (1)	197
8	1(0.45)	1(0,15)	1 (0.075)	-1 (1)	184
9	-1 (0.15)	-1(0.05)	-1 (0.025)	1 (2)	304
10	1(0.45)	-1(0.05)	-1 (0.025)	1 (2)	308
11	-1 (0.15)	1(0,15)	-1 (0.025)	1 (2)	240
12	1(0.45)	1(0,15)	-1 (0.025)	1 (2)	375
13	-1 (0.15)	-1(0.05)	1 (0.075)	1 (2)	254
14	1(0.45)	-1(0.05)	1 (0.075)	1 (2)	290
15	-1 (0.15)	-1(0.05)	1 (0.075)	1 (2)	180
16	1(0.45)	1(0.15)	1 (0.075)	1 (2)	306
17	-2 (0)	0 (0.1)	0 (0.05)	0 (1.5)	129
18	2(0.6)	0 (0.1)	0 (0.05)	0 (1.5)	250
19	0 (0.3)	-2 (0)	0 (0.05)	0 (1.5)	260
20	0 (0.3)	2(0.2)	0 (0.05)	0 (1.5)	310
21	0 (0.3)	0 (0.1)	-2 (0)	0 (1.5)	315
22	0 (0.3)	0 (0.1)	2(0,1)	0 (1.5)	348
23	0 (0.3)	0 (0.1)	0 (0.05)	-2 (0.5)	157
24	0 (0.3)	0 (0.1)	0 (0.05)	-2 (0.5)	430
25	0 (0.3)	0 (0.1)	0 (0.05)	-2 (0.5)	320
26	0 (0.3)	0 (0.1)	0 (0.05)	-2 (0.5)	310
27	0 (0.3)	0 (0.1)	0 (0.05)	-2 (0.5)	320
28	0 (0.3)	0 (0.1)	0 (0.05)	-2 (0.5)	345

* Order of the randomized trials conducted.

Table 4 Summary of the ANOVA of the second-order mathematical model for xylanase.

Source	**SQ**	**DF**	**MS**	***F* Value**	***F* tab**
Regression	94,679.90	5	18,935.98	8.24	2.66
Residual	50,555.50	22	2,297.98		
Total	145,235.40	27			

R^2=0.70; p-value <0.05; *SQ*: sum of squares of model parameters, *DF*: degree of freedom, *MS*: mean square of model parameters, *F* tab: *F* tabulated.

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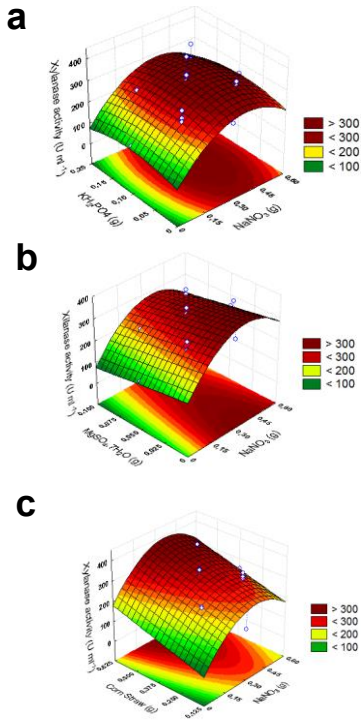
The application of multiple regression analysis to the experimental data using the statistical program allowed the generation of a second-order mathematical model to optimize the composition of the medium to improve the production of xylanase by *A. fumigatus* (OI-1R-T). Eq. 2 corresponds to the obtained second-order equation that represents the mathematical model to predict the amount of enzyme produced by the optimized parameters, in which $\hat{X}$ represents the expected amount of enzyme and N and K represent the coded values for the parameters $NaNO_3$ and KH_2PO_4.

$$\hat{X} = 312{,}8 - 9{,}6125N^2 + 34{,}58K^2 \qquad \textbf{(Eq. 2)}$$

To determine the optimum levels of the variables, response surface plots were generated to analyze the activity of xylanase (Fig. 2).

Figure 2 Response surface plots for xylanase production by A. fumigatus. (a) NaNO3 and KH2PO4, (b) NaNO3 and MgSO4.7H2O and (c) NaNO3 and corn straw.



The optimum concentration ranges of sodium nitrate and potassium phosphate were 0.30 – 0.45 g and 0.05 – 0.15 g, respectively, which were close to the central points of the ranges tested for these two factors (Fig. 2a, b). The maximum production of xylanase occurred at the highest tested concentrations of corn straw, 2% - 2.5% (w/v), and showed a clear tendency of increased enzyme production with increased concentration of the residue in the culture medium (Fig. 2c).

Magnesium sulfate (Fig. 2b) showed no significant influence on xylanase activity. The tested concentrations indicated that the reduction of magnesium sulfate in the culture medium would result in an increase in enzyme production, suggesting that its presence in association with other elements inhibited enzyme production by the fungus. Finally, our data strongly indicate that the most significant elements for the induction of xylanase activity were corn straw, sodium nitrate and potassium phosphate.

4.3 OPTIMIZATION AND VALIDATION OF THE MODEL

Statistica 8.0 software was used to design a set of numerical optimizations predicted values for the data encoded according to the quadratic model. These data indicated the optimized concentrations of sodium nitrate, potassium phosphate, magnesium sulfate and corn straw required to achieve an optimized production of xylanase. In these mathematical simulations, the most suitable conditions for increasing the production of xylanase were 0.37 g of sodium nitrate, 0.1 g of potassium phosphate, total absence of magnesium sulfate and 0.625 g (2.5%, w/v) of corn straw.

To confirm the theoretical model, these conditions were tested experimentally in triplicate. The average xylanase activity obtained in these experiments was 354 U mL^{-1}, a result consistent with the predicted value of 302.4 U mL^{-1}. Thus, by validating the optimization model proposed by CCRD, it was possible to increase xylanase activity by 117%.

4.4 OPTIMIZED PRODUCTION OF XYLANASE BY INCREASING THE AMOUNT OF CARBON SOURCE

After confirmation of the statistical model, a new experiment was conducted with incremental increases in the concentration of the carbon source (corn straw) in order to determine whether the xylanase activity of *A. fumigatus* could be increased further. The increase in the corn straw biomass concentration led to a further increase in the enzyme activity when used in combination with other optimized elements, such that the addition of 1.625 g (6.5%, w/v) of corn straw resulted in a xylanase activity of 530 U mL^{-1} (Fig. 3).

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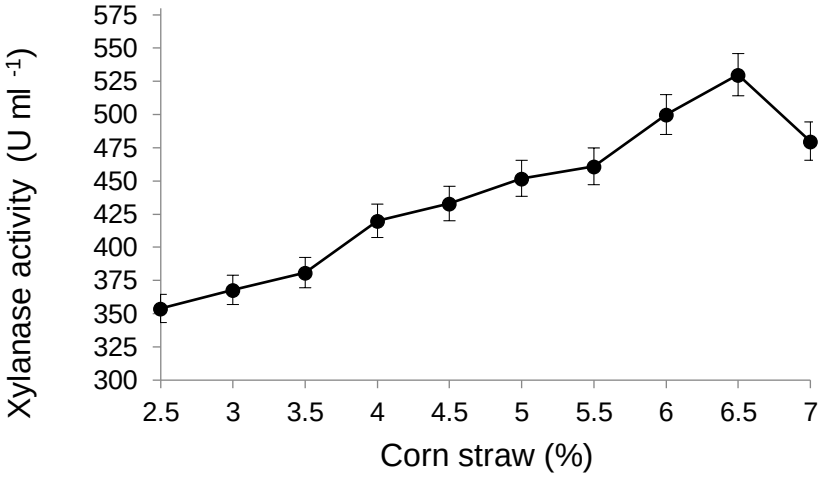
Figure 3 Xylanase activity of *A. fumigatus* after three days of stationary culture at 42 °C in 25 mL optimized liquid Czapek medium. NaNO3 (0.37 g), KH2PO4 (0.1 g), yeast extract (0.1 g), pH (6.0) and corn straw (2.5-7%).

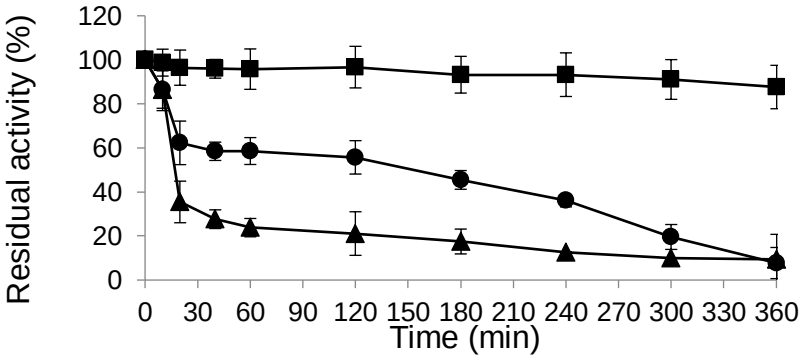


The most effective medium used in this work for the production of xylanase by *A. fumigatus* was the optimized liquid Czapek medium (25 mL) that contained $NaNO_3$ (0.37 g), KH_2PO_4 (0.1 g), yeast extract (0.1 g), pH (6), corn straw (6.5%) and distilled water. The ideal culture conditions were at 42 °C for 3 days.

4.5 THERMOSTABILITY ASSAY OF THE XYLANASE

The thermostability test showed that the xylanase activity in crude extract has greater stability at 50 ºC, decreasing by only 10% after 6 h of incubation at this temperature (Fig. 4). In addition, the xylanase had a half-life of 3 h at 55 ºC. At the temperature of 60 °C, the enzyme lost more than 70% of its hydrolytic capacity during the first hour of incubation.

Figure 4 Thermal stability of xylanase activity in the crude extract of *A. fumigatus*. Tests to verify the thermostability were carried out with the crude extract incubated at three different temperatures: 50 (■), 55 (●) and 60 °C (▲) at pH 7.0 for up to 360 min. After this incubation, the samples were used for the standard measurement of activity. The thermal stability is shown as a residual activity compared to the activity at time zero.



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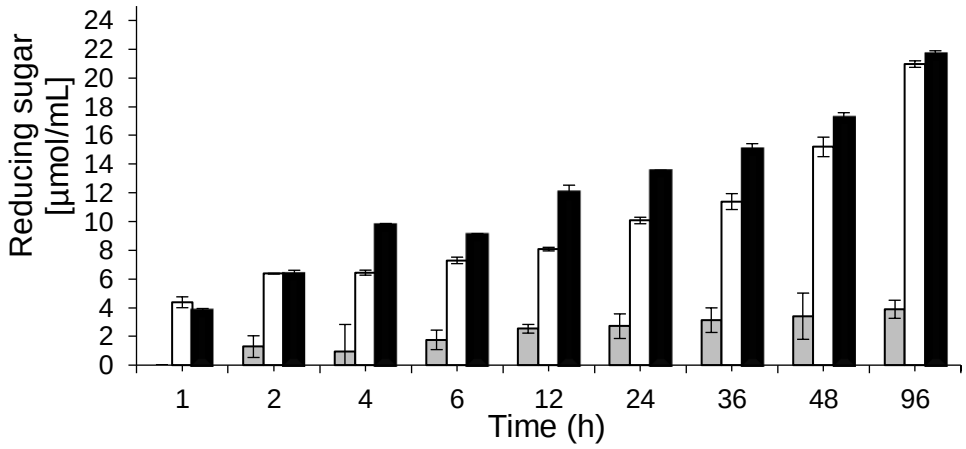
4.6 ENZYMATIC HYDROLYSIS

Enzymatic hydrolysis of the crude extract produced using the optimized xylanase conditions showed that the enzyme remained active for 96 h at 50 °C and led to the formation of reducing sugars incrementally over time (Fig. 5). The increase in the amount of reducing sugars produced from hydrolyzed corn straw was similar to the increase in xylan hydrolysis over 96 h. In general, a production of 3.89, 20.96 and 21.64 µmol mL^{-1} reducing sugars was observed for crude corn straw, pre-treated corn straw (hemicellulose) and xylan from beechwood, respectively, using an enzyme extract containing 2 U mL^{-1} of xylanase.

Figure 5 Hydrolysis of compounds by the action of enzymes present in the crude enzymatic extract produced by the fungus *A. fumigatus* using the optimization process for the production of xylanase. The compounds used are indicated by colored bars: gray for untreated corn straw, white for pretreated corn straw (hemicellulose) and black for xylan from beechwood (Sigma). Hydrolysis was performed by incubation of crude enzyme extract expressing xylanase activity (2 U mL-1), previously sterilized by filtration, mixed with each of the three compounds (1%, w/v) prepared in vials with 20 mL sodium phosphate buffer (pH 7.0). The samples were incubated at 50 °C between 1 and 96 h. Aliquots of the samples were collected for measurement of the total reducing sugars produced.

5



DISCUSSION

The optimized xylanase biosynthesis was 530 U mL-1 in the presence of 6.5% (w/v) of the residual biomass, which was 1,157% higher than that obtained with only the PBD (45.8 U mL-1). This result represents an 11-fold increase in total xylanase production compared to the highest value obtained in the initial experiments with PBD (45.8 U mL^{-1}). These results are very interesting from the biotechnological point of view, as they indicate that microorganisms isolated from unexplored ecosystems can present interesting characteristics when properly subjected to experiments based on experimental design. Another important factor is the use of inexpensive and abundant agro-industrial residues in the production of compounds of biotechnological interest, indicating the advantage of the economic gain in the production chain, and the environmental advantage of reusing

plant biomass that would otherwise be deposited in the environment, leading to pollution of the soil or water resources.

A comparison of the highest optimized enzyme production found in the present report using PBD (45.8 U mL-1) with that found after confirmation of the CCRD mathematical model (354 U mL-1) showed a 7.72-fold increase in xylanase production, a higher value than that observed for the optimization of xylanase from the fungal *T. lanuginosus* strain SDYKY-1 using PBD and planning central composite (CCD) (Su et al. 2011) *T. lanuginosus* xylanase activity after optimization by CCD was only 1.42 times higher than that obtained using PBD.

In the present report, the crude corn straw showed lower hydrolysis than the other substrates used, which may be explained in part by the rigid structure of lignin that reduced access of the active site of the enzyme xylanase to the polymer chain of xylose in lignocellulose (Anderson and Akin, 2008). Although the compounds tested have different chemical compositions the experiment was standardized with pH 7.0 because the goal of this work initially hydrolysis capacity of the produced enzyme. Future studies may be done in order to verify the ideal condition of hydrolysis for each compound.

The improvement in enzyme production by fungi through the optimization of culture media is an interesting and rapid approach to achieve high levels of production of certain strains of microorganisms. Thus, experimental design techniques assist in statistical modeling optimization, involving a minimum number of experiments with a large number of variables. Furthermore, the optimal level of each parameter for a particular target can be calculated (Fang et al. 2010; Su et al., 2011).

Similar of our data, the optimization by PBD of xylanase from *Thermomyces lanuginosus* SDYKY-1 also showed the level of significance (5% significance) among other factors: KH_2PO_4, corn straw and $FeSO_4$ (Su et al. 2011). However, contrary to what we observed in the present work, KH_2PO_4 showed a negative influence on xylanase production from *T. lanuginosus* (-120.67), $FeSO_4$ had a positive influence, and the effect of $MgSO_4 \cdot 7H_2O$ was not significant. Corn straw also acted as a positive factor for the induction of the xylanase activity, but the pH was not significant. In another study with PBD, the optimization process of the production of xylanase by the *Aspergillus fumigatus* strain ABK9 through solid state fermentation (SSF) showed significant effects by pH, the ratio of substrate (wheat bran: pretreated rice straw) and the concentration (g mL^{-1}) of substrate (Das et al. 2013). These data indicate that the production of enzyme by various types of fungi may vary widely relative to the medium composition. However, the results also demonstrate the benefits of using a waste material, as corn straw, is an available resource and one of the most abundant types

of agricultural biomass in the American continents, representing an application of an economical source of carbon.

Substrate selection and identification of suitable crops for the production of target enzymes are key factors for the efficient development of biotechnology because one of the strategies of metabolic engineering, process optimization, aims to increase productivity, reduce costs, increase energy efficiency and use renewable raw materials to provide a more environmentally friendly process than conventional chemical approaches (Wuest et al. 2011).

The optimization of the production of xylanase by *Aspergillus carneus* using CCD and PBD showed an increase of 227% compared with that obtained before the application of the experimental design (Fang et al. 2010). The xylanase production by PBD and Box-Behnken in solid state fermentation (SSF) of *A. fumigatus* ABK9 was 1.18 times higher when compared to the total value optimized by Plackett-Burmann design (Das et al. 2013). Both studies indicate optimization results that were lower than those obtained in this report, as with the highest amount of corn straw tested, *A. fumigatus* produced 530 U mL^{-1} of xylanase activity. This result represents an increase of 1,157% or 11.57 times compared to the highest value obtained using PBD (45.8 U mL^{-1}).

To the best of our knowledge, there are no previous reports on the optimization of xylanase production by the thermo-tolerant strain *A. fumigatus* or other fungi isolated from the Atlantic Forest biome using statistical approaches to the fermentation conditions based on abundant and low-cost agricultural by-products, such as corn straw. Most studies on the production of xylanase by *A. fumigatus* using waste have been performed in solid-state fermentation (SSF). In the case of *A. fumigatus* SK1, significant levels of xylanase activity were obtained using palm trunk oil (Ang et al. 2013). The two strains of *A. fumigatus* studied by Sherief et al. (2010) and *A. fumigatus* ABK9 (Das et al. 2013) were cultured using a mixture of rice straw and wheat bran to optimize xylanase production. For *A. fumigatus* P40M2, a significant increase in the xylanase activity was also shown using wheat bran, soybean meal, crushed cane sugar, sugarcane bagasse treated with steam explosion, orange peel, and mixtures of some of these (Delabona et al. 2013). The ability of *A. fumigatus* using a large number of lignocellulosic materials has also been reported by other authors (Stewart and Heptinstall, 1998), however, there are no references in the literature to date for the use of corn straw to produce xylanase, and this residue is produced abundantly in the American continents.

Consistent with our results concerning the thermostability of *A. fumigatus* xylanase, Mander et al. (2014) also reported a high thermal stability of xylanase produced by *Streptomyces* sp. CS624 at 50 °C. The xylanases from *Aspergillus terreus* (Moreira et al. 2013) and *Streptomyces* sp. CS428

(Pradeep et al. 2013) were also shown to be stable at 50 °C. The xylanase of *A. terreus* was able to retain 68% of its maximal activity in the temperature range of 40 to 55 °C (Moreira et al.2013). The biochemical characteristics of the xylanase of *A. fumigatus* were determined as part of a preliminary study with the ultimate aim to exploit the biotechnological application of the xylanase. Thermal stability is a key property for good enzyme performance in practical applications.

Xylanase Xynl10g of *Gloeophyllum trabeum*, cloned and expressed in *Pichia pastoris* GS115, was also shown to be effective in hydrolyzing pretreated corn straw (hemicellulose), producing reducing sugars at 50 °C and pH 4 (Mosier et al. 2005). With *G. trabeum*, mixtures of different enzymes were even more effective in optimizing the hydrolysis of lignocellulosic materials. The practical demonstration of the ability of the xylanase of *A. fumigatus* to hydrolyze polymeric substrates, even when using unpurified enzyme, highlights the importance of bioprospecting to identify new species or isolates of microorganisms that produce xylanase because the biochemical characteristics of each enzyme may vary in different isolates belonging to the same species, thereby opening the door to different biotechnological applications. In addition, the strain *A. fumigatus* (OI-1R-T) exhibits a negligible cellulase activity (not shown), suggesting a favorable application of this fungus to processes that depend on the absence of cellulose degrading activity, such as paper manufacturing (Ang et al. 2013).

According to Mosier et al. (2005), corn straw residue consists of 35.1 to 39.5% cellulose, 20.7 to 24.6% hemicellulose and 11 to 19.1% lignin and contains a high amount of cellulose and hemicellulose for possible biotechnological applications dependent on the generation of 5- and 6-carbon sugars, such as bioethanol production.

There are two keys points to the application of enzymes produced in processes using waste. First, it allows the use of optimized crude enzyme extract with the xylanase of *A. fumigatus* (OI-1R-T) in biotechnological processes aimed at the production of lower molecular weight sugars, thus eliminating steps of enzyme purification that could lead to an increased cost fort the processes (Corrêa et al. 2016). In addition, xylo-oligosaccharides and xylose produced by xylanase and β-xylosidases can be used by pentose-fermenting yeast and represent an important resource for cellulosic ethanol production (dos Santos et al., 2019). According to Farinas et al. (2010), the production of ethanol from plant biomass is a promising alternative for the production of renewable and sustainable energy, but the high cost of the enzymes needed for converting biomass presents a barrier (Berrin and Juge, 2008).

Second, important to the growing market aimed at the use of alternative sources of low-cost substrates for the culture of microorganisms, the agro-industrial residues may be a source of

low molecular weight fermentable sugars of that can be applied in other fields, such as food production. For example, the purified xylanase of *Streptomyces* sp CS624 (Mander et al., 2014), in cultures induced with wheat bran, is capable of producing xylooligosaccharides through the hydrolysis of xylan and wheat bran, demonstrating that agricultural residues can, through microbial clean technology, be used in many applications, thereby adding value to the product and generating new compounds for various applications.

6 CONCLUSIONS

In the present work we shown that the culture conditions of the thermo-tolerant fungus *Aspergillus fumigatus* (OI-1R-T) to achieve high levels of xylanase activity were successfully optimized combining two statistical methods, the factorial Plackett-Burman design and the central composite rotational design, with the analysis of response surface plots. The data showed that xylanase reached a maximal activity of 530 U mL^{-1}. This represents an 11-fold increase in total enzymatic production compared to the best enzyme activity obtained in the initial tests using PDB (45.8 U mL^{-1}). Enzymatic hydrolysis experiments showed that the crude extract of *A. fumigatus* expressing xylanase under optimized conditions was able to hydrolyze crude corn straw, pre-treated corn straw (hemicellulose) and xylan from beechwood. The hydrolytic ability of the enzyme was measured using the generation of reducing sugars, and the enzyme remained active for more than 96 h at 50 ºC. Our data indicate that the xylanase activity of *A. fumigatus* (OI-1R-T) could be applied to the production of low molecular weight sugars for use by pentose-fermenting yeast for the production of fuels and chemicals, among other products.

COMPLIANCE WITH ETHICAL STANDARDS

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

Ethical approval: L. Graciano, D. Jacomini, and B. Simioni were fellow from CAPES. R.C.G. Simão (process 630/2014) and M.K. Kadowaki (process 395/2013) were supported by Productivity Scholarships from the Araucaria Foundation. K.L. Candido was supported by SESA-PR. This work was partially supported by CNPq (563260/2010-6 /Brazil).

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