PRODUCTION AND CHARACTERIZATION OF CARBOXYMETHYL CELLULASE BY SUBMERGED FERMENTATION OF *STACHYLIDIUM BICOLOR*

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ABSTRACT

Microorganisms that are capable of degrading lignocellulolytic materials can produce extracellular cellulase complexes. Fungal are good producers of cellulolytic complex, since these microorganism sources have a high capacity of multiplication and production of various enzymes, enabling industrial production. At this work, we researched the production of the enzyme carboxymethyl cellulase (CMCase) by the fungus *Stachylidium bicolor*. The pH and temperature optimum were studied by Response surface methodology with different temperatures (30, 50, 70ºC) at different pHs, ranging from 2 to 6. The effect of substances (sodium chloride, sodium benzoate and monosodium phosphate) was studied as stabilizer CMCase using a simple-centroid mixture design. The enzyme showed optimum pH and temperature at 4.0 and 50 ˚C, respectively. Monosodium phosphate showed higher and positive EP, suggesting that the mixture with a higher proportion of this component provides a higher enzymatic activity.

Keywords: Fermentation. Fungal. Production. Response surface methodology. Characterization.

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INTRODUCTION

Microorganisms are an excellent alternative for production of cellulolytic complex, since these sources have a high speed of multiplication, are adaptable to various nutrient media few expansive, produce enzymes of commercial (Loureiro et al., 2018, Santana et al., 2023). Microorganisms that are capable of degrading lignocellulolytic materials normally produce extracellular cellulase complex. Microorganisms, including fungi, produce cellulases, an inducible biocatalyst, at different phases of their development on cellulose substrates (Sutaoney et al., 2024).

Utilization of microbial enzymes has been widely reported for centuries, but the commercial use of enzymes has been recently adopted (Ejaz et al., 2021). Cellulases has applications in various commercial sectors including agriculture, brewing, laundry, pulp and paper and textile industry. Cellulases can be used to tenderize fruits, clarify the fruit juices, reduce roughage in dough, hydrolyze the roasted coffee, extract tea polyphenols and essential oils from olives and can increase aroma and taste in food items (Ejaz et al., 2021).

The microbial conversion of cellulose to soluble products requires the action of various types of glycosidic hydrolytic enzymes, such as endoglucanases or carboxymethyl cellulase (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.91 and β-glucosidase (EC 3.2.121) (Henrrissat 1991, Lynd et al., 2002; Hua-Li et al., 2007, Santana et al., 2023). The use of cellulase-producing microorganisms and the development of new technological routes for cellulase production remain a strategic issue to be considered during the development of a sustainable process for ethanol production from biomass (dos Reis et al., 2015; Juturu and Wu, 2014, Pirota etal., 2016; Loureiro et al. 2018). Sugarcane bagasse has potential for industrial use to ethanol production due to its great abundance in countries as Brazil generating a large amount of agro-industrial waste which can be used and transformed into a value-added product employing cellulases enzymes (Santana et al., 2023).

The diversity of fungi in the Caatinga is also high, with conidial fungi being important components of the mycobiota (Cruz; Gusmão, 2009). *Stachylidium bicolor* is a fungus that is part of the class Chaetosphaeriales, phylum Ascomycota (Almeida et al., 2017).

This study describes the optimization of pH and temperature using response surface methodology and stabilization of carboxymethyl cellulase (CMCase) from *Stachylidium bicolor.*

MATERIAL AND METHODS

MICROORGANISMS

The microorganism *Stachylidium bicolor* was obtained from the Bahia Microorganism Culture Collection (CCMB) of the State University of Feira de Santana (UEFS) and reactivated in Batata-Dextrose Agar (BDA) medium for 10 days in a B.O.D. incubator at 28ºC.

FERMENTATION AND OBTAINING THE ENZYME EXTRACT

The enzyme extract was obtained according to the methodology described by Delabona et al. (2012; 2019). The composition of the pre-culture medium was adapted from Mandels and Weber (1969) using 10 g/L of glucose as a carbon source. The composition of the pre-inoculum medium was: 1 mL of Tween 80; 0.3 g/L of Urea; 2.0 g/L of KH_2PO_4; 1.4 g/L of $(NH_4)_2SO_4$; 0.4 g/L $CaCl_2.2H_2O$; 0.3 g/L of $MgSO_4.7H_2O$; 1.0 g/L of peptone; 5.0 mg/L of $FeSO_4.7H_2O$; 1.6 mg/L of $MnSO_4.4H_2O$; 1.4 mg/L of $ZnSO_4.7H_2O$; 2.0 mg/L $CoCl_2.6H_2O$. The culture medium was prepared with the same pre-culture medium.

To prepare the inoculum, a conidia suspension of the fungus was prepared by adding 20 mL of distilled water and Tween 80 (0.1%) to the cultured BDA plates until approximately 10^{-7} cells. This suspension was transferred to Erlenmeyer flasks containing 50 mL of the pre-culture medium, incubating 72 h at 29°C in a rotary shaker at 200 rpm.

To fermentation to produces CMCase enzyme a volume of 20 mL of the inoculum was then transferred to Erlenmeyer flasks containing 180 mL of the production medium containing sugarcane bagasse in natura as inducer. The assays were incubated for 10 days at 29°C and 200 rpm in a refrigerated shaker incubator. The samples were centrifuged at 10,000 rpm, 10°C for 15 min and analyzed for enzymatic activity obtained.

CARBOXYMETHYL CELLULASE (CMCASE) ENZYME ACTIVITY

The reaction tubes were prepared with 0.25 mL of the substrate carboxymethylcellulose (CMC) at 0.5% (w/v) prepared in a buffer solution citrate phosphate 50mM and 0.25 mL of enzymatic extract (crude extract). To control the reaction, 0.25 mL of buffer solution and 0.25 mL of enzymatic extract were added. The reaction white was composed of 0.25 mL of buffer solution and 0.25 mL of the substrate carboxymethyl cellulase (CMC). The samples were incubated at 50° C for 30 minutes. After incubation, the tubes were placed in an ice bath, and then 0.5 mL of the DNS reagent was added. Subsequently, the tubes were submerged in boiling water (95ºC) for 15 minutes and, after cooling, 2.5 mL of distilled water was added to the tubes. The absorbance reading was

performed at 540 nm (Santos et al., 2011). The assay was carried out in replicate. This was chosen to conduct the next essential experiments for the development of the work.

PH AND TEMPERATURE OPTIMUM

The effect of temperature on the activity of carboxymethyl cellulase was determined by assays of enzymatic activity in reactions with different temperatures (30, 50, 70ºC) at different pHs, ranging from 2 to 6. As shown in Table 3. Doehlert experimental design was used, with two variables, equivalent to 9 experiments, having its central point in experiments 4, 5 and 6 (Table 1), with tests conducted in replicate.

The enzymatic activity of the carboxymethyl cellulase obtained at each point was determined as previously described and used to plot the response surface graph using the Statistic 7.0 program.

MULTIVARIATE OPTIMIZATION FOR ACTIVITY STABILIZATION

The experimental conditions were optimized using a simplex-centroid mixture design. A total of nine experiments were performed randomly, as shown in Table 2, including repetitions of the central point to estimate the experimental error. The mixture was composed of sodium chloride, sodium benzoate and monosodium phosphate, modeled in the proportion from 0 to 100% (concentration of 1 mol L^{-1}). The enzymatic activity (U mL^{-1}) was used as an experimental response to obtain the mathematical models that were evaluated from the analysis of variance (ANOVA). The statistical significance of the regression, the coefficients of determination (R^2) and the absence of lack of fit were considered (Azcarate; Pinto; Goicoechea, 2020; Bezerra et al., 2020). Data were processed using the Statistic 12 software (StatSoft, Tulsa, United States) at a confidence level of 95% ($p = 0.05$).

STATISTICAL ANALYSIS

The data obtained were submitted to analysis of variance (ANOVA) obtained with the aid of the Statistica program, as well as a response surface graph, analysis of variance, regression index, R^2, among other statistical parameters.

RESULTS AND DISCUSSION

OBTAINING THE ENZYME EXTRACT

The results showed the use of nature (fresh) sugarcane bagasse showed an activity of 0.12 μmol $min^{-1.}$ These result evidence the utilization of sugarcane bagasse in production of carboxymethyl cellulase (CMCase) by the fungus *Stachylidium bicolor.*

PH AND TEMPERATURE OPTIMUM

pH and temperature are among the most important parameters, which directly affect the production of enzymes (Behera et al., 2017). Then, to analyze these variables, the Doehlert experimental design was applied to evaluate the effect of these parameters on the enzyme CMC obtained from *Stachylidium bicolor* (Table 1).

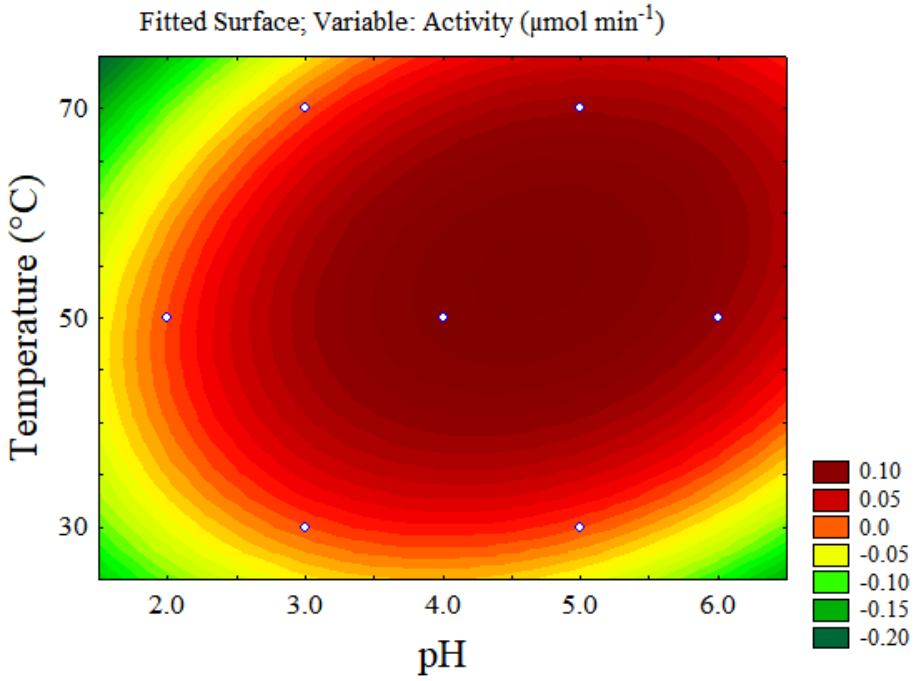
Table 1. pH and Temperature Characterization: Doehlert Experimental Design and Specific Activity

Sample	pH	Temperature (°C)	Activity (μmol min^{-1})
1	3 (-0.5)	70 (+0.866)	0
2	5 (+0.5)	70 (+0.866)	0.077
3	2 (-1.0)	50 (0.0)	0
4 (CP*)	4 (0.0)	50 (0.0)	0.1152
4 (CP*)	4 (0.0)	50 (0.0)	0.1150
4 (CP*)	4 (0.0)	50 (0.0)	0.1151
5	6 (+1.0)	50 (0.0)	0.0821
6	3 (0.0)	30 (-0.866)	0
7	5 (+0.5)	30 (-0.866)	0

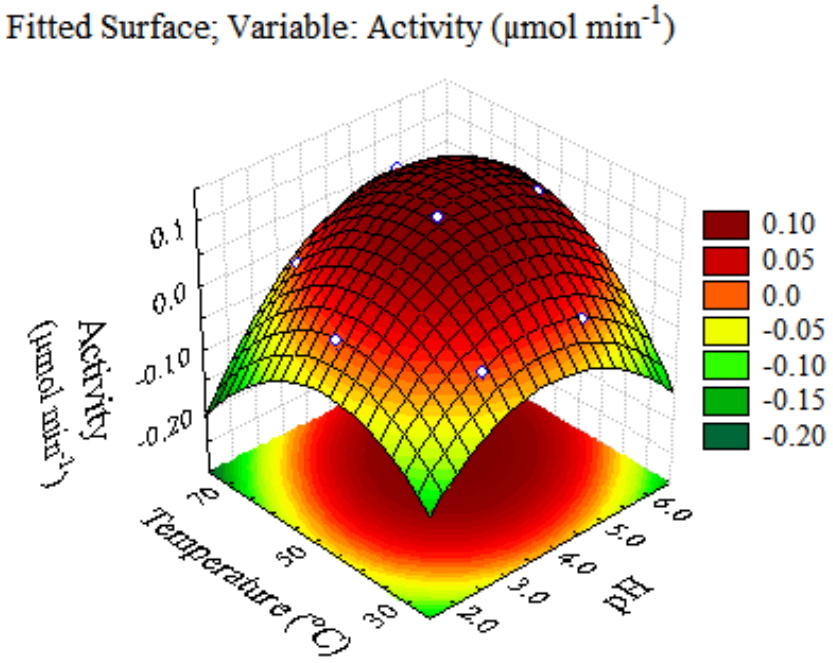
*CP = Central Point

The Statistica program was used to generate the response surface graph, which shows the influence of pH and temperature on the production of cellulase from the microorganism *Stachylidium bicolor* (Figure 1, A and B).

Figure 1. A-pH and Temperature Characterization: Area Graph. B-Response surface chart.



A



B

According to the analysis of the data obtained, the activity was higher at pH 4.0 at 50ºC, with an activity of approximately 0.115 µmol min^{-1}.

The experimental result obtained with the Doehlert design had its equation adjusted to the data obtained in the enzymatic characterization which describes the response surface, namely:

(UA) = -0.6004 + 0.1200 x (pH) – 0.0185 x (pH)² + 0.0164 x (Temperature) – 0.00019 x (Temperature)² + 0.0009625 x (pH) x (Temperature).

Table 2 shows the validity of the models obtained through experimental design can be observed by analysis of variance (ANOVA), obtaining a value of R^2 equal to 0.99. The model obtained for the activity on cellulase was validated with a calculated F of 3284.2, higher than the tabulated F (9.01), showing that the function is well adjusted to the answers obtained.

pH and temperature values influenced activity, showing higher activity at pH 4.0 and 50°C, with the critical values presented by the model in the study of 4.65 and 54.11°C. The Predicted value was 0.1236 μmol min^{-1}.

Table 2. pH and Temperature Characterization: analysis of variance (Anova)

Variation source	QS[a]	DF[b]	QM[c]	F calculated	F Tabulated (CI[d] 95 %)
Regression	0.0241	5	0.0048	3284.2517	9.01
Residual	0.0000	3	0.0000		
Lack of Fit	0.000004	1	0.000004	0.0000	0.00
Pure Error	0.000000	2	0.000000		
SQ total	0.024146	8			

a = QS, Quadratic Sum; b = DF, Degrees of Freedom; c = QM, Quadratic Mean; d = CI, Confidence Interval.

The Pareto chart was also generated, which aims to describe an order under which factors will present better results in reducing losses.

Figure 2. pH and Temperature Characterization: Pareto Graph.

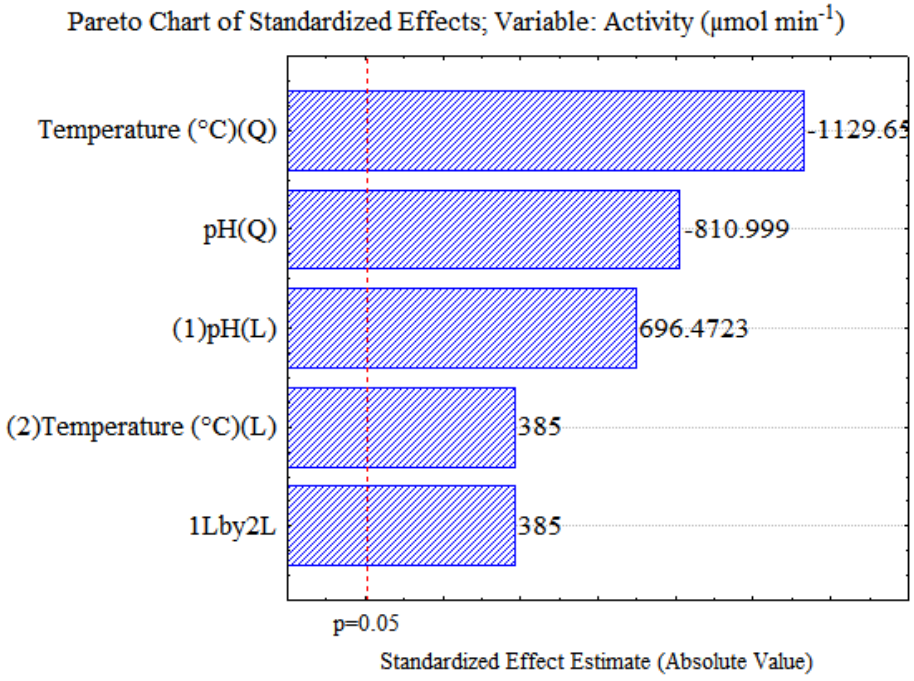


Figure 2 - Cellulase enzyme area chart – influence of temperature under pH
Source: The author himself.

In general, the cellulases produced by filamentous fungi have optimal pH values in the acid range (3.6-5.0) (Castro; Pereira JR, 2010). Para Sales et al., (2010) the pH with the best result was 5.0, according to Sohail et al., (2009). The best results for obtaining fungal cellulase occurred at pH 4.0. Buntić et al. (2016) described better results obtained at a pH ranging between 5.0 and 7.0, reaching their maximum values closer to pH 7.0 for the fungi used.

To determine the best temperature of filamentous fungi activity to obtain cellulosic enzymes, Castro; Pereira Jr. (2010) described that the best results were obtained in the temperature range was 50ºC, while Santos (2014) reports better results in the production of fungal cellulase at a temperature of 35ºC. According to Sales et al., (2010) the best results were found at a temperature of 33ºC and for Buntić et al. (2016) the best result was achieved at a temperature of 45ºC. Thus, as described in Table 1, for the present study the best result obtained for obtaining fungal cellulosic enzymes was at a temperature of 50ºC at a pH of 4.0.

The results showed similarity with several studies with endoglucanases from different organisms, which have pH 7.0 and optimum activity at temperature of 50°C (Romaniec, 1992; Saha 2004; Wen et al., 2005; Dutta et al, 2008; Heidorne et al, 2006). Results showed that in *Bacillus amyoliquefaciens* the temperature and pH optimum cellulase activity was 50°C and pH 7.0, respectively (Lynd et al., 2002). Padilha *et al* (2015) studied the production of cellulase from thermophilic strain *Bacillus* sp. C1AC5507 and found that the optimum temperature and pH for the CMCase production were 70 °C and 7.0, respectively.

Production of cellulases by *Ceriporiopsis subvermispora* cult on wood chips of *Eucalyptus grandis* and *Pinus taeda* was studied. The biochemical characteristics of cellulases produced in both wood species were almost the same. The optimum pH for these enzymes was between 4.0 and 5.0 and the optimum temperature was 60 °C (Romaniec et al., 1992).

Aspergillus niger endoglucanase activity showed an increase in activity when incubated at temperatures of 30 and 35°Cat pH 4 with a reduction in pH 5 (Sohail et al, 2009). Characterization studies of cellulase from *Phanerochaete chrysosporium* BKM-F-1767 showed values of pH 4.6 and 60°C as great to the maximum activity of cellulose (Khalil 2002). Studies show that pH and temperature of purified endoglucanase has similarity with endoglucanase of crude extract. The cellulases of *Bacillus* were optimally active in the pH range of 5-6.5. The optimum temperature was 65 and 70°C for the endoglucanase of CH43 and HR68, respectively Mawadza et al (2000).

Then, is it possible to conclude that CMC cellulase shows pH and temperature optimum that are in the range observed in the literature.

MULTIVARIATE OPTIMIZATION OF THE STABILIZING MIXTURE

To analyze the effect of substances in enzyme activity, the stabilizing mixture composed of sodium chloride (A), sodium benzoate (B) and monosodium phosphate (C) was investigated in a multivariate manner using a simple-centroid mixture design. The experimental matrix resulting from the combination of the components in proportions ranging from 0 to 1.00 mL and the experimental response (enzymatic activity) are presented in Table 9.

Table 3 - Experimental matrix with experiments with the combination of components and evaluated experimental response (Enzymatic activity).

Experiment	Sodium chloride (mL)	Sodium benzoate (mL)	Monosodium phosphate (mL)	Activity (U mL^{-1})
1	1.00	0.00	0.00	0.025
2	0.00	1.00	0.00	0.019
3	0.00	0.00	1.00	0.030
4	0.50	0.50	0.00	0.025
5	0.50	0.00	0.50	0.034
6	0.00	0.50	0.50	0.032
7 CP*	0.33	0.33	0.33	0.035
8 CP*	0.33	0.33	0.33	0.033
9 CP*	0.33	0.33	0.33	0.034

*CP = Central Point
Source: The author himself.

The mathematical models were obtained using experimental data and evaluated using ANOVA (Table 4).

Table 4 - Analysis of variance for the quadratic model

Source of variation	SS	Df	MS	Fcalculated	p-value
Regression	$2.35E^{-4}$	5	$4.69E^{-5}$	26.7	0.011
Lack of fit	$3.28E^{-6}$	1	$3.28E^{-6}$	3.3	0.212
Pure error	$2.00E^{-6}$	2	$1.00E^{-6}$		
Total	$2.40E^{-4}$	8	$3.00E^{-5}$		

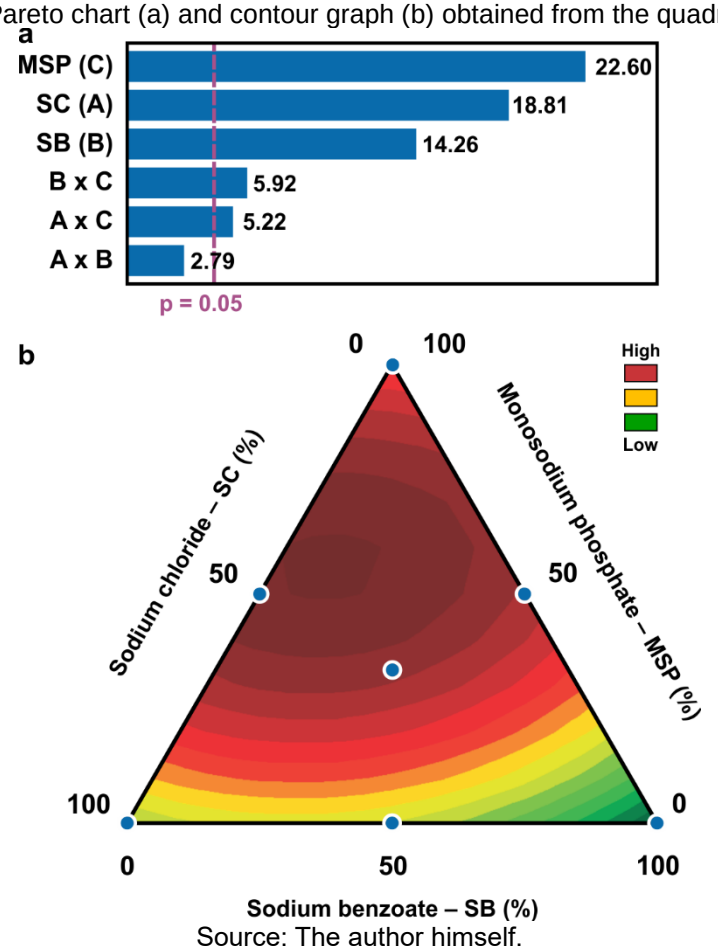
Quadratic sum (SS); degree of freedom (Df); mean square (MS); coefficient of determination (R^2).
Source: The author himself.

Considering the possible mathematical models, the quadratic had better descriptive potential. The regression was significant ($p < 0.05$), that is, it can be used consistently to describe the behavior of the experimental response as a function of the variation in the

proportion of the components of the mixture. In addition, the quadratic model did not provide evidence of misfit, indicated by the lack of statistical significance for the lack of fit (p = 0.212). Thus, a model has been obtained whose regression was significant and the lack of fit was not significant, being considered adequate for evaluating the optimal condition (Novaes et al. 2017). Also, the high descriptive capacity of the model is evidenced by the value of $R^2 = 0.978$.

The model obtained was adequate and from this, it was possible to estimate and evaluate the impact of the standardized effect (SE) of the mixture components and their interactions in the variation of the experimental response. The SE of the components and interactions are shown in the Pareto chart (Figure 3a). Monosodium phosphate presented a higher and positive EP, suggesting that the mixture with a higher proportion of this component provides a greater enzymatic activity. In addition, the other components have effects of the same magnitude and positive. Only the interaction effect between sodium chloride and sodium benzoate was not significant.

Figure 3. Pareto chart (a) and contour graph (b) obtained from the quadratic model



Source: The author himself.

Finally, considering the high predictive capacity, the mathematical equation (Equation 1) was obtained, which describes the behavior of the experimental response as a function of the proportion of components in the mixture shown by the contour graph (Figure 3b).

$$Enzymatic\ activity\ (U\ mL^{-1}) = 0.025A + 0.019B + 0.030C + 0.016AB + 0.030AC + 0.034BC \quad \textbf{(1)}$$

The model presented a maximum condition, enabling the calculation of the coordinates of the critical points, that is, the region in the contour graph that provided the greatest experimental response. Maximum enzymatic activity was obtained using a stabilizing mixture composed of 34% sodium chloride (A), 10% sodium benzoate (B), and 56% monosodium phosphate (C). Under these conditions, the enzymatic activity predicted by the model was 0.036 U mL^{-1}.

Junqueira et al. (2023) studied the effect of preservatives in the activity of CMCase enzyme produced fungus *Aspergillus niger* ATCC 1004. These authors tested sodium chloride, monosodium phosphate and sodium benzoate at various concentrations during 72 hours on the activity of the CMCase enzyme by incubating the enzyme with these salts, in citrate-phosphate buffer pH 5.0 at 50 mM, at a temperature of 50 °C. They found the substances studied show that are good options to preserve the CMCase enzyme.

Sodium chloride is used in the food industry, in the agricultural and chemical industries as chemical preservative (Ravishankar and Juneja, 2014).

Sodium benzoate is widely used in industries as a preservative, especially in the food industry (Junqueira et al. 2023). The effect of postharvest applications of sodium benzoate on physico-chemical properties and enzymatic activities of pear fruit cv was studied by Kaur et al. (2019) that found higher efficacy in maintaining less enzymatic activity of cellulase, pectin methyl esterase and polyohenol oxidase.

Monobasic sodium phosphate or monosodium phosphate is applied in food industries as an acidity regulator. In addition, it may contribute to the stabilization of some proteins (Junqueira et al. 2023). Monosodium phosphate is used as a buffer in several stages of enzyme production by fermentative processes, such as substrate preparation, enzymatic extraction, determination of enzyme activity, etc., and thus used in various studies with this approach (Junqueira et al. 2023).

FINAL CONSIDERATIONS

The results obtained showed that it is possible to produce the enzyme cellulase from the fungus *Stachylidum bicolor*, demonstrating the viability of the fungus for use, since it is possible to be acquired in some common regions of the semi-arid region of Bahia.

The enzyme cellulase produced by *S. bicolor* showed better activity at pH 4.0 at a temperature of 50ºC.

Tests were conducted, with the addition of stabilizing mixtures which should enhance or decrease the enzymatic activity. The results obtained showed that a stabilizing mixture composed of different proportions of sodium chloride, sodium benzoate and monosodium phosphate enabled an efficient enzymatic activity in the process, which can be used together or even in mixtures in pairs: monosodium phosphate + sodium benzoate or monosodium phosphate + sodium chloride.

Therefore, the feasibility of using *Stachylidiym bicolor* in obtaining and characterizing cellulase is noted, and its use as a microorganism that produces this enzyme is possible.

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