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Optimization of the Production of α-L-Rhamnosidase by *Aspergillus niger* in Solid State Fermentation Using Agro-Industrial Residues

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

The enzyme α-L-rhamnosidase specifically cleaves terminal rhamnose residues from a wide variety of natural products. This property endows this enzyme with important biotechnological potential as α–L-rhamnosidase could be employed in a variety of applications, including removing bitterness from citrus fruit juices, improving the aroma of wines and converting clinically important steroids. This work optimized α-L-rhamnosidase solid-state fermentation production from *Aspergillus niger* 426 using statistical methods. Firstly, a statistical mixture-design with three components to determine the best ratio of nutrients for enzyme production was carried out. The optimal conditions consisted of growing the fungi in media containing 0.14 g of cane sugar bagasse, 1.25 g of soybean hulls and 3.05 g of rice straw; these conditions achieved a maximum α-L-rhamnosidase activity of 1.92 U / mL. Next, a 3² Box-Behnken design to optimize culture moisture levels and nutrient solution pH values for enzyme production was carried out. α-L-rhamnosidase activity increased to 3.02 U / mL when medium moisture was 75.5% and pH value of 4.0.

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Keywords: α-L-rhamnosidase; Aspergillus niger; mixture design; box-behnken design; solid-state fermentation.

1. INTRODUCTION

α-L-rhamnosidase is an enzyme that catalyzes the hydrolysis of glycosidic linkages between rhamnose and other compounds [1]. This enzyme is part of an enzyme complex called naringinase that hydrolyzes naringin by its α-L-rhamnosidase activity (EC 3.2.1.40) to produce rhamnose and prunin, which are then converted into glucose and naringenin via its β-D-glucosidase activity (EC 3.2.1.21) [2]. In the citrus juice industry, α-L-rhamnosidase potentially could be used to decrease bitterness [3] and to avert the presence of hesperidin crystals in citrus products [4], helping to prevent their precipitation in industrial fruit juice tanks [5]. In the wine industry, α-L-rhamnosidase could be employed to promote an increase in the aroma of grape juice and wine [1,6]. Additionally, α-L-rhamnosidase could be used to increase the bioavailability of glycosidic flavonoids in the functional beverage industry [7]. Lastly, in the pharmaceutical industry, α-L-rhamnosidase could be utilized to achieve the derhamnosylation of a wide variety of natural products containing terminal L-rhamnose residues [8].

Naringinase enzyme complex production has been studied by solid-state fermentation (SSF) [9,10]. In SSF, microorganisms grow in the absence of free water on both natural substrates and inert supports [11]. Additionally, SSF represents an alternative use for agro-industrial residues [12,13].

For fungal enzyme production, factorial design has been used extensively to establish optimal fermentation conditions in SSF [14,15,16]. However, more recently, mixture design has been applied to optimize the ratio of three or more substrate components [17,18].

This paper aimed to optimize, through both mixture design and factorial design, the SSF production of α-L-rhamnosidase from *Aspergillus niger* using agro-industrial residues.

2. MATERIALS AND METHODS

2.1 Microorganism

Aspergillus niger strain 426 was isolated from dried prunes and identified by the Institute of Food Technology (Instituto de Tecnologia de Alimentos - ITAL), UNICAMP, Brazil.

2.2 Fermentation Conditions

According to the statistical design sugar cane bagasse, soybean hulls and rice straw were used as substrates for SSF. Cultures were carried out in 125 mL Erlenmeyer flasks. The solid materials were moistened with 12 mL of a nutrient solution (adjusted pH 4.5) comprised of 5.0 g/L $(NH_4)H_2PO_4$, 1.5 g/L K_2HPO_4, 0.5 g/L $MgSO_4.7H_2O$, 0.5 g/L KCl, 5.0 g/L yeast extract and 10.0 g/L naringin. For each liter of nutrient solution, we added 1 mL of a micronutrient solution comprised of 0.8 g/L $FeSO_4.7H_2O$, 0.8 g/L $MnSO_4.7H_2O$, 0.008 g/L $ZnSO_4.7H_2O$, 0.8 g/L $Na_2MoO_4.2H_2O$, 0.4 g/L $CuSO_4.5H_2O$ and 0.04 g/L $NaB_4O_7.10H_2O$. The initial pH of the cultures was adjusted to 4.5. The cultures were inoculated with 10^9 spores/g. The culture flasks were incubated at 28°C with 97% controlled atmospheric humidity.

2.3 Experimental Design and Statistical Analyses

2.3.1 Substrate selection for α-L-rhamnosidase production

A simplex-centroid design (Fig. 1), with 2^{q-1} combinations of mixtures, where *q*, the number of components or variables whose sum is 1 or 100%, to 3 components (sugar cane bagasse, soybean hulls and rice straw) and two replicates at the central point were used [18]. The amount of each substrate used corresponded to the amount required to absorb 12 mL of the nutrient solution without drainage [9]. Thus, in the tests with a single substrate (100%), 1 g of sugar cane bagasse, 5 g of soybean hulls or 5 g of rice straw was used.

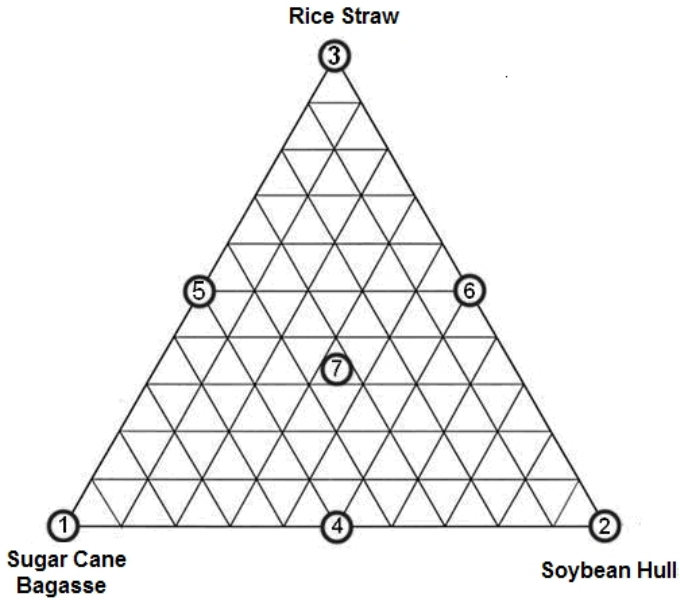

Fig. 1. Simplex-centroid experimental design for the three components

A special cubic model was used to represent the mean of the response variable as a function of the factors described in Equation 1,

$$y = \sum_{1 \le i \le q} \overset{\circ}{\gamma}_i x_i + \sum_{1 \le i \le j \le q} \overset{\circ}{\gamma}_{ij} x_i x_j + \overset{\circ}{\gamma}_{123} x_1 x_2 x_3 \quad (1)$$

Where, Y is the α-L-rhamnosidase activity (U/mL), x_1 the cane sugar bagasse, x_2 the soybean hulls, x_3 the rice straw and γ the estimated parameters. All statistical analyses were performed using Statistica v.9.0 software [19].

2.3.2 SSF variable optimization for α-L-rhamnosidase production

Variable optimization for SSF was achieved by proportioning the selected substrates using a mixture design. The pH of nutrient solution was maintained using a phthalate-HCl or phthalate-NaOH buffer, according to the pH levels to be tested in the experimental design.

Firstly, an incomplete 3^3 Box-Behnken factorial design was used to determine the optimal conditions for α-L-rhamnosidase production. Three independent variables, culture humidity, substrate grain size and nutrient solution pH, were analyzed at three levels (-1, 0 and 1).

Next, a complete 3^2 Box-Behnken factorial design was employed to optimize α-L-rhamnosidase production. We analyzed two independent variables, culture humidity and nutrient solution pH, at three levels (-1, 0 and 1) [20].

2.4 Analytical Methods

With the fermentation flasks placed in iced bath, the cell-free enzymatic extracts were recovered by adding 5mL sodium acetate buffer 50mM, pH 4 and further mixing using a glass rod. Subsequently, the suspension was filtered and centrifuged 7700g for 30 min at 4ºC. The supernatants were collected, and the α-L-rhamnosidase and invertase activity levels and total sugar concentrations were assessed. α-L-Rhamnosidase activity was determined using an adaptation of the method presented by Koseki et al. [21]. The assay mixture contained 0.8mL of naringin (5 g/L) in 1.6mL of sodium acetate buffer (50 mM, pH 4) and 0.5mL of the enzymatic extract. After incubation at 40ºC for 20 min. the reaction was stopped by the addition of 0.1mL of 1 M NaOH and then neutralized with 0.1mL of 1 M HCl. Aliquots were then removed to quantify rhamnose levels by HPLC with detection by refractive index (HPLC-RI) using an Aminex® HPX-87C column with an ultra-pure water mobile phase at a flow rate of 0.6 mL/min at 55ºC. One unit of activity of α-L-rhamnosidase was defined as the amount of enzyme required to release 1 μmol of rhamnose per minute under the reaction conditions described. Invertase activity levels were determined according to the Miller method [22]. A 0.1mL portion of enzyme extract was added to the reaction mixture in final volume of 0.5mL. It contained 0.1mL sucrose in 0,1M acetate buffer (pH 5.0). The reaction mixture in final volume of 0.5mL and incubated for 10 min at 37ºC. The reaction was stopped by the addition of 1.5mL of 3, 5-dinitrosalicylic acid (DNS) and the released reducing sugars were measured by reference to a calibration graph plotted from results obtained with glucose: Fructose standard solution. The total sugar level in each culture was determined using the phenol-sulfuric method described by Dubois et al. [23].

The soluble sugars impregnated in the substrates were quantified by HPLC-RI using an Aminex® HPX-87C column with an ultra-pure water mobile phase with a flow rate of 0.6mL/min at 55ºC. A 30mL volume of ultra-pure water was added to 1g of each substrate; the mixtures were then homogenized for 60 min. Following filtration, chromatographic analyses were performed.

3. RESULTS AND DISCUSSION

3.1 Substrate Selection for α-L-rhamnosidase Production

During substrate selection, the fermentation kinetics for the central point of the mixture design was carried out to determine the optimal time for enzyme production. The central point was composed of a 33.33% ternary mixture of each supporting substrate (sugar cane bagasse, soybean hulls and rice straw). As depicted in Fig. 2, at 120, 144 and 168 hours of culture, the maximal enzymatic activities were 1.71, 1.67 and 1.65 U/mL, respectively. In submerged fermentation, these were also the best times to evaluate carbon and nitrogen source selection according to studies by Puri et al. [2], Rosa et al. [24] and Machado et al.

[25], who used a complex medium and by Custodio et al. [26], who used a defined medium. In SSF, similar fermentation time were found for rhamnosidase production in a study by Elinbaum et al. [9], who investigated substrate selection and other fermentation factors.

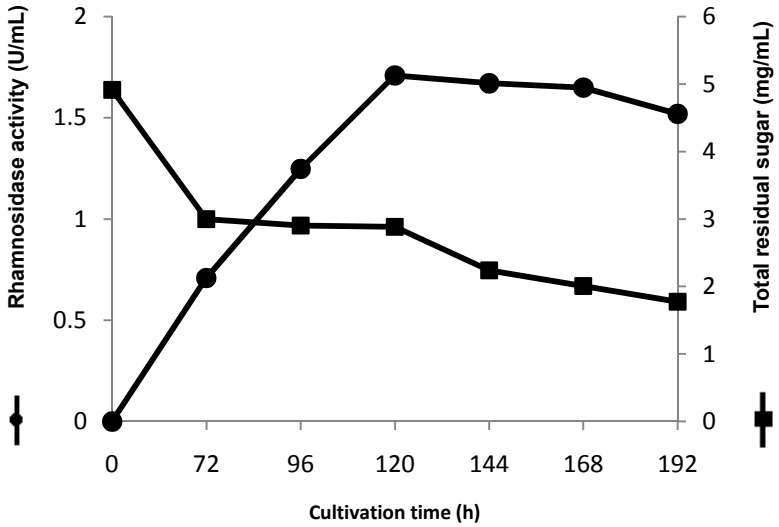

Fig. 2. Kinetics of the central point of the mixture design for α-L-rhamnosidase production

The statistical analysis using the Tukey test showed no significant difference between the first two time points for the enzyme production media (P<0.05). Therefore, the mixture design tests were conducted at the 120-hour time point because of the higher productivity (0.014 U/mL per h). These results are presented in Table 1.

Table 1. α-L- rhamnosidase activity on different substrates according to a mixture design

Run	Coded mixture (x_1; x_2; x_3)**	Original mixture* (x_1; x_2; x_3)**	α-L-Rhamnosidase activity (U/mL)
1	(1; 0; 0)	(1; 0; 0)	0.362
2	(0; 1; 0)	(0; 5; 0)	0.648
3	(0; 0; 1)	(0; 0; 5)	1.94
4	(½; ½; 0)	(0.5; 2.5; 0)	2.005
5	(½; 0; ½)	(0.5; 0; 2.5)	1.575
6	(0; ½; ½)	(0; 2.5; 2.5)	1.937
7	(⅓; ⅓; ⅓)	(0.33; 1.66; 1.66)	1.988
8	(⅓; ⅓; ⅓)	(0.33; 1.66; 1.66)	1.959
9	(⅓; ⅓; ⅓)	(0.33; 1.66; 1.66)	1.828

**amount (g) of support required to absorb 12 mL of the nutrient solution*
***x_1 sugar cane bagasse, x_2 soybean hulls and x_3 rice straw*

We statistically analyzed the data to select the optimal ratio of substrates for enzyme production and adjusted the data to the special cubic model. This analysis is presented in equation 2, in which terms with an asterisk are significant (P<0.05).

$$Y= 0,362x_1+0,648*x_2+1,940*x_3+6,000*x_1x_2+1,8696x_1x_3+2,572*x_2x_3-5,379x_1x_2x_3 \quad (2)$$

Equation 2 revealed that the parameters for x_2, x_3, x_1x_2 and x_2x_3 were significant ($P < 0.05$). With an R^2 adjusted value of 0.989, these data indicate that the model explains 98.9% of the variation in the experimental data. Further, as the variance analysis showed that the model had a 1.37% level of significance, the equation can be used for predictive purposes.

The ternary combination region between the independent variables x_1, x_2 and x_3 can be observed through the contour lines shown in Fig. 3. The figure shows the boundary regions of the surface response for the dependent variable (α-L-rhamnosidase production) that were obtained using equation 2. These results revealed a broad region of elevated rhamnosidase production. The analysis also demonstrated that the regions with increased proportions of both cane sugar bagasse and soybean hulls exhibited decreased rhamnosidase activity.

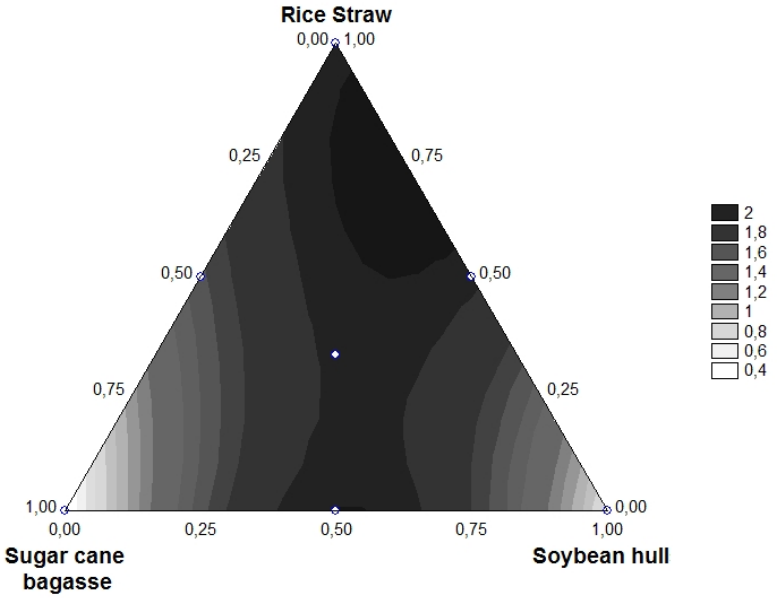

Fig. 3. Curve contour plot resulting from the influence of the substrates on α-L-rhamnosidase activity

The rhamnosidase production response could be classified into two groups: cultures with low rhamnosidase activity (tests 1 and 2) and cultures with high rhamnosidase activity. Tests 1, 2, 3 and 4 will be discussed further and the determinations of rhamnosidase, invertase and sugar consumption are presented in Table 2.

In test 1, which examined sugar cane bagasse as a substrate, sugar consumption was satisfactory. The invertase activity values were the highest (Table 2), suggesting that higher quantities of sucrose were impregnated in the sugar cane bagasse, causing the inhibition of rhamnosidase activity. In test 2, which examined soybean hulls as a substrate, sugar consumption was the lowest. These results suggest that the variety of sugars impregnated in the soybean hulls inhibited rhamnosidase activity. Invertase activity was lower in test 2 supernatants compared to test 1 supernatants (Table 2). Thus, the quantity of sucrose was moderate and insufficient to inhibit rhamnosidase activity. The largest consumption of sugar

was observed in test 3, which utilized rice straw as a supporting substrate. The invertase activity was the lowest value (Table 2), suggesting both a lower quantity of sucrose impregnated in the substrate and absence of rhamnosidase inhibition so that resulted in its increased activity. In test 4, which examined a mixture of sugar cane bagasse and soybean hulls, the sugar consumption was similar to that in test 1. However, increased rhamnosidase activity was observed due to the decreased amount of sugar in the soybean hulls and the moderate quantity of sucrose. In fact, soybean hull sugar levels were approximately half of the values observed in test 2. In test 4, the invertase activity levels were similar to that in test 2.

Table 2. Values for α-L-rhamnosidase activity, initial sugar, sugars consumption and invertase activity for tests 1, 2, 3 and 4

Run	α-L-Rhamnosidase activity (U/mL)	Initial sugar (mg/mL)	Sugar consumption (%)	Invertase activity U/mL)
1	0.362	8.6	67	7.200
2	0.648	5.4	41	5.425
3	1.940	6.2	85	1.426
4	2.005	7.2	61	5.810

These results demonstrate that the α-L-rhamnosidase activity was related to the quantity and variety of sugars impregnated in the substrates. Elevated concentrations of sucrose repressed the naringinase complex which contains α-L-rhamnosidase activity [3,25]. Also, Kumar et al. [27] reported that low levels of carbohydrates induced the production of this enzyme. A qualitative HPLC analysis of the sugars contained in the substrates found that the soybean hulls contained glucose, sucrose, arabinose, galactose and stachyose. Meanwhile, the cane sugar bagasse contained sucrose and glucose. In contrast, rice straw only contained sucrose. The variety of sugars present in soybean hulls might suggest a change in the metabolism of *A. niger* and a subsequent decrease in α-L-rhamnosidase activity. Monti et al. [28] reported that the choice of carbon source dictated the metabolism of the microorganism in terms of α-L-rhamnosidase production and prevented the production of other enzymes. Mendoza-Cal et al. [10] reported that naringinase activity was elevated in cultures containing a smaller number of monosaccharide naringinase inhibitors.

Using our data, a predictive analysis estimated the maximum α-L-rhamnosidase activity to be 2.03 U/mL in cultures that contained 14% cane sugar bagasse, 25% soybean hulls and 61% rice straw (Fig. 4). An additional culture that was performed to validate the proposed model yielded an α-L-rhamnosidase activity level of 1.92±0.02 U/mL. This result corresponded to 94.7% of the expected value, validating the effectiveness of the predictive model and confirming the substrate proportions.

The production of α-L-rhamnosidase with the mixture of the three substrates in our study was higher than that observed in Elinbaum et al. [9]. In the substrate selection step with cultures using washed sugar cane bagasse, these authors demonstrated an α-L-rhamnosidase production of 1.7 U/mL from *Aspergillus terreus.* Mendoza-Cal et al. [10] also reported a lower production (1.46 U/mL) of naringinase from *A. niger* in SSF studies that used only grapefruit peels as substrate.

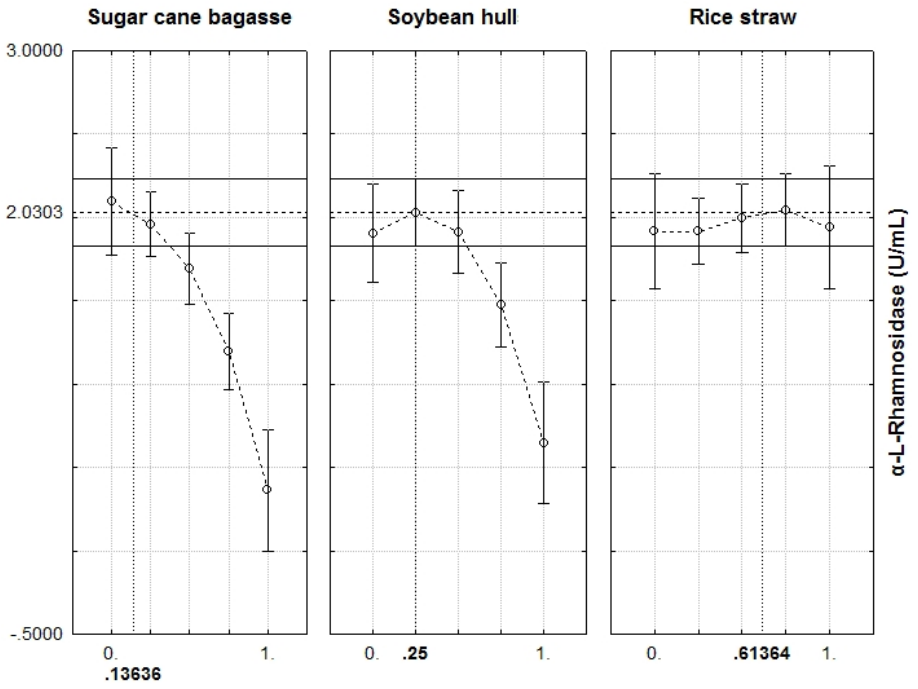

Fig. 4. Optimization of α-L-rhamnosidase production using Statitica v.9.0

The search for improved enzyme production in SSF has predominately investigated substrate characteristics such as porosity, grain size and particle shape [12]. However, culture humidity and pH are also important. Based on the results obtained from the mixture design (Fig. 4), Box-Behnken designs were performed to optimize SSF conditions for α-L-rhamnosidase production.

3.2 Optimization of SSF Conditions for α-L-rhamnosidase Production

Experiments were performed according to a 3^3 Box-Behnken design to analyze α-L-rhamnosidase production. The levels of variables and enzyme activity results are presented in Table 3.

The data from rhamnosidase production were subjected to an analysis of variance (ANOVA). The analysis revealed that two independent variables, culture humidity and nutrient solution pH, were significant (P<0.05). In contrast, substrate particle size was not significant, indicating that any of the particle sizes included in the study could be utilized. Although other authors have reported that substrate particle size affected the production of other enzymes from *A. niger* [29,30,31], no study has reported particle size to be a key variable for naringinase complex production.

Subsequent to the analysis of the results from the incomplete 3^3 Box-Behnken design, we performed the tests for the complete 3^2 Box-Behnken design with a fixed 1.5 mm particle size. The enzymatic activities are presented in Table 4. The maximal α-L-rhamnosidase activity obtained was 2.96 U/mL in a culture with 73% humidity and pH 4.0.

Table 3. α-L-rhamnosidase activity for the incomplete 3^3 Box-Behnken design

Run	Decoded variables			Rhamnosidase (U/mL)
	Humidity (%)	Granulometry (mm)	pH	
1	43	0.8	4.5	1.949
2	73	0.8	4.5	2.194
3	43	2.2	4.5	1.988
4	73	2.2	4.5	2.234
5	43	1.5	4.0	2.165
6	73	1.5	4.0	2.619
7	43	1.5	5.0	1.928
8	73	1.5	5.0	1.934
9	58	0.8	4.0	2.426
10	58	2.2	4.0	2.324
11	58	0.8	5.0	1.742
12	58	2.2	5.0	1.848
13	58	1.5	4.5	2.105
14	58	1.5	4.5	2.081
15	58	1.5	4.5	2.118

Table 4. α-L-rhamnosidase activity levels for the complete 3^2 Box-Behnken design

Run	Decoded variables		α-L- Rhamnosidase
	Humidity (%)	pH	activity (U/mL)
1	73	3.4	0
2	73	3.7	2.823
3	73	4.0	2.960
4	78	3.4	0
5	78	4.0	2.603
6	83	3.4	0
7	83	3.7	0
8	83	4.0	2.416
9	78	3.7	2.260
10	78	3.7	2.299
11	78	3.7	2.273

The statistical analysis of Table 4 data has resulted in a predictive equation which is presented below:

$$Y= 2.086 - 0.561x_1+1.330x_2 - 0.388x_1^2 - 0.498x_2^2 - 0.136x_1x_2 \quad (3)$$

The significance of the effects of the variables on α-L-rhamnosidase production was analyzed using ANOVA; these data are presented in Table 5. Culture humidity and pH were highly significant for enzyme production ($P<0.05$). These results are consistent with studies by Singhania et al. [11] and Hamidi-Esfahani et al. [32], who emphasized the importance of these variables for the production of enzymes by *A. niger*.

Table 5. Analysis of variance (ANOVA) for the α-L-rhamnosidase activity levels from the complete 3^3 box-behnken design

	Sum of square	Degree of freedom	Mean square	F	*p*-value
(1) Humidity (%) L+Q	2.27036	2	1.135180	2878.73	0.000347
(2) pH L+Q	11.23842	2	5.619210	14249.90	0.000070
1*2	0.07398	1	0.073984	187.62	0.005288
Lack of Fit	2.69271	3	0.897568	2276.17	0.000439
Pure Error	0.00079	2	0.000394		
Total SS	16.63422	10			

The response surface analysis (Fig. 5) revealed that, at the levels studied, α-L-rhamnosidase activity was higher for cultures with lower humidity levels (73%) and higher pH values (4.0).

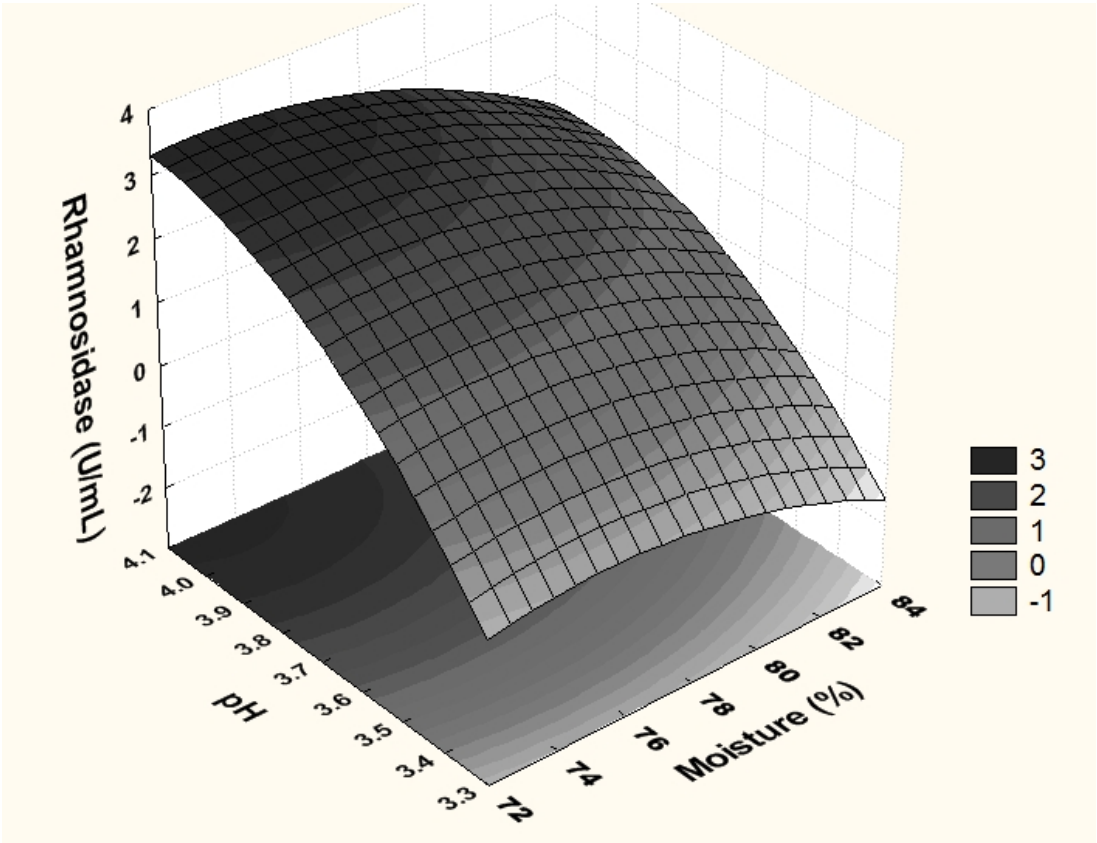

Fig. 5. Surface response plot resulting from the influence of the interaction between pH and humidity on α-L-rhamnosidase activity according to 3^3 Box-Behnken design

These results were submitted to a predictive analysis according to equation 3, which estimated a maximum α-L-rhamnosidase activity of 3.10 U/mL in a culture with 75.5% humidity and a pH of 4.0. A validation culture exhibited a maximal α-L-rhamnosidase activity of 3.02±0.03 U/mL, which corresponded to 97.4% of the expected value. This result validated

the effectiveness of the predictive model and confirmed the optimal cultivation conditions that were determined.

For filamentous fungi, low humidity (40-60%) has been reported to be optimal for enzyme production [33]. However, our results demonstrated that 75.5% humidity was optimal for α-L-rhamnosidase production. This finding is consistent with that of Singhania et al. [11], who reported that low humidity reduces nutrient accessibility and results in decreased growth.

The optimal pH value was determined to be pH 4.0. Recent studies have reported that α-L-rhamnosidase production by Aspergillus is favored at pH values between 4 and 6.5 [7,31,34].

The maximum α-L-rhamnosidase activity value of 3.02 U/mL in this study was higher than that reported by Mendoza-Cal et al. [10], who used grapefruit peels as substrate. These authors obtained 2.58 U/mL of production after optimizing culture pH, water activity and temperature. Additionally, the α-L-rhamnosidase productivity obtained in this study was higher compared with the findings from Elinbaum et al. [9], who used sugar cane bagasse as a substrate.

By applying a statistical methodology, we achieved a 37% increase in the production of this potentially biotechnologically important enzyme compared with a mixture design.

4. CONCLUSION

Rhamnosidase production by A. niger under state solid fermentation has been investigated. When solid substrate selection was carried out, according to a mixture statistical design, the ternary mixture of each solid substrate (sugar cane bagasse, soyabean hull and rice straw) achieved better values of rhamnosidade activity and was selected for further investigation. The tests of culture humidity, nutrient solution pH and solid substrate particle size have shown that only the latter was not significant, and lastly both the optimal pH value of 4,0 and culture humidity of 75% achieved the best values of enzyme activity.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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