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Optimization of culture conditions for tannase production by *Aspergillus* sp. gm4 in solid state fermentation

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ABSTRACT. Tannase is an industrially important enzyme produced by a large number of microorganisms. This study analyzed the production of tannase by *Aspergillus* sp. GM4 under solid-state fermentation (SSF) using different vegetable leaves (mango, jamun and coffee) and agricultural residues (coffee husks, rice husks and wheat bran). Among the substrates used jamun leaves yielded high tannase production. The Plackett-Burman design was conducted to evaluate the effects of 12 independent variables on the production of tannase under SSF using jamun leaves as substrate. Among these variables, incubation time, potassium nitrate and tannic acid had significant effects on enzyme production. A lower incubation time was fixed and supplementation with potassium nitrate and tannic acid were optimized using the Central Composite Design. The best conditions for tannase production were: incubation time of 2 days; tannic acid at 1.53% (w w^{-1}) and potassium nitrate at 2.71% (w w^{-1}). After the optimization process, tannase production increased 4.65-fold, which showed that the statistical experimental design offers a practicable approach to the implementation of optimization of tannase production.

Keywords: surface response methodology, enzyme, jamun, agricultural residues.

Otimização das condições de cultivo para produção de tanase por *Aspergillus* sp. gm4 em fermentação em estado sólido

RESUMO. Tanase é uma enzima industrialmente importante produzida por um grande número de microrganismos. Este estudo analisou a produção de tanase por *Aspergillus* sp. GM4 em fermentação em estado sólido (FES) utilizando diferentes vegetais como folhas de manga, de jambolão, de café e resíduos agrícolas, como a casca de café, casca de arroz e farelo de trigo. Entre os substratos utilizados, as folhas jambolão renderam alta produção de tanase. O planejamento de Plackett-Burman foi conduzido para avaliar os efeitos de 12 variáveis independentes sobre a produção de tanase em FES usando folhas jambolão como substrato. Entre estas variáveis, tiveram efeitos significativos na produção da enzima o tempo de incubação, o nitrato de potássio e o ácido tânico. O menor tempo de incubação foi fixado e a suplementação de nitrato de potássio e ácido tânico foi otimizada utilizando o planejamento composto central rotacional. As melhores condições para a produção de tanase foram o tempo de incubação de dois dias, a concentração de ácido tânico de 1,53% (g g^{-1}) e de nitrato de potássio 2,71% (g gw^{-1}). Após o processo de otimização, a produção tanase aumentou 4,65 vezes**,** o que mostrou que o delineamento experimental foi um método viável para a otimização da produção de tanase.

Palavras-chave: metodologia de superfície de resposta, enzima, jambolão, resíduos agrícolas.

Introdução

Tannase or tannin acyl hydrolase (EC 3.1.1.20) is an inducible enzyme produced by different microorganisms, such as bacteria (OSAWA et al., 2000; MONDAL et al., 2001) and fungi (BANERJEE et al., 2001; SAXENA; SAXENA, 2003; MUKHERJEE; BANERJEE, 2006). It catalyzes the breakdown of hydrolysable tannins, as for example tannic acid, as well as gallic acid esters (LEKHA; LONSANE, 1997). In addition, tannase is applied commercially in the food industry where it is used as clarifying agent in some wines and fruit juices, and in the production of instant tea (LEKHA et al., 1993). In the pharmaceutical industry, it is used in the production of gallic acid, a substrate applied in the chemical or enzymatic synthesis of the propyl gallate, a potent antioxidant and in the manufacture of Trimethoprim. Tannase is also used to treat tannery effluents and to reduce the non-

nutritional effects of tannins in animal feed (AGUILAR; GUTIÉRREZ-SÁNCHEZ, 2001).

Studies on tannase production have been done in liquid surface, submerged or solid-state fermentation (SSF) (SABU et al., 2005; MUKHERJEE; BANERJEE, 2006; BANERJEE et al., 2007; BATTESTIN; MACEDO, 2007; RODRIGUES et al., 2007; MANJIT et al., 2008; SELWAL et al., 2011). SSF is a bioprocess in which microorganisms are grown on solid substrates in the absence or near absence of free water (RODRIGUES et al., 2008). Tannase production by SSF shows advantages over submerged or liquid surface fermentation (AGUILAR; GUTIÉRREZ-SÁNCHEZ, 2001; LEKHA; LONSANE, 1994). In this system of enzyme production by fungal sources, the obtaining of tannase is cheaper and less technology oriented. In this respect, tannin-rich plant residues could be a cost-effective substrate in SSF and are advantageous for countries where these materials are generated in abundance (JANA et al., 2012). In addition, this system produces only a negligible amount of liquid effluents and thereby creates less pollution (MANJIT et al., 2008).

Among the microorganisms, the genus *Aspergillus* has been widely used for the production of tannase (LEKHA; LONSANE, 1997). Species have been studied to produce tannase by fermentation, such as *Aspergillus niger* (LEKHA; LONSANE, 1994; AGUILAR; GUTIERREZ-SANCHEZ, 2001; SABU et al., 2005), *Aspergillus foetidus* (MUKHERJEE; BANERJEE, 2006), *Aspergillus aculeatus* (BANERJEE et al., 2007), *Aspergillus ruber* (KUMAR et al., 2007), *Aspergillus fumigatus* MA (MANJIT et al., 2008) and *Aspergillus oryzae* (RODRIGUES et al., 2007, 2008).

Fermentation processes for the production of tannase are influenced by several parameters, besides the microorganism used, that should be optimized to achieve greater enzyme yield. The main parameters that influence the production of tannase are tannic acid concentration, humidity, type of substrate used, temperature, pH, incubation time and addition of different sources of carbon and nitrogen (LEKHA; LONSANE, 1997). Few studies use experimental designs to optimize the production of tannase, with the majority of optimization experiments evaluating the effect of each variable separately (KAR; BANERJEE, 2000; SABU et al., 2005; MANJIT et al., 2008; RODRIGUES et al., 2008). However, as in fermentation processes each variable can interact with and influence the effect of other, it is essential that an optimization method capable of detecting possible interactions be developed, so that an optimal point is chosen to obtain high yields of enzyme (JANA et al., 2012).

Thus, in the present study the best substrate and optimized the medium conditions were selected for tannase production by *Aspergillus* sp. GM4 under SSF using experimental design associated to the response surface methodology.

Material and methods

Microorganism and inoculum preparation

The strain *Aspergillus* sp. GM4, from the collection of the Bioprospecting and Fungal Genetics Laboratory of the Federal University of Lavras, was used. The initial inoculum of *Aspergillus* sp. GM4 was prepared from 7 day-old cultures grown at 30°C on potato dextrose agar (PDA) medium. The spores were then scraped and transferred to distilled water and counted in a Neubauer chamber until the desired concentration for each experiment was reached.

Selection of substrates for tannase production under SSF

Leafy vegetables, such as mango (*Mangifera indica*L.), jamun (*Syzygium cumini*) and coffee (*Coffea arabica* L.), and agro industrial residues like coffee husks, rice husks and wheat bran, were used as substrates for tannase production by *Aspergillus* sp. GM4 under SSF. The mature leaves of each substrate and the agro industrial wastes were dried at 60°C, finely powdered and used in SSF. Ten grams of each substrate were added to 250 mL Erlenmeyer flasks, moistened with 10 mL of mineral salt solution at pH 5.0 containing (g L^{-1}): $NaNO_3$, 3; KCl, 0.5; KH_2PO_4, 1; $MgSO_4.7H_2O$, 0.5; $FeSO_4$, 7; H_2O, 0.01. The obtained contents were autoclaved at 121°C for 20 min., cooled to room temperature and inoculated with 10^7 spores g^{-1} to each substrate. After that, all contents were incubated at 30°C for 96 hours for growth and tannase production by *Aspergillus* sp. GM4. Each substrate was investigated in duplicate and average values were reported. The Sisvar 5.3 program was used to compare the data by the Tukey test ($p \leq 0.05$). The substrate that presented higher enzyme production was selected for optimization of tannase production in SSF.

Estimation of tannin and centesimal composition of the substrates

The tannin content of substrates was estimated by using the Folin and Denis method (FOLIN; DENIS, 1912).

Moisture content was determined by drying them at 105°C until constant weight. The ethereal

extract, which corresponds to every fraction of the soluble substrate in ether primarily comprising fats, was determined using the continuous type Soxhlet extractor, according to AOAC (1990). The remainder of analyzes were determined in dried and degreased samples: crude protein and amino acid polymers were determined by total nitrogen, according to the method of Kjeldahl, and multiplied by a factor 6.25 for conversion into protein. The ash content, which corresponds to inorganic fraction of the substrate, was determined by burning in a muffle at 550ºC for 3 hours.

Crude fiber (CF) is the residue of acid digestion of the substrate and was determined by the Weende method, based on the difference of weight of a funnel pore before and after receiving the sample digested in acid. In addition, the carbohydrate portion of the substrate was determined by subtraction:

Carbohydrate = 100 - (moisture + protein + ether extract + ash + fiber) (AOAC, 1990).

Effect of tap water as moisturizing agents of jamun leaves in SSF

Tap water was evaluated as moisturizing agent of jamun leaves (substrate selected for next experiments) to replace mineral salt solution. Ten grams of jamun leaves were added to 250 mL Erlenmeyer flasks, moistened with 10 mL of tap water (pH = 5.0). The contents were autoclaved at 121ºC for 20 min., cooled to room temperature and inoculated with 10^7 spores g^{-1} of substrate. The contents were incubated at 30ºC for 96 h (KUMAR et al., 2007).

Plackett–Burman design

For identifying the influence of the different variables for tannase production, 12 factors were evaluated using the PB experimental design of Plackett and Burman (1946) with 16 trials. The total number of trials to be carried out according to PB is k + 4 where k is the number of variables (RODRIGUES; IEMMA, 2005). Each variable is represented at two levels, high and low, which are denoted by "+1" and "−1", respectively; and by one central point denoted by "0". Table 1 illustrates the 12 factors investigated, as well as the levels of each factor used in the experimental design. The software Statistica® 5.0 was used to analyze the PB design. The independent variables with p value < 0.1, at confidence level of 90%, were considered statistically significant.

Table 1. Range of different variables studied in the Plackett–Burman design.

	Variables	Levels		
		-1	0	+1
1	Temperature (ºC)	25	30	35
2	Tap water : Substrate (v w^{-1})	0.5:1	1:1	2:1
3	pH	4.0	5.0	6.0
4	Inoculum	1 x 10^5	1 x 10^6	1 x 10^7
5	Tanic acid% (w w^{-1})	0	2.5	5
6	Glucose% (w w^{-1})	0	2.5	5
7	Potassium nitrate% (w w^{-1})	0	0.6	1.2
8	Substrate quantity (g)	2	6	10
9	Incubation time (days)	2	4	6
10	Gallic acid% (w w^{-1})	0	2.5	5
11	Yeast extract% (w w^{-1})	0	0.6	1.2
12	Ammonium sulfate% (w w^{-1})	0	0.6	1.2

Central composite design (CCD), response surface methodology, experimental design and statistical analysis

The response surface methodology was used to optimize the screened components for tannase production using the CCD (MONTGOMERY, 1997). The complete experimental design was performed with 2^2 experiments plus four axial points and three central points to estimate the experimental error. Each factor in the design was studied at five different levels (−1.41, −1, 0, +1, +1.41). A set of 13 experiments was performed.

Upon completion of the experiment, tannase production was taken as the dependent or response variable (Y). The independent variables are coded for statistical calculation according to the following equation: $Xi = xi - x0/\Delta xi$, where Xi is the dimensionless coded value (-1.41 or 1.41) of the independent variable xi; xi is the real value of that independent variable; x0 is the real value of that independent variable xi at the center point; Δxi is defined by $(Xi_{(+1)} - Xi_{(-1)}/2)$.

The role of each variable, their interactions, and statistical analysis to obtain predicted yields is explained by applying the quadratic equation: $Y = \beta 0 + \beta i Xi + \beta ii Xi^2 + \beta j Xj + \beta jj Xj^2 + \beta ij XiXj$, where Y is the predicted response, β0 is the offset term, βi and βj are the linear effects, βii and βjj are the squared effects, βij is the interaction effect, X_i and X_j are the levels of the independent variables. Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA).

Statistical significance of the model equation was determined by Fisher's test value, and the proportion of variance explained by the model was given by the multiple coefficient of determination, R squared (R^2) value. The quadratic models were represented as contour plots (3D) and response surface curves were generated. The software Statistica® 5.0 was used in this investigation.

Obtainment of tannase and enzymatic assay

After incubation, the fermented substrates were mixed properly by adding 0.1 M phosphate buffer (pH 6.0) to the flasks (MANJIT et al., 2008). Then, the flasks were kept on the rotary shaker at 200 rpm for one hour. The crude enzyme was extracted by passing the extract through muslin cloth and later through Whatman no. 1 filter paper. Then it was centrifuged at 10,000 *g* for 5 min. The filtrate was collected in test tubes and used for determination of tannase activity.

Tannase activity was estimated by a modified protein precipitation method (DESCHAMPS et al., 1983). The reaction mixture (1 mL) contained 250 μL of 1% tannic acid (in phosphate buffer, pH 6.0), 500 μL of phosphate buffer (pH 6.0) and 250 μL of the culture filtrate. The mixture was incubated at 40◦C for 30 min. in water bath. The reaction was stopped by adding 1 mL of 2% bovine serum albumin (BSA) solution. In the control, BSA was added to the incubation mixture prior to incubation. All tubes were left for 20 min. at room temperature to precipitate residual tannins and they were centrifuged at 3000 *g* for 20 min. The tannase activity in the supernatant was estimated after appropriate dilution and the absorbance directly read at 260 nm (this wavelength corresponds to the optimal absorption of gallic acid) against the control (as blank) in a UV spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme that liberates 1 μmol gallic acid per min under assay conditions.

Protein determination

Total soluble protein was determined by the method of Bradford (1976), using bovine serum albumin (BSA) as standard.

Results and discussion

Production of tannase by *Aspergillus* sp. GM4 under SSF

Different agricultural residues and leaves of different vegetables were studied for production of tannase by *Aspergillus* sp. GM4 in SSF, as shown in Table 2. Among the different substrates used, the high yield of tannase was obtained using in jamun leaves (1.44 U mg^{-1}) as substrate, followed by mango leaves (0.99 U mg^{-1}), after 4 days of incubation. There was no significant difference in the production of this enzyme between the jamun leaves and mango leaves ($p < 0.05$). On the other hand, jamun and mango leaves presented higher maximum tannin content at 98.58 and 92.43 mg g^{-1}, respectively, compared to the other surveyed substrates. Hence, tannase activity was related to tannin content on the respective substrates. This observation suggested that these substrates could act as an inducer for tannase production (MACEDO et al., 2005). In addition, the high enzyme activity with these substrates may be due to the presence of nutrients required by the fungus (KUMAR et al., 2007).

In respect to the centesimal composition, it was observed that both jamun and mango leaves had high levels of carbohydrates (54.33 and 41.35 %, respectively), fats (5.9 and 4.4 %, respectively) and fiber (21.20 and 30.60%, respectively) compared with others studied substrates. Based on our findings, it was suggested a stimulatory effect on fungus growth (*Aspergillus* sp. GM4) and, consequently, in its enzyme production (KUMAR et al., 2007).

From these previous results, our study was directed to the jamun leaves, which were selected as a substrate more appropriated for optimization of tannase production on SSF by *Aspergillus* sp. GM4.

Effect of tap water as moistening agent

The tannase production by *Aspergillus* sp. GM4 was higher in jamun leaves using tap water as moistening agent (3.07 U mg^{-1}) than using mineral salt solution (1.44 U mg^{-1}). The ability of the microorganism to produce a higher enzyme yield with tap water without addition of mineral salt in SSF could lead to reduction in enzyme production costs (KUMAR et al., 2007). Thus, tap water was used as moistening agent of jamun leaves in optimization experiments.

Table 2. Tannin content, centesimal composition and tannase activity in different substrates used for SSF.

Substrate	Tannin content (mg g^{-1})	Moisture (%)	Protein (%)	Fat (%)	Fiber (%)	Ash (%)	Carbohydrate (%)	Tannase activity (U mg^{-1})⋆
Jamun leaves	98.58	5.14	8.49	5.90	21.20	6.94	52.33	1.44 ±0.43^{a}
Mango leaves	92.43	4.89	8.49	4.40	30.60	10.27	41.35	0.99 ± 0.16$^{a\,b}$
Coffee husks	31.17	10.37	11.46	2.50	23.60	6.68	45.39	0.36 ± 0.09^{b}
Coffee leaves	30.70	14.17	18.73	3.30	21.00	11.24	31.57	0.34 ± 0.03^{bc}
Wheat bran	5.92	13.27	16.63	1.90	12.20	4.10	51.90	0.23± 0.04^{c}
Rice husks	2.75	5.54	2.98	0.00	51.00	16.08	24.41	0.13 ± 0.04^{c}

⋆Values are means of duplicates and those with different letters are significantly different at $p < 0.05$.

Plackett–Burman (PB) design

With the purpose of increasing tannase production by *Aspergillus* sp. GM4 using jamun leaves as substrate under SSF, 12 independent variables were studied (Table 1). Based on Table 3, it may be observed the PB experimental design for 16 trials with two levels of each variable, three trials on central points of each variable and the corresponding tannase production in the course of the experiments.

Among the variables tested, the statistically significant variables ($p < 0.1$) were tannic acid, potassium nitrate and incubation time. Tannic acid and incubation time had negative effects on tannase production and potassium nitrate had a positive effect (Figure 1).

Tannic acid has been used as inducer for tannase production; however, a great increase in its concentration do not promote an equivalent increase in enzyme synthesis (BATTESTIN; MACEDO, 2007). Actually, tannic acid at a high concentration may produce complexes with membrane protein of the organism, thereby both growth and enzyme production may be inhibited (BANERJEE et al., 2007). Considering the influence of potassium nitrate, similar results were observed for tannase production by *A. niger* ATCC 16620 (SABU et al., 2005).

This probably occurred due to the distinct assimilation of inorganic ions and the possibility of formation of complexes between tannins and protein structures of the yeast extract (RODRIGUES et al., 2007). Therefore, significant variables were subsequently evaluated.

The other variables studied in PB design such as temperature, amount of substrate , pH, inoculum concentration, glucose, gallic acid, ammonium sulfate, water: substrate ratio and yeast extract had no significant effect over tannase production ($p < 0.1$) (Figure 1).

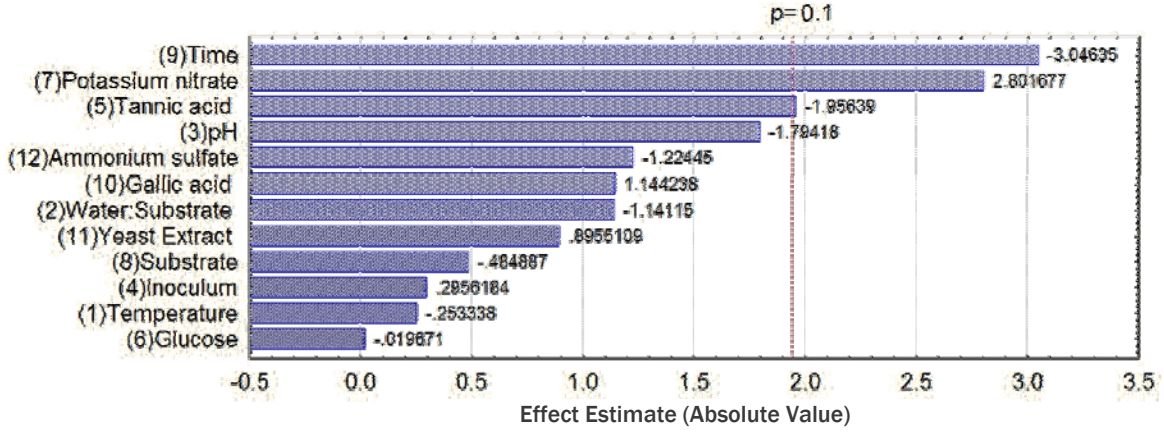

Figure 1. Pareto Chart of standardized effects of variables in PB design on tannase production by *Aspergillus* sp. ($R^2 = 0.83$).

Table 3. Placket–Burman design variables (in coded levels) with tannase production as response.

Trials	Variables												Activity
	1	2	3	4	5	6	7	8	9	10	11	12	(U mg^{-1})
1	1	1	1	1	1	1	1	1	1	1	1	1	0.80
2	1	1	1	-1	1	1	-1	1	-1	-1	1	-1	1.04
3	1	1	-1	1	1	-1	1	-1	1	-1	-1	1	1.24
4	1	1	-1	-1	1	-1	-1	-1	-1	1	-1	-1	3.08
5	1	-1	1	1	-1	1	1	-1	-1	1	-1	-1	6.12
6	1	-1	1	-1	-1	1	-1	-1	1	-1	-1	1	0.47
7	1	-1	-1	1	-1	-1	1	1	-1	-1	1	-1	8.94
8	1	-1	-1	-1	-1	-1	-1	1	1	1	1	1	1.58
9	-1	1	1	1	-1	-1	-1	1	1	1	-1	-1	0.68
10	-1	1	1	-1	-1	-1	1	1	-1	-1	-1	1	3.52
11	-1	1	-1	1	-1	1	-1	-1	1	-1	1	-1	2.49
12	-1	1	-1	-1	-1	1	1	-1	-1	1	1	1	7.29
13	-1	-1	1	1	1	-1	-1	-1	-1	1	1	1	3.86
14	-1	-1	1	-1	1	-1	1	-1	1	-1	1	-1	1.34
15	-1	-1	-1	1	1	1	-1	1	-1	-1	-1	1	1.08
16	-1	-1	-1	-1	1	1	1	1	1	1	-1	-1	4.81
17 (C)	0	0	0	0	0	0	0	0	0	0	0	0	0.59
18 (C)	0	0	0	0	0	0	0	0	0	0	0	0	0.95
19 (C)	0	0	0	0	0	0	0	0	0	0	0	0	1.45

(C) Central Points.

Thus, the non-significant variables in the PB design were fixed on following levels: temperature (30ºC); water:substrate ratio (v w^{-1}) (1:1); pH (4.0); inoculum concentration (10^5 spores g^{-1} of substrate); amount of substrate (5g). As the incubation time had negative and significant effects (Figure 1) on tannase production, this variable was fixed at the lowest level (2 days) (RODRIGUES; IEMMA, 2005).The variables glucose, gallic acid, ammonium sulfate and yeast extract were removed from the CCD experiments.

Central composite design (CCD), RSM, experimental design and statistical analysis

The final medium optimization and interaction amongst the selected factors on PB design was studied. The variables tannic acid (%) and potassium nitrate (KNO_3) (%) were optimized using CCD. The levels of the factors chosen were based on the previous PB analysis. Each variable was studied at five coded levels (-1.41, -1, 0, 1, 1.41).

The design matrix of CCD is a 2^2 design combined with three central points and four axial points. The matrix for this design along with the experimental and predicted results is shown in Table 4.

Table 4. CCD matrix of two variables with experimental and predicted values of tannase production.

Trials	Tannic Acid (%)	KNO_3 (%)	Tannase Activity (U mg^{-1})	Predicted Activity (U mg^{-1})
1	(-1) 0.4	(-1) 1.6	4.75	3.79
2	(+1) 2.4	(-1) 1.6	1.97	1.25
3	(-1) 0.4	(+1) 3.6	1.83	1.19
4	(+1) 2.4	(+1) 3.6	5.06	4.67
5	(-1.41) 0	(0) 2.6	1.78	2.63
6	(1.41) 2.82	(0) 2.6	2.79	3.30
7	(0) 1.41	(-1.41) 1.19	1.33	2.24
8	(0) 1.41	(1.41) 4	2.36	2.81
9 (C)	(0) 1.41	(0) 2.6	5.03	6.15
10 (C)	(0) 1.41	(0) 2.6	7.74	6.15
11 (C)	(0) 1.41	(0) 2.6	5.70	6.15

(C) Central points.

An empirical relationship between the response and the selected variables was expressed by the following fitted second-order polynomial equation: $Y = 6.1545 + 0.2366\ X_1 - 1.60438\ X_1^2 + 0.20377 X_2 - 1.826\ X_2^2 + 1.5044\ X_1 X_2$, where Y is the predicted response of tannase production (U mg^{-1}), and X_1 and X_2 are the coded values of tannic acid and KNO_3, respectively. This polynomial equation was tested for adequacy by ANOVA. The results of the ANOVA showed that the F value of 4.44 was significant. The R^2 value indicated that 82% of the total variation was explained by the model. Table 4 shows that all the predicted values of the model are located nearby the experimental values. This supports the hypothesis that the model equation is sufficient to describe the response of the experimental observations (RODRIGUES; IEMMA, 2005). Thus, one response surface curve was plotted to determine the optimum concentration of each factor for maximum tannase specific activity (Figure 2).

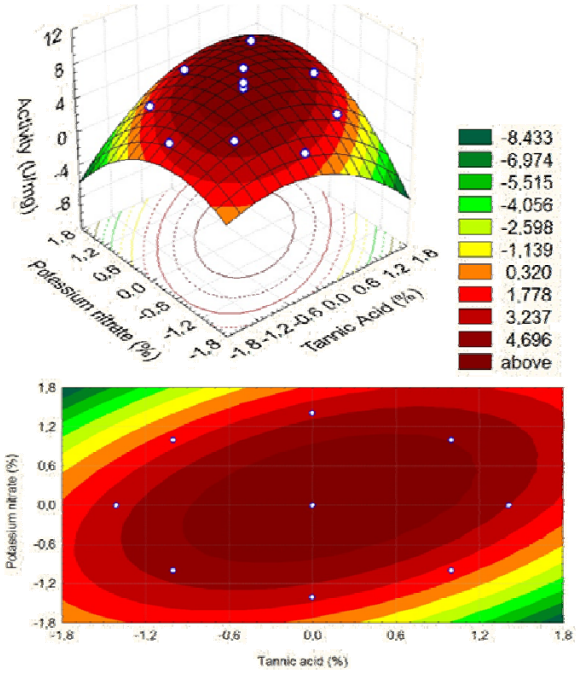

Figure 2. Response surface and contour diagrams for tannase activity considering tannic acid and potassium nitrate.

The location of the optimum (Figure 2) was determined to be 0.12 (tannic acid) and 0.11 potassium nitrate (coded values) or 1.55% (w w^{-1}) tannic acid and 2.71% potassium nitrate (w w^{-1}) (real values), obtained by the software Design Experts 7.0. Although tannic acid presented negative effect on tannase production by *Aspergillus* sp. GM4 as observed in PB design, this compound was not removed from the medium since it induces tannase production under SSF as reported in other investigations (LEKHA; LONSANE, 1994; RANA; BHAT, 2005; SABU et al., 2005; BATTESTIN; MACEDO, 2007; RODRIGUES et al., 2008).

These results indicate that although jamun leaves present high tannin content, substrate supplementation with tannic acid was necessary for increasing tannase production. In addition, the presence of nitrogen sources has a positive effect on microbial growth and, consequently, on enzyme production. Tannase production depends on the

availability of carbon and nitrogen sources in the medium and both have regulatory effects on enzyme synthesis (BATTESTIN; MACEDO, 2007). Thus, under these conditions, the enzyme production was increased 4.65-fold.

The model was validated by three experiments using 5g of jamun leaves, 1.55% tannic acid and 2.71% KNO_3. The predicted response for tannase production was 6.18 U mg^{-1},while an experimental response of 6.69 ± 0.29 U mg^{-1} was found, confirming the validity of the model (Figure 2) (RODRIGUES; IEMMA, 2005). The good agreement between the predicted and the experimental data indicated that RSM was a powerful tool for optimization of tannase production by *Aspergillus* sp. GM4.

We are reporting that the fungus *Aspergillus* sp. GM4 was able to produce high yields of tannase under SSF conditions with jamun leaves. After the optimization process, the tannase production for *Aspergillus* sp. GM4 using this substrate increased 4.65-fold, which indicates that the statistical experimental design offers a practicable approach to the implementation of medium optimization. Our optimized medium for tannase production is mainly composed of a cheap and accessible substrate (jamun leaves), an inexpensive inorganic salt (potassium nitrate) and low supplementation of tannic acid (1.55%).

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