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Research Article

# Production and Characterization of Carboxymethylcellulase by Submerged Fermentation of *Moniliophthora perniciosa*

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**Abstract**

Microorganisms that are capable of degrading lignocellulolytic materials can produce extracellular cellulase complexes. Microorganisms are an excellent alternative for the production of cellulolytic complex, since these sources have a high power of multiplication. In this work, we researched the production by the fungus *Moniliophthora perniciosa*. The production and pH and temperature optimum optimization were studied by Response surface methodology and carboxymethylcellulase (CMCase) characterization. Thermal stability was evaluated at 60, 70, 80 and 90°C. Doehlert experimental design was employed using inductor concentration in five levels (3.0, 4.5, 6.0, 7.5 and 9.0 g $L^{-1}$ of yeast extract) and fermentation time was studied in three levels (7, 14 and 21 days). The production of CMC enzyme was higher in the concentration of 7.0 g $L^{-1}$ of yeast extract and 19 days fermentation time. CMCase showed optimum pH and temperature at 7.2 and 47°C, respectively. The CMCase retained 88.66% of residual activity after 30 minutes of incubation at 90°C. Due to the characteristic of

thermal stability, this enzyme will be studied to be expressed in recombinant microorganisms.

**Keywords**
Fermentation; fungal; inducers; production; response surface methodology; characterization

## 1. Introduction

The filamentous fungus *Moniliophthora perniciosa* [1] is a hemibiotrophic Basidiomycota (*Agaricales, Tricholomataceae*) that causes witches' broom disease of cocoa (*Theobroma cacao* L.). It has been claimed as one of the most important phytopathological problems that have afflicted the Southern Hemisphere in recent decades. In Brazil, this phytopathogen is endemic in the Amazon region [1]. However, since 1989, this fungus has been found in the cultivated regions in the state of Bahia, the largest production area in the country. The fungus caused a severe decrease in Brazilian cocoa production reducing Brazil from the second-largest cocoa exporter to a cocoa importer in just a few years [2].

Microorganisms that are capable of degrading lignocellulolytic materials normally produce extracellular cellulase complexes. This set of enzymes is reflected in different sequences, structures and their hydrolytic mechanism, and performing an inversion or retention in the configuration of anomeric carbon [3]. Enzymes with similar sequences have different specificities (Exo or endo hydrolysis), which suggests that this activity results from 3D structure modifications [4].

Cellulose is the main constituent of plant cell walls and comprises units of β-D-glucopyranosyl, connected by glycosidic bridges β-1, 4. For microorganisms to hydrolyze and metabolize insoluble cellulose, extracellular cellulases must be produced that are either free or cell-associated [5]. The microbial conversion of cellulose to soluble products requires the action of various types of glycosidic hydrolytic enzymes, such as endoglucanases (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.91 and β-glucosidase (EC 3.2.121) [4, 6, 7]. Sugarcane bagasse has potential for industrial use in ethanol production due to its great abundance in countries such as Brazil generating a large amount of agro-industrial waste which can be used and transformed into a value-added product employing cellulases enzymes.

Microorganisms are an excellent alternative for the production of cellulolytic complex, since these sources have a high power of multiplication, are adaptable to various nutrient media, synthesize various chemicals, among them enzymes, and may have low cost of cultivation.

This study describes production optimization using response surface methodology and characterization of carboxymethylcellulase (CMCase) from *M. perniciosa.*

## 2. Materials and Methods

### *2.1 Chemicals Reagents*

Carboxymethylcellulose (CMC), glucose, and bovine serum albumin were purchased from Sigma (Sigma Chemical Co., St Louis, MO, USA). All the chemicals used were of high-quality analytical grade.

### *2.2 Culture Conditions of Moniliophthora perniciosa*

The microorganism used in this study was *M. perniciosa* (CCMB0257). It was obtained from the Collection of Cultures of Microorganisms of Bahia (CCMB).

To produce CMCase, mycelium inoculums with 1 cm of the diameter of the fungus (The fungus was grown for 10 days on potato dextrose broth) were transferred to Erlenmeyer flasks with 100 mL half submerged containing the following: wheat bran (40.0 g $L^{-1}$), yeast extract ranging from 3-9 (g $L^{-1}$); $K_2HPO_4$ $3H_2O$, (1.0 g $L^{-1}$); $MgSO_4$ $7H_2O$ (0.2 g $L^{-1}$); KCl (0.2 g $L^{-1}$) dissolved in distilled water. The incubation was conducted at 28°C for 7 to 21 days and 120 rev $min^{-1}$ in a rotary shaker. The liquid culture medium, on which *M. perniciosa* was grown, was filtered and centrifuged (Centrifuge 5804R – Eppendorf, S.o Paulo, Brazil) at 8,000 x g for 15 min at 4°C, and the supernatant, containing the proteins from *M. perniciosa*, was used as a crude enzymatic extract [8].

### *2.3 Doehlert Experimental Design for Enzyme Production*

The most important parameters affecting enzyme production are the time of fermentation and concentration of the inducer; in this case, yeast extract was used as enzyme inducer. Different means of propagation should be used in time to reduce production costs and increase enzyme activity to induce cellulolytic enzymes [9]. The yeast extract has an excellent nitrogen source responsible for synthesizing nucleic acids, proteins, and other compounds [9].

Response surface methodology was used to predict the production of enzymes under any conditions of time and concentration of the inducer in the experimental domain [10]. The Doehlert experimental design, with two variables (concentration of inductor and fermentation time) and three replicates at the center of the domain leading to a total of 9 experiments (Table 1) was used to obtain the knowledge of the effect of time of fermentation and concentration of the inducer on the production of enzyme Carboxymethylcellulase. The concentration of the inductor was studied in five levels (3.0, 4.5, 6.0, 7.5 and 9.0 g $L^{-1}$) and fermentation time was studied in three levels (7, 14 and 21 days). The experimental errors were evaluated from the replication of the central point. The experimental data were processed by using the STATISTICA software. All the experiments in this step were carried out in random order.

**Table 1** Doehlert matrix used for the optimization of production of cellulase *M. perniciosa.*

| Experiment | Concentration of inducer (g/L) | Time (days) |
|---|---|---|
| 1 | 3 (-1) | 14 (0) |
| 2 | 4.5 (-0.5) | 7 (-0.866) |
| 3 (C) | 6 (0) | 14 (0) |
| 4 (C) | 6 (0) | 14 (0) |
| 5 (C) | 6 (0) | 14 (0) |
| 6 | 7.5 (0.5) | 7 (-0.866) |
| 7 | 9 (1) | 14 (0) |
| 8 | 4.5 (-0.5) | 21 (0.866) |
| 9 | 7.5 (0.5) | 21 (0.866) |

*(C): central point; coded values are presented.*

### *2.4 Obtaining the Extract Cellulolytic*

After the fermentation, the medium was centrifuged at 15,000 x g for 15 minutes at 4°C. The supernatant was used as an enzymatic extract and stored in a freezer [8].

### *2.5 Protein Determination and Enzyme Analysis*

Protein concentration was determined by the method of Bradford [10] using bovine serum albumin (BSA) as a standard. The DNS method determined the enzymatic activity by quantifying reducing sugars [11]. As substrate, the enzyme activity was measured with CMC (from, Sigma). CMC was dissolved in 0.05 mol $L^{-1}$ citrate buffer (pH 5.8). The reaction mixture containing 250 µL of 0.1% CMC and 250 µL of enzyme solution was incubated at 50°C for 15 min [9].

The reaction was stopped by adding 500 µL of 1% 3.5-dinitro salicylic acid reagent and boiled for 15 min. According to Miller, the amount of reducing saccharides released from laminarin was measured spectrophotometrically at 540 nm [12]. One unit (U) of activity (µmol/min) was defined as the amount of the enzyme that catalyzes the conversion of 1 micromole of substrate per minute under the above experimental conditions.

### *2.6 pH and Temperature Optimum Determination*

A double-variable, three-level central composite design (CCD) leading to 9 sets of experiments (assays), performed in triplicate, was used to verify the optimum pH and temperature (Table 2). The STATISTICA 6.0 software (StatSoft, Tulsa, OK) was used to generate the design matrix and to analyze the results. Table 2 shows the design matrix and responses (results) obtained for CMCase activity.

**Table 2** Optimizing the effect of pH and temperature on cellulase activity of *M. perniciosa.*

| N° | pH | Temperature (°C) |
|---|---|---|
| **1** | 6.0 | 80 |
| **2** | 8.0 | 80 |
| **3** | 5.0 | 60 |
| **4** | 7.0 | 60 |
| **4** | 7.0 | 60 |
| **4** | 7.0 | 60 |
| **5** | 9.0 | 60 |
| **6** | 6.0 | 40 |
| **7** | 8.0 | 40 |

### *2.7 Thermal Stability*

Samples of CMC in test tubes (selected to be equal in weight, volume and size) were incubated in water at different temperatures (60, 70, 80 and 90°C) and for various times (0, 5, 10, 15, 20, 25 and 30 min). After the heating process the tubes were cooled in melting ice and the residual activity measurement was carried out at pH 5.8 and a temperature of 50°C.

### *2.8 Effect of Salts*

The effect of the salts (potassium bromide, calcium chloride, ammonium chloride and potassium chloride) in enzyme activity was evaluated in different concentrations (0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 mmol $L^{-1}$). The CMC activity was determined as described previously.

### *2.9 Preparation of Sugarcane Bagasse*

The treatment was performed using 100 g of cane sugar bagasse washed and ground, autoclaved (121°C for 30 minutes) with 2 L of 4% sodium hydroxide. Then the material was filtered and neutralized with 10% phosphoric acid and dried in an oven at 65°C until constant weight (adapted from Sukumaran et al. [13].

### *2.10 Tests for Hydrolysis of Sugarcane Bagasse using Crude Extract of M. Perniciosa*

The efficiency of hydrolysis of sugarcane-based ethanol was done in sodium phosphate buffer 50 mmol $L^{-1}$, pH 7.0 at 50°C, at two concentrations of enzyme extract (0.5 mg $mL^{-1}$ and 0.75 mg $mL^{-1}$). The control hydrolysis of sugarcane bagasse was carried out by quantifying the reducing sugars DNS method for overtime 0-168 minutes [14].

### *2.11 Replications and Statistical Analysis*

The enzyme tests were repeated at least three times. Significant differences between sample means were tested.

## 3. Results and Discussion

### *3.1 CMCase Production*

Several factors can be studied for cellulases, such as nutrients, carbon source and condition of incubation. The optimum conditions for the production of cellulase *M. perniciosa* were evaluated using the study of two variables through the Doehlert matrix (Table 1).

The equation below illustrates the relationship between two variables and the response, where R is the response of enzyme activity, IC is the concentration and T is time:

$$UA = 2.1819 + 0.0829(T) – 0.010196(T)^2 + 0.9329(YE) – 0.12162(E.L)^2 + 0.0425(T)(YE)$$

UA: activity; T: time fermentation; YE: yeast extract.

The results obtained from the response surface correspond to the graphics level curve for the equation described in Figure 1. According to the response surface plot shown in Figure 1, the yeast extract significantly influences cellulase activity over fermentation time. However, it can be observed that both parameters influence cellulase activity.

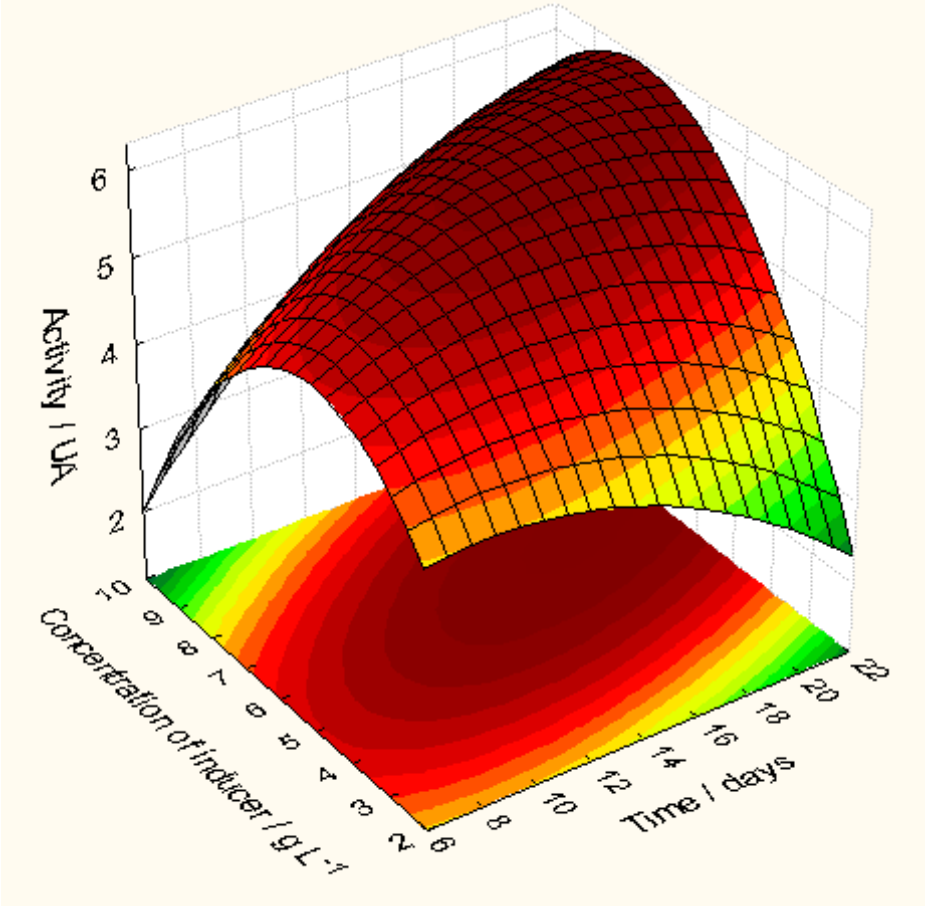


**Figure 1** Response surface from the substrate concentration versus time of incubation. The central red circle indicates the influence of fermentation time and concentration of inducer (substrate).

Analysis of variance was applied to evaluate the experimental data of the quadratic model.

Based on F test, the equation is statistically significant, yielding a calculated F of 33.16 and one F tabulated of 9.01. From the plotting of graphs for testing the concentration of yeast extract and fermentation time, it was observed that concentrations of yeast extract above 7 g $L^{-1}$ and below 5 g $L^{-1}$ showed a reduction in cellulolytic activity. Concentrations of yeast extract, between 6 and 07 g $L^{-1}$ and fermentation time of 14 and 20 days showed higher values for the production of cellulolytic complex, with a maximum activity of cellulase in a concentration of 7.15 g $L^{-1}$ yeast extract in a time in 19 days of fermentation resulting in 6.33 AU.

Microorganisms such as bacteria and fungi are good producers of cellulolytic enzymes, although fungi are more suitable for cellulase producers due to their extracellular properties that facilitate obtaining [14].

Extracellular cellulolytic enzymes were produced under solid-state cultivation by the thermophilic fungus *Thermoascus aurantiacus* and characterized [15]. High levels of endoglucanase and β-glucosidase activities were produced simultaneously by optimizing growth factors and under ideal growth conditions, the authors obtained 1572 units of endoglucanase [15].

Cellulase production by the fungi *Trichoderma reesei* was studied using dairy manure as a substrate. Using a reaction time of 6-8 days, a maximum cellulase production activity of 1.74 IU/ml of filter paper activity, 12.22 IU/ml of CMCase activity, and 0.0978 IU/ml of beta-glucosidase was

obtained [16].

Several factors may influence the production of the cellulolytic complex. Cultivation techniques in submerged medium have greater advantages in producing extracellular enzymes, mainly due to the interaction of nutrients with the microorganism.

### *3.2 pH and Temperature Optimum Determination*

The results of the tests optimization of temperature and pH of the samples of crude enzymes extracted by the software Statistica 10.0. The data shows that pH values below 7.0 and above 8.0 causes reduction in cellulolytic activity. The pH values between 7.0 and 8.0 and temperatures ranging 40-55°C showed the best activity results. The optimum pH and temperature values were 7.22 and 47°C (Figure 2). The results obtained by the response surface chart show a great influence of the effect of pH on cellulase activity.

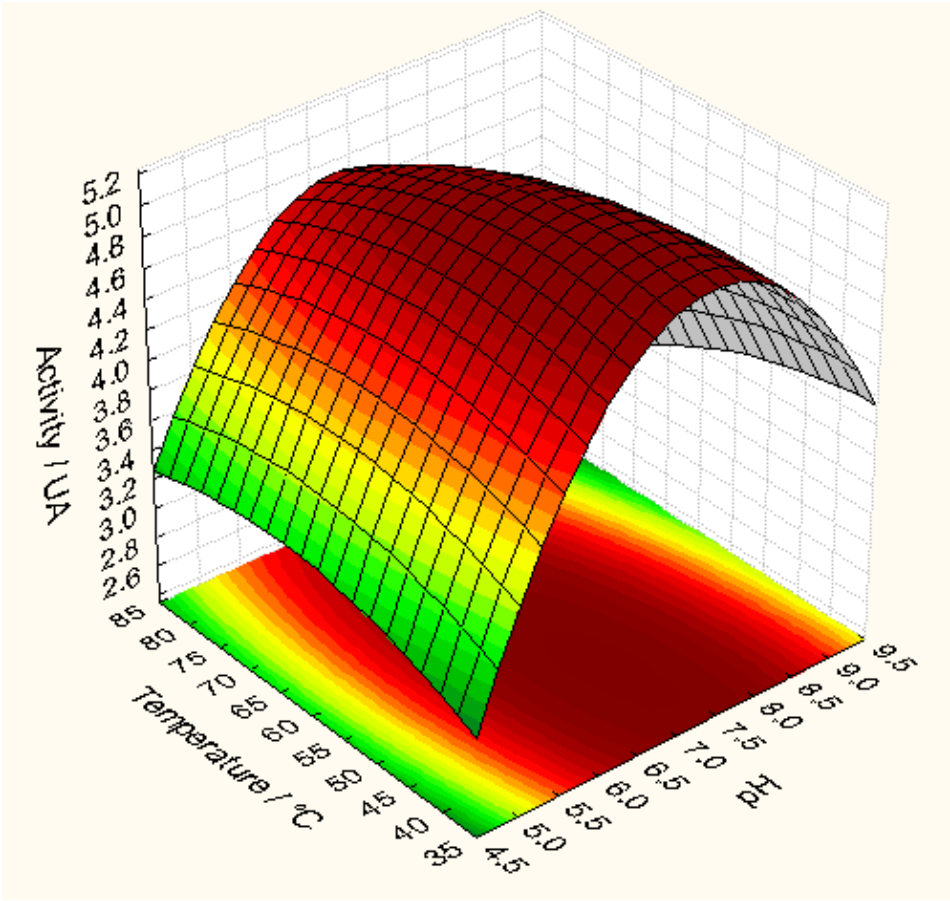


**Figure 2** Temperature and pH optimums of cellulase *M. perniciosa*. The deep red central circle indicates the central point of optimum pH and optimum temperature.

The results showed similarity with several studies with endoglucanases from different organisms, which have pH 7.0 and optimum activity at 50°C [16-20]. Bagga [21] found that the optimal assay conditions for all forms of cellulase components ranged from pH 5.0 to 6.0 and 50°C and 65°C for exo-glucanases and endo-glucanases but 35°C and 65°C for β-glucosidases. Results showed that in *Bacillus amyoliquefaciens* the temperature and pH optimum cellulase activity was 50°C and pH 7.0, respectively [3]. Padilha *et al.* [22] studied cellulase production from the 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 identical. The optimum pH for these enzymes was between 4.0 and 5.0 and the optimum temperature was 60°C [19].

Similar results were also found in *A. nidulans*, *Aspergillus Niger*, and *Clostridium cellulolyticum* with an optimum temperature of 51°C and pH 7.2 [22-26]. *Aspergillus niger* endoglucanase activity increased when incubated at temperatures of 30 and 35°C at pH 4 with a reduction in pH 5 [23]. 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 [27]. Studies show that purified endoglucanase's pH and temperature are similar to the endoglucanase of crude extract. The optimum temperature was 65 and 70°C for the endoglucanase of CH43 and HR68, respectively [28].

Studies with *P. enchinulatun* and *Thermoascus aurantiacus* showed maximum endoglucanase activity at 60°C [28, 29]. For the multi-enzyme complex cellulolytic the optimum temperature of 60°C is often cited in the literature [30].

### *3.3 Thermal Stability of Cellulase*

The effects of thermal stability on the residual activity of cellulase activity showed 99%, 95.05% and 94.80% of residual activity after 30 minutes of incubation at 60, 70 and 80°C, respectively (Figure 3). After 30 minutes of incubation at 90°C, the complex cellulolytic showed 88.66% of residual activity (Figure 3).

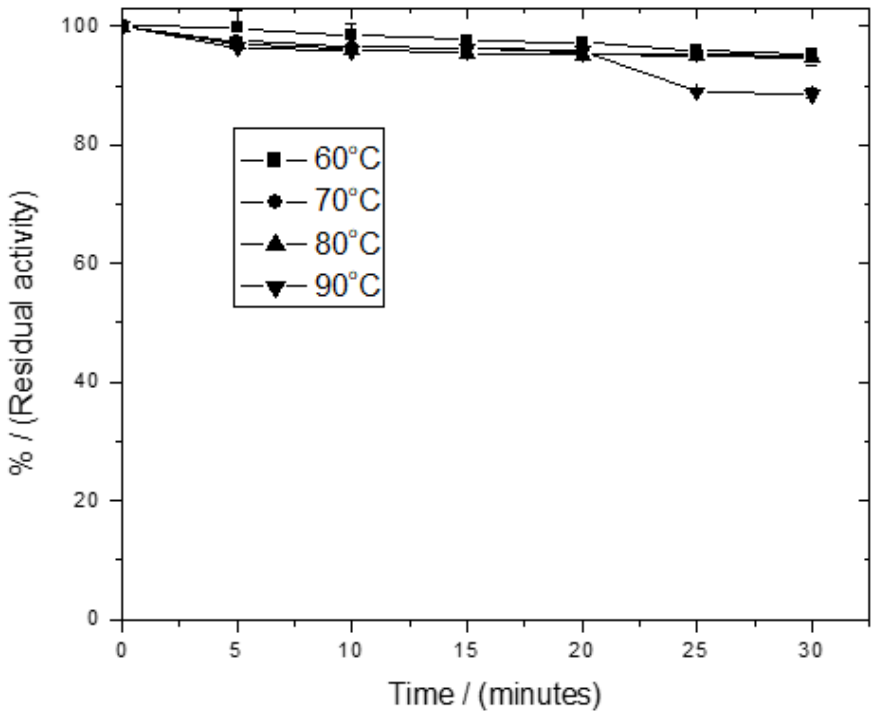


**Figure 3** Effect of thermal stability of cellulase produced by *M. perniciosa.* 60 (■), 70 (●), 80 (▲) and 90°C (▼).

The presence of cellulosic enzymes stable at 50 and 60°C is shown in other microorganisms [28, 29]. Enzymes that exhibit stability at temperatures above 40°C are called thermostable, it is common to find that they grow at mesophilic temperatures of 28 and 32°C and produce enzymes that act at 60°C, for example, glucoamylase of *Neosartorya fischeri* and *Aspergillus fumigatus* [31].

The crude extract containing endoglucanase obtained from the actinomycete *Streptomyces drozdowiciczii*, showed thermal stability over an hour and a half to 50°C, and at 60°C the enzyme lost 100% activity at the same time [11].

A study from thermophilic fungi *Thermoascus aurantiacus* with endoglucanase and cellobiase showed both enzymes remained stable at 42 and 18 minutes at 80°C, respectively [15].

Thermostable enzymes are highly specific and thus have considerable potential for many industrial applications [32]. The enzymes can be produced from the thermophiles through either optimized fermentation of the microorganisms or cloning of fast-growing mesophiles by recombinant DNA technology. The review of Haki [33] discusses the source microorganisms and properties of thermostable starch hydrolyzing amylases, xylanases, cellulases, chitinases, proteases, lipases and DNA polymerases. The enzymatic hydrolysis of cellulose has potential economic and environment-friendly applications. Therefore, discovering new extremophilic cellulases is essential to meet industry requirements [34].

### *3.4 Effect of the Influence of Salts on the Activity of Cellulase*

Studies indicate that the presence of different salts and concentrations can influence the activity of CMC. The enzyme activity was observed from the effect of potassium bromide, calcium chloride, ammonium chloride and potassium chloride were studied on cellulase activity at different concentrations.

The enzyme retained 96% and 94% activity when incubated with 100 mM of potassium chloride and bromide respectively (Figure 4). In the presence of 100 mM calcium chloride and ammonium chloride, there was retention of 74% and 71% residual activity, respectively.

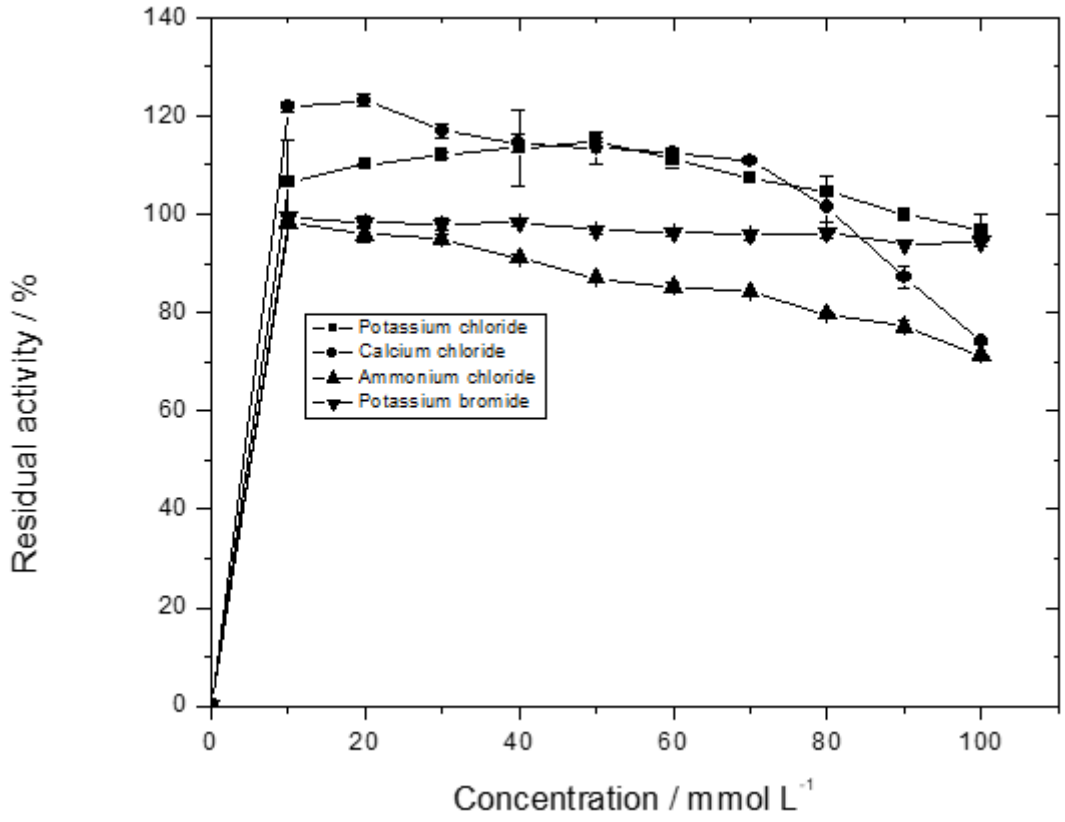


**Figure 4** Effect of the influence of salts on the activity of cellulase produced by *M. perniciosa.* Potassium chloride (■), Calcium chloride (●), Ammonium chloride (▲) and Potassium bromide (▼).

In *Clostridium thermocellium* the presence of $CaCl_2$ 5 mmol $L^{-1}$ caused one increase of 50% of endoglucanase activity [18].

### *3.5 Celulose Conversion*

The results show that after three days of reaction using 0.75 mg $L^{-1}$ of enzyme extract, a higher conversion when compared with the reaction using 0.5 mL of enzyme extract for the same period. The percentage of conversion obtained using 0.5 mg $L^{-1}$ remained stable over time, after four days. However, when we used 0.75 m $L^{-1}$ of enzymatic extract there was a conversion of 50% after 4 days, and this value remained stable over time (Figure 5).

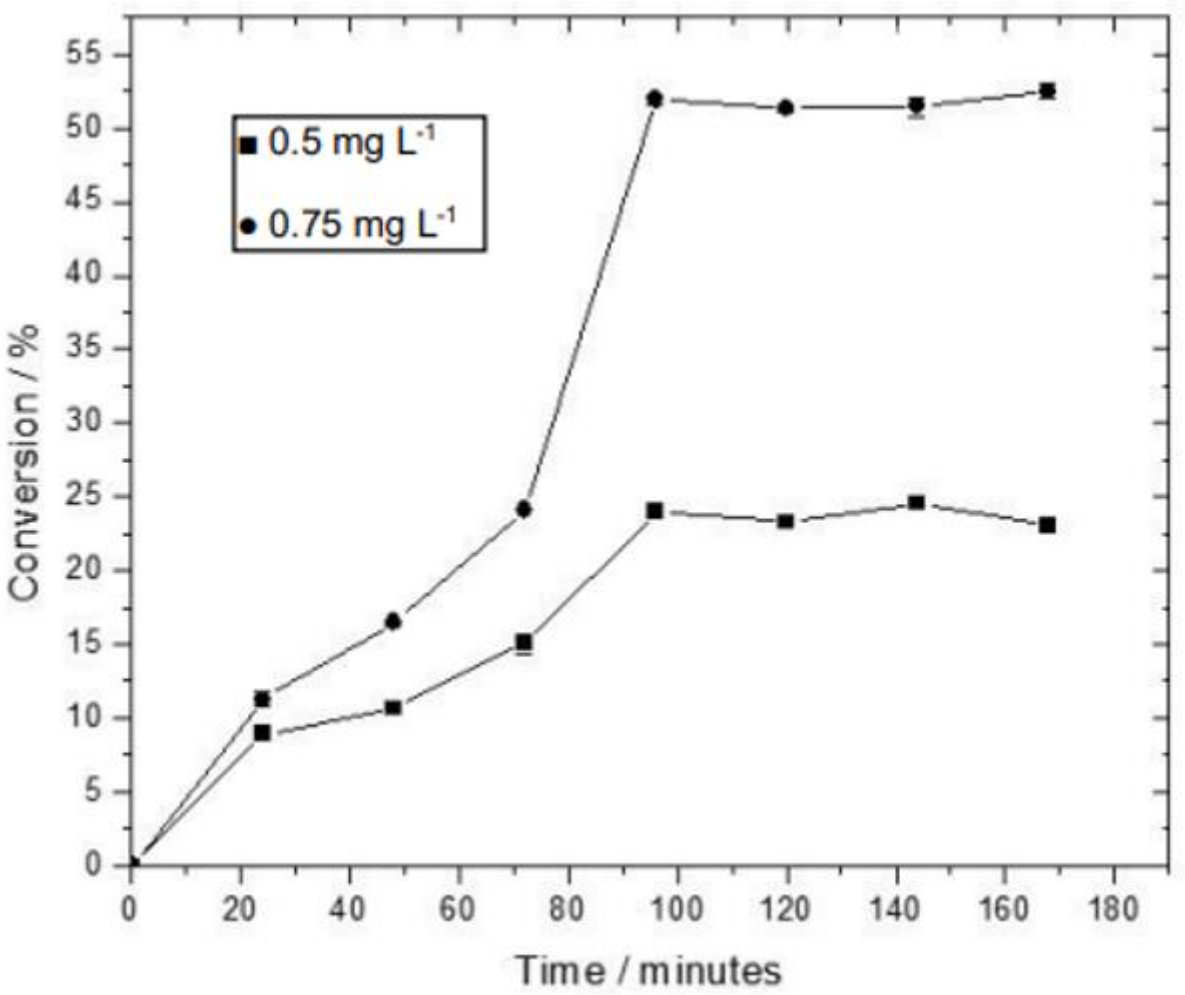


**Figure 5** Conversion of sugarcane bagasse with crude extract a 0.5 mg $L^{-1}$ (■) and 0.75 mg $L^{-1}$ (●).

Almeida et al. [35] related that the maximum production of reducing sugars (89.15%) was *Moniliophthora perniciosa* in the hydrolysis of pretreated sugarcane bagasse (3% NaOH + 6% $Na_2SO_3$).

Methods of pretreatments used to depolymerize, solubilize or remove the lignin present in the materials lignocellulolytic, the process of steam explosion followed by alkaline delignification has been one of the most promising for the purification of the three main constituents of biomass in parallel with a significant increase susceptibility of cellulose to enzymatic saccharification [35, 36]. By the experiments obtained from the hydrolysis of sugarcane bagasse, it is possible to evaluate the efficiency of substrate show the applicability of the complex, if possible, also obtain efficiency in the characterization of enzymes. Applying the enzyme produced on the same substrate may benefit some substrates [3].

Due to the use of an enzyme complex, can occur to the action of another enzyme, such as xyloglucanases act on the hydrolysis of cellulose. The stability of the reaction rate in both concentrations may be related to its operational stability, since after a long period thermal stability of the enzyme will be decreased. A major problem in enzymatic hydrolysis is the need for high thermal stability of the enzymes to work with high temperatures and thus achieve high reaction rates and solubility of reagents.

## 4. Conclusions

The production of CMC enzyme was higher in the concentration of 7.0 g $L^{-1}$ of yeast extract and 19 days fermentation time. CMC showed optimum pH and temperature at 7.2 and 47°C, respectively and the results show a great influence of the effect of pH on cellulase activity. The CMC retained 88.66% of residual activity after 30 minutes of incubation at 90°C.Now, we are working to obtain this enzyme in recombinant way and the enzyme will use in different applications with industrial interest.

The results showed that the CMC from *Moniliophthora perniciosa* has high thermal stability, making it interesting for application in industrial processes such as cellulose conversion. Also, the application of the Doehlert matrix as a multivariate optimization strategy in optimizing enzyme production.

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## Author Contributions

Mona Liza Santana, Aline Santos e Santos, Gildomar Lima Valasques Júnior and Sandra Aparecida de Assis are responsible for data acquisition, organization and discussion of the manuscript.

## Competing Interests

The authors have declared that no competing interests exist.

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