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Production of Cellulolytic Enzymes by *Aspergillus phoenicis* in Grape Waste using Response Surface Methodology

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Abstract The production of cellulolytic enzymes by the fungus *Aspergillus phoenicis* was investigated. Grape waste from the winemaking industry was chosen as the growth substrate among several agro-industrial byproducts. A 2×2 central composite design was performed, utilizing the amount of grape waste and peptone as independent variables. The fungus was cultivated in submerged fermentation at 30 °C and 120 rpm for 120 h, and the activities of total cellulases, endoglucanases, and β-glucosidases were measured. Total cellulases were positively influenced by the linear increase of peptone concentration and decrease at axial concentrations of grape waste and peptone. Maximum activity of endoglucanase was observed by a linear increase of both grape waste and peptone concentrations. Concentrations of grape waste between 5 and 15 g/L had a positive effect on the production of β-glucosidase; peptone had no significant effects. The optimum production of the three cellulolytic activities was observed at values near the central point. *A. phoenicis* has the potential for the production of cellulases utilizing grape waste as the growth substrate.

Keywords Agro-industrial waste · Factorial design · Cellulase · β-Glucosidases · *Aspergillus phoenicis*

Introduction

Cellulases comprise a complex of enzymes involved in the natural degradation of cellulose, the major polysaccharide of plant cells. The enzymatic complex can convert the cellulose to oligosaccharides and glucose. Microorganisms such as fungi and bacteria are important producers of cellulases, and the microbial cellulases have been used in diverse industries, such as the detergent, textile, pulp and paper, feed, wine, and beverage industries [1].

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Three major types of enzyme activities are found in cellulolytic complexes: (a) endoglucanases (endo-1,4-D-glucanohydrolase, EC 3.2.1.4), hydrolyzing at random the internal glycosidic linkages of amorphous cellulose chain, (b) exoglucanases (1,4-β-D-cellobiohydrolase, EC 3.2.1.91), acting in a progressive manner on the reducing or nonreducing ends of cellulose chains, releasing either glucose or cellobiose, and (c) β-glucosidases (EC 3.2.1.21), which hydrolyze soluble cellodextrins and cellobiose to glucose [2]. The genus *Aspergillus* has been studied for the production of many extracellular enzymes, including amylases, proteases, and cellulases [3–5]. *A. phoenicis* is described as a good producer of β-glucosidases [6–8]. However, the description of endoglucanases and exoglucanases from *A. phoenicis* has not been emphasized.

The use of agro-industrial residues as the basis for cultivation media is a matter of great interest, aiming to decrease the costs of enzyme production and meeting the increase in awareness on energy conservation and recycling. The conversion of lignocellulosic biomass to useful byproducts has long been recognized as a desirable enterprise but has been neglected over the years, and there is an increasing concern on utilization of cellulosic waste as a promising alternative source to fuel production [9]. Enzymatic hydrolysis of cellulose has a lower utility cost compared to acid or alkaline hydrolysis because the former process is usually conducted under mild conditions and does not have a corrosion problem [10]. The breakdown of cellulosic biomass is a significant and advantageous alternative to land filling and incineration, since the hydrolysis products can be potentially converted into fuels, chemicals, and food. A diversity of agro-industrial residues, such as rice straw, beat pulp, and wheat bran, has been used for the production of cellulases [11]. Large amounts of grape are processed worldwide generating a huge quantity of available grape waste as a byproduct of winemaking and the juice industry [12]. In this context, the use of grape waste can be an interesting alternative for enzyme production.

Factorial design and response surface methodology have been utilized as reliable techniques for studies on the influence of multiple factors on production of enzymes [13, 14] and other bioactive metabolites with biotechnological interest [15, 16]. The aim of this investigation was to evaluate the utilization of grape waste for the production of cellulose-degrading enzymes by *A. phoenicis* in submerged fermentation (SmF), utilizing a factorial design approach.

Materials and Methods

Microorganism and Conidia Suspension

The fungi *A. phoenicis* was maintained on potato dextrose agar (PDA; Biobras, Montes Claros, Brazil) plates at 4 °C and subcultured periodically. The culture was reactivated by transferring onto fresh PDA plates and cultured at 30 °C for 5–7 days. After, the conidia were suspended with sterile distilled water and collected with a Pasteur's capillary. The material was centrifuged at 7,000×*g* for 7 min. The supernatant was discharged, and the spores were suspended again in distilled water. The conidia concentration was determined with a Neubauer's chamber.

Selection of Growth Substrates

Carboxymethylcellulose (CMC) and agro-industrial residues (grape waste, sugar cane bagasse, rice hulls, and *Acacia mearnsii* husk) were tested with a combination of two

different sources of organic nitrogen: peptone and soy protein. The proportion of CMC and agro-industrial wastes was 10 g/L and peptone or soy protein 5 g/L. Grape waste from the wine industry was used as a carbon source, with bacteriological peptone as the nitrogen source. Grape waste consisted of grape peels and seeds that were dried for 48 h at 45 °C in a stove and then milled. The chemical composition of grape waste was determined following standard procedures [17].

Erlenmeyer flasks (500 mL) with 100 mL of medium were inoculated with 10^6 conidia per milliliter. The duration of the microorganism growth was 96 h in each growth medium. The SmF was developed at 30 °C at 120 rpm. The samples were vacuum filtered through Whatman filter paper no. 1, to separate the supernatant from the mycelia. After this, total cellulases and endoglucanase activities were measured in supernatants.

Enzyme Assays

Total cellulases and endoglucanase activities were assayed using filter paper and CMC (Sigma, St. Louis, MO, USA), respectively, as substrates [18]. The β-glucosidase activity was assayed in a reaction mixture containing 100 μL sodium citrate buffer (50 mM, pH 4.8), 10 μL of *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG; 4 mg/mL), and 100 μL of the culture supernatant. After the incubation at 50 °C during 20 min, the reaction was stopped by adding 1 mL of sodium carbonate (500 mM). The activity was estimated spectrophotometrically by reading the absorbance of the liberated *p*-nitrophenol at 405 nm (ε=18.700). One unit of β-glucosidase was defined as the amount of enzyme required for the hydrolysis of 1 μmol of substrate (*p*NPG) per minute, under the assay conditions.

Statistical Analysis

Analysis of variance (ANOVA) and Tukey's test were utilized to detect significant differences ($P<0.05$) for endoglucanases and total cellulases activities among the supernatants from the different growth media.

Second-order Factorial Design

A 2×2 plus star configuration factorial central composite design was developed for the two independent variables (grape waste and peptone concentration). Each variable was studied at five levels, with four star points and four replicates at the central point for pure error. This design was employed to fit a second-order polynomial model in which 12 experiments were required for this procedure [19]. The actual levels corresponding to the coded settings, the treatment combinations, and responses are shown in Table 1. This design is represented by a second-order polynomial regression model described by Eq. 1 to generate contour plots:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_{11}X_1^2 + b_{22}X_2^2 + b_{12}X_1X_2 \quad (1)$$

where Y predicted response; b_0, constant; X_1, grape waste (g/L); X_2, peptone (g/L); b_1 and b_2, linear coefficients; b_{11} and b_{22}, quadratic coefficients; and b_{12}, interaction coefficient.

Statistica 5.0 software (Statsoft, Tulsa, USA) was used for regression and graphical analysis of the data. The significance of the regression coefficients was determined by Student's t test, and the second-order model equation was determined by Fisher's test. The variance explained by the model is given by the multiple coefficient of determination (R^2).

Table 1 Coded values (in parentheses) and real values for factorial design and responses.

Run	X_1	X_2	FPU	Endoglucanase (UE/mL)	β-glucosidase (IU/mL)
1	(−1) 5	(−1) 2.5	0.053	0.463	45.45
2	(−1) 5	(+1) 7.5	0.059	0.545	35.31
3	(+1) 15	(−1) 2.5	0.028	0.463	40.33
4	(+1) 15	(+1) 7.5	0.095	0.666	43.56
5	(0) 10	(0) 5	0.090	0.564	44.88
6	(0) 10	(0) 5	0.106	0.615	46.41
7	(0) 10	(0) 5	0.090	0.573	46.29
8	(0) 10	(0) 5	0.079	0.574	50.36
9	(0) 10	(−1.41) 1.5	0.045	0.466	48.59
10	(0) 10	(+1.41) 8.5	0.081	0.567	51.76
11	(−1.41) 3	(0) 5	0.046	0.483	34.72
12	(+1.41) 17	(0) 5	0.070	0.572	47.79

X_1=Grape waste (g/L); X_2=peptone (g/L)

Results and Discussion

Characterization of Grape Waste as Growth Substrate

A. phoenicis was grown in several agro-industrial byproducts. Among the media tested, the fungus produced increased cellulase activity in CMC, grape waste, and sugarcane bagasse (Fig. 1). However, grape waste and sugarcane bagasse yielded elevated filter paper activity only in combination with peptone and soybean protein, respectively. Low activity was observed in rice hulls and *Acacia* husk. Grape waste was selected for further investigation, since there is limited information about potential applications for this residue.

The grape waste used in this study showed to be an alternative for the production of cellulolytic enzymes by *A. phoenicis*. Grape waste has close to one third of total fibers in its composition, the presence of about 210 g/kg of starch as other polysaccharide, and small amounts of glucose and sucrose, beside the presence of proteins and fat, both close to 100 g/kg (Table 2). This residue was previously used for the cultivation of *Monascus purpureus*, resulting in good production of pigments [16] and β-glucosidase [20]. Another grape residue, which comes from the process of pressure extraction of the grape juice industry, showed to be adequate for the production of xylanases and exo-polygalacturonase [21]. However, according to those authors, the production of cellulases was not satisfactory. It has been recently reported that grapefruit peel waste can be hydrolyzed to monomeric sugars using a combination of cellulase, pectinase, and β-glucosidase [22].

Central Composite Design

The investigation for the best conditions for the production of enzymatic activities of total cellulases, endoglucanases, and β-glucosidases as a function of the grape waste and peptone concentrations was performed using tools of experimental design and response surface methodology. The experimental conditions and results are shown in Table 1. It was possible to estimate the effects of growth substrates (grape waste and peptone) concerning to the enzymatic activities obtained in the development of the process, both varying from a bottom level (−1) to a top level (+1) of the corresponding factors [19]. Both values from the statistical *t* test and *P* values were used to confirm the relevance of studied factors.

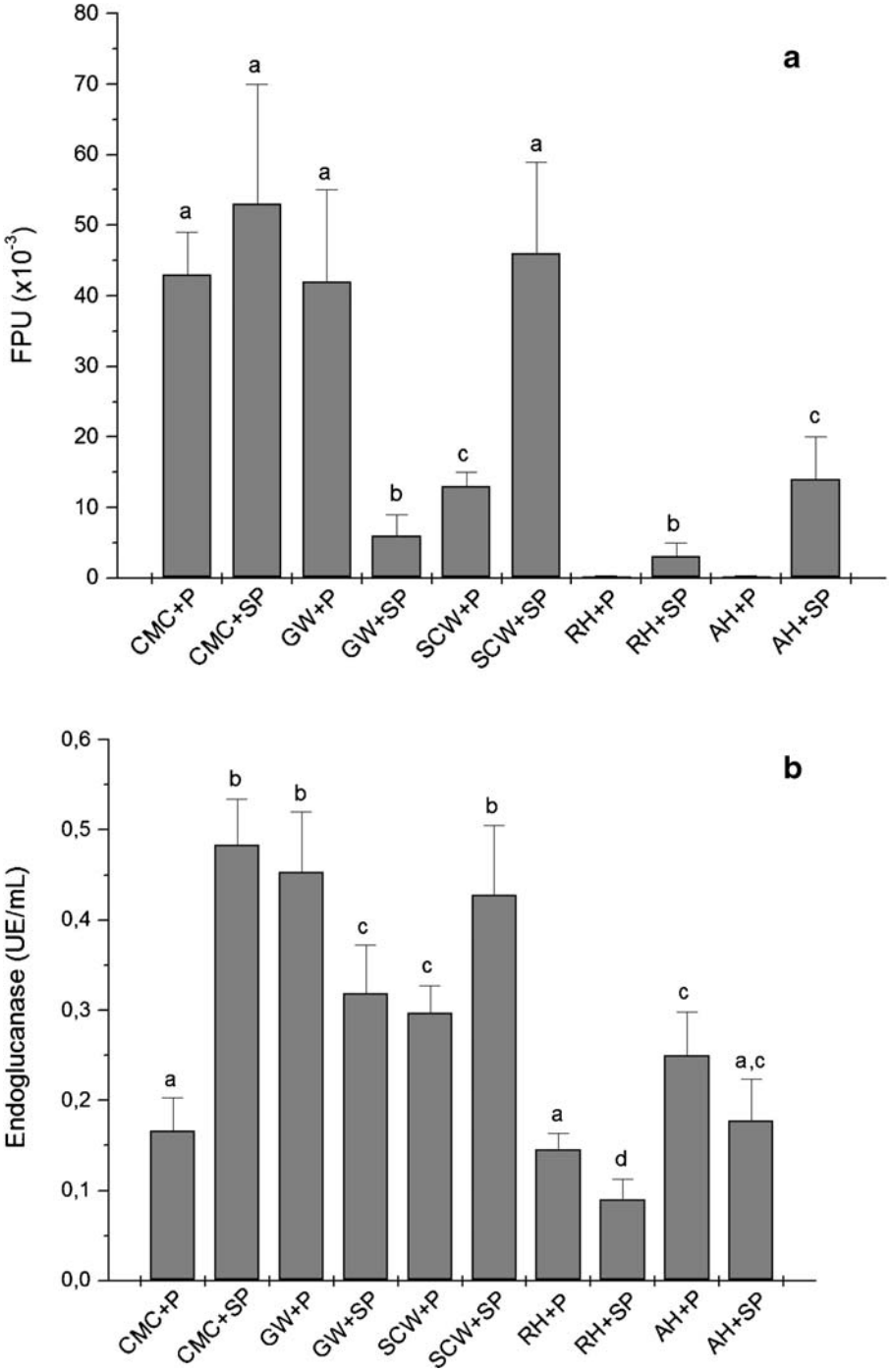

Fig. 1 Production of FPU (**a**) and endoglucanase (**b**) by *A. phoenicis* growing in different byproducts. *P* Peptone, *SP* soy protein, *CMC* carboxymethylcellulose, *GW* grape waste, *SCW* sugarcane waste, *RH* rice hull, *AH A. mearnsii* husk

Table 2 Chemical analysis of waste grape.

Component	Composition (g/kg)
Protein	109.8
Glucose	18.8
Sucrose	26.0
Starch	214.4
Total fibers	329.8
Total lipids	91.0
Ash	43.0
Moisture	81.3
Other	85.9

Production of Total Cellulases

For total cellulases, the highest activity was observed in run 4 (0.094 filter paper units [FPU]) and in the average of the four central points (0.091 FPU), while the least activity was observed in run 3 (Table 1). The effects of each independent variable and their interactions on FPase activity is presented in Table 3. The change in the concentration of grape residue from the axial level (−1.41) to the top axial level caused a reduction in the enzymatic activity, about 0.034 FPU, which was significant to a confidence level of 90%. The effect of the grape residue varying from −1 to + 1 was not significant at the level of 90% (P=0.247).

The concentration of peptone, varying between 2.5 and 7.5 g/L, increased the enzymatic activity around 0.031 FPU. However, when the peptone concentration decreases to 1.5 g/L or increases to 8.5 g/L, a decrease of 0.029 FPU was observed (Table 3). The combined effect of the grape residue with peptone promoted the increase of the enzymatic activity, around 0.030 FPU (P=0.074). To build a second-order model, which can describe the enzymatic activity (dependent variable) concerning to grape waste and peptone amounts, the ANOVA was employed for the determination of significant parameters. The regression equation obtained indicated the R^2 value of 0.88 (a value of R^2>0.75 indicates the aptness of the model). This value ensured a satisfactory adjustment of the quadratic model to the experimental data and indicated that the model could explain 88% of the variability in the response. Based on the F test, the sample is able to describe the enzymatic activity related to the independent variables. The F value was calculated as 14.01, while the tabulated F was 2.96 at the confidence level of 90%. The higher value of the calculated F related to the tabulated F allowed the establishment of Eq. 2, with enzymatic activity according to the

Table 3 Main effects and interaction analysis for FPU in submerged cultivation of *A. phoenicis*.

Factor	Effect FPU	Standard error	t value	P value
Mean	0.091	0.005	16.41	0.0005*
Grape waste (L)	0.011	0.008	1.43	0.247
Grape waste (Q)	−0.034	0.009	−3.87	0.030*
Peptone (L)	0.031	0.008	3.97	0.028*
Peptone (Q)	−0.029	0.009	−3.29	0.046*
Grape waste×peptone	0.030	0.011	2.69	0.074*

R^2 =0.88

*Significant factors, P<0.1

independent variability. The codified sample was used to generate the response surface graph showed in Fig. 2a.

$$Y(FPU) = 0.091 + 0.015X_2 - 0.017X_1^2 - 0.014X_2^2 + 0.015X_1X_2 \quad (2)$$

Production of Endoglucanase

Production of endoglucanase activity (CMCase) followed a similar behavior to that observed for total cellulases. Maximum activity was obtained in run 4 (0.666 unit of enzyme activity [UE]/mL), while runs 1, 3, 9, and 11 had the lowest activities (Table 1). For this enzymatic activity, the independent variables had a significant value for $P<0.1$ (Table 4). As observed for the total cellulase activities, the concentration of grape residue and peptone at the axial levels decreased the enzymatic activity in 0.048 and 0.058 UE/mL, respectively. However, the linear effect of grape waste concentration was positive (0.062 UE/mL) to a significant level of 90%, and $P=0.03$, which contrasted with the P value observed for the same variability in the total cellulases. For peptone, a positive effect was observed in the interval of 2.5 to 7.5 g/L (Table 4). Information about the effects of the nitrogen source on the production of fungal cellulases is relatively scarce. Stewart and Parry [23] verified that the use of ammonium sulfate or ammonium nitrate as a source of nitrogen were more effective for the production of endoglucanase and exoglucanases by *Aspergillus fumigatus* at the end of 16 days of submerged cultivation. *Aspergillus niger* synthesized exoglucanases during growth on corn steep liquor associated to different nitrogen sources, like ammonium nitrate, ammonium sulfate, sodium glutamate, sodium nitrate, and urea [24]. In *Pleurotus dryinus*, the production of lignocellulosic enzymes did not have a dependent effect in relation to the different nitrogen sources tested namely, potassium nitrate, ammonium sulfate, ammonium nitrate, and peptone [25].

The results from Table 1 were used to build a second-order model, with the endoglucanase activity according to the grape residue and peptone. The ANOVA of the regression model showed its significance ($R^2=0.90$), with the calculated F value as 12.57, four times higher than the tabulated $F=3.11$, for a significance level of 90%. The codified sample was used to draw a response surface graph (Fig. 2b), as well as Eq. 3, which describes the endoglucanase activity according to the independent variables.

$$Y(\mathrm{UE/mL}) = 0.581 + 0.030X_1 + 0.053X_2 - 0.024X_1^2 - 0.029X_2^2 + 0.030X_1X_2 \quad (3)$$

Production of β-Glucosidase

Concentrations of grape waste between 5 and 15 g/L had a positive effect on the production of β-glucosidase (Table 5). However, when the concentrations of the grape residue went to axial levels, the activity decreased to 8.06 UE/mL. The lowest activity was observed in run 11, containing 3 g/L of grape waste. The effect of peptone on the production of β-glucosidase activity was not significant, with the P value calculated as 0.739 at levels −1 to + 1 and 0.66 at levels −1.41 to +1.41. Even though peptone does not cause any significant effect in the enzyme production, the interaction between the independent variables showed to be significant in a confidence level of 90% ($P=0.066$). The model generated by ANOVA showed that the higher levels of β-glucosidase activity were obtained with grape waste and peptone concentrations close to those observed for total cellulases and endoglucanase

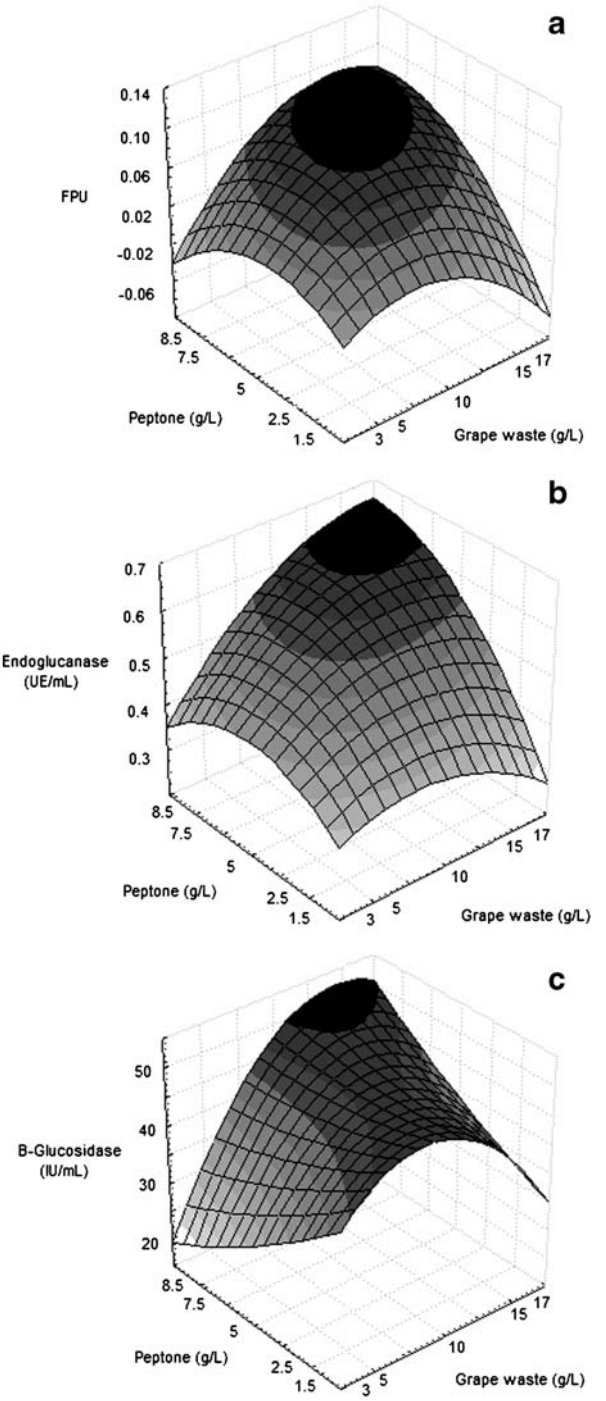

Fig. 2 Response surface for production of FPU (**a**), endoglucanase (**b**), and β-glucosidase (**c**) as a function of grape waste and peptone concentrations

Table 4 Main effects and interaction analysis for endoglucanases activity in submerged cultivation of *A. phoenicis*.

Factor	Effect (UE/mL)	Standard error	*t* value	*P* value
Mean	0.581	0.011	51.34	<0.001
Grape waste (*L*)	0.062	0.016	3.86	0.030*
Grape waste (*Q*)	−0.048	0.018	−2.66	0.076*
Peptone (*L*)	0.107	0.016	6.67	0.007*
Peptone (*Q*)	−0.058	0.018	−3.25	0.047*
Grape waste×peptone	0.061	0.022	2.68	0.075*

R^2 =0.90

*Significant factors, P<0.1

activities (Fig. 2c). The calculated F value was 7.14, higher than the tabulated F=2.92. Equation 4 was obtained, which describes the enzymatic activity for β-glycosidase according to the independent variables:

$$Y(\mathrm{UE/mL}) = 47.36 + 2.70X_1 - 4.12X_2 + 3.34X_1X_2 \quad (4)$$

A number of works describe β-glucosidase in the genus *Aspergillus*. Hang and Woodams [26] showed the use of apple residues for enzyme production in solid-state fermentation by *Aspergillus foetidus*. The importance of β-glucosidase for ethanol production has been shown during the fermentation process by *Aspergillus oryzae*, a fungus utilized in the rice solid fermentation for sake production [27]. *Botrytis cinerea* and *Penicillium brasilianum* produced higher β-glucosidase and endoglucanase activities than *A. niger* and *Trichoderma reesei* when cultivated in alkali-treated wheat husk [28]. The use of different carbon sources, such as Solka Floc, spruce, willow, and corn stover, showed to be an important factor for β-glucosidases production by *Trichoderma reesei* [29]. However, β-glucosidase production by *Trichoderma* is not as high as *A. phoenicis*, which justifies the use of mixed cultures for the production of an efficient cellulolytic complex [7, 30], reinforcing the capability of *A. phoenicis* as a good enzyme producer .

The use of agro-industrial wastes may represent an alternative to the added value to the industries and confront it with the search of renewable sources of raw material. Among

Table 5 Main effects and interaction analysis for β-glucosidase activity in submerged cultivation of *A. phoenicis*.

Factor	Effect (UE/mL)	Standard error	*t* value	*P* value
Mean	47.0	1.18	39.90	<0.001*
Grape waste (*L*)	5.40	1.67	3.24	0.048*
Grape waste (*Q*)	−8.06	1.87	−4.31	0.023*
Peptone (*L*)	−0.61	1.67	−0.36	0.739
Peptone (*Q*)	0.91	1.87	0.49	0.660
Grape waste×peptone	6.69	2.35	2.84	0.066*

R^2 =0.670

*Significant factors, P<0.1

several agro-industrial residues, rice straw [11], corn stover [31], beet pulp [32], and manure [8] have been studied with the goal of obtaining cellulases. The factorial design made in this paper showed the possibility of producing a cellulolytic complex in SmF, using grape waste as the carbon source. SmF is characterized to be a process that allows a standard and a rationalization of the bioprocess, which is necessary for industrial purposes [33].

Conclusions

In this work, factorial design and response surface methodology were employed for the production of cellulolytic enzymes by *A. phoenicis* growing on an agro-industrial byproduct, grape waste. Cultivation on grape waste resulted similar enzyme yields to those obtained with CMC as the carbon source. The enzymatic complex has prominent β-glucosidase activity, but all of the three activities measured had their highest values near the central point, suggesting that both endo- and exoglucosidases are maximally produced under the same conditions. The utilization of grape waste from the wine industry, which is highly available in Southern Brazil, represents a potential alternative for cellulase production by *A. phoenisis*.

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