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Stabilization by chemical preservatives of carboxymethyl cellulase from fungus *Aspergillus niger* produced by State Solid fermentation in fruit residues using chemical preservatives

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ABSTRACT
The residues of fruticulture as acerola, guava, passion mango can be efficiently used to produce enzymes by Solid state fermentation. In this work we used these residues and filamentous fungus Aspergillus niger ATCC 1004 to obtain carboxymethyl cellulases (CMCase). The preservation of enzyme activity is a fundamental for provide commercial enzymes with good price and high enzyme activity. Three substances (sodium chloride, sodium benzoate and monosodium phosphate) were tested as enzyme activity preservative using a simplex-centroid mixture design was applied to obtain the better concentrations of this substances. The effect of preservatives was tested for 72 hours on the activity of the CMCase enzyme by incubating the enzyme with the salts of sodium chloride, monosodium phosphate and sodium benzoate at various concentrations, in citrate-phosphate buffer pH 5.0 at 50 mM, at a temperature of 50 °C. The results showed that the mixture that favored the optimum condition simultaneous response was composed of 42% sodium chloride, 38% sodium benzoate, and 20% monosodium phosphate. Then, the substances studied shows that are good option to preserve the CMCase enzyme produced fungus Aspergillus niger ATCC 1004.

Keywords: Solid state fermentation, Cellulases, Immobilization.

1 INTRODUCTION

Millions of tons of fruit waste from agro-industrial activities are generated annually. Some of these residues are used as animal feed or disposed of in the field, however, most of it is still discarded without treatment, causing damage to the environment.

Fruits are usually processed for the production of juices, pulps and sweets, resulting in large amounts of residues, which are most often discarded in the environment, although they have high potential for production of important bio commodities as enzymes and organic solvents (Galvão, 2014; Brito et al., 2015, Panda et al., 2016, Panda et al., 2017). These residues can contain sugars, minerals,

and proteins, and can provide a low cost of possible raw material. Besides, recycling these wastes is important to contribute with the environment (Matei et al, 2021, Orlandelli et al., 2017).

Solid state fermentation (SSF) is a biotechnological process in which microorganisms are cultivated in environments without free water or with water present in small amounts (Singh et al., 2020), however, it is necessary that the substrate used has sufficient moisture to support the growth and the metabolism of the microorganism (Mandari et al., 2020). SSF has high productivity in short periods, facilitated oxygen circulation, simpler technology, reduced operational problems, resembles the natural habitat of several microorganisms, the enzymes produced are less susceptible to substrate inhibition problems and are stable against changes of temperature and pH, in addition to having some environmental importance, since SSF has the ability to use substrates from agro-industrial residues, which serve as a source of carbon and energy for enzymatic growth (Almanaa et al., 2020; Verma et al., 2020), due to these advantages, it is commonly used in the production of microbial enzymes (Das Neves et al., 2020; Xu et al., 2020).

Carboxymethyl cellulases consists in a multi-enzyme complex comprising; endoglucanases, exoglucanases and β-glucosidases (BGL) which act together to hydrolyze completely the cellulose molecule (Olelunke et al., 2021). The global enzyme market of cellulases is expressive due to possibility of uses in many industrial processes such as textile, laundry detergents, paper and pulp, brewing and wine and biomass refinery (Olunkule et al., 2021).

The stabilization of cellulases can help overcome these limitations, as it allows easy recovery of the enzyme and increases its operational life, thus reducing storage and catalyst costs (Shen; Xia, 2004). In this sense, this work studied the stabilization by use of preservatives chemicals simultaneously of four crude enzymatic extracts of CMCase obtained a fermentation using fruits residues (passion fruit, acerola and guava).

2 MATERIALS AND METHODS

2.1 PREPARATION OF SAMPLES

The fruit residues (passion fruit, acerola and guava) were provided by an agro-food processing industry located in the South region of Bahia, which were dried in an oven at 50 ºC for 24 h (SL 102; Solab). They were crunched in a mill of knives of the Willey-type (ACB LABOR) type down to a particle size of 2 mm and then stored in plastic container until they were ready to use.

2.2 MICROORGANISM AND INOCULUM

The filamentous fungus *Aspergillus niger* ATCC 1004 was provided by Fundação Oswaldo Cruz (FIOCRUZ, Rio de Janeiro, RJ, Brazil) and also deposited at the Instituto Nacional de Controle de Qualidade em Saúde (INCQS, Rio de Janeiro, RJ, Brazil). This fungus was preserved in silica and

glycerol and maintained in an ultra freezer at a temperature of -80ºC. The microorganism was cultivated in Potato-Dextrose-Agar (Himedia, Mumbai, India), which were placed in Erlenmeyer flasks 250 mL, over a period of 7 days at 35 °C in a temperature control incubator (SL 222; Solab, Piracicaba, Brazil). For the preparation of the inoculum, the sporulated culture in PDA medium was suspended in Tween 80 (0.01%) and counting the number of spores was performed using Neubauer chamber and binocular microscope (L1000; Bioval, São Paulo, Brazil).

2.3 STATE SOLID FERMENTATION (SSF)

The enzymatic production was carried out by solid state fermentation. 10 g of food waste were autoclaved (121 ºC/1 atm/15 min) in Erlenmeyer flasks of 125 mL. Sterile water has added to the flasks until reaching 61% humidity, and the inoculation was performed with 108 spores. g^{-1} dry residue. The cultures were cultivated in a BOD (TE-317, TECNAL) with controlled temperature at 35 °C for 96 h (Santos et al., 2013; Do Santos et al., 2015).

2.4 ENZYMATIC EXTRACTION

After fermentation, 50 mL of sodium phosphate buffer (0.1 M/pH 7.0) were added to the fermented substrate and the mixture was stirred (shake incubator, TECNAL) at 35 ºC and 200 rpm for 20 min. The liquid phase was separated by mechanical pressing, followed by centrifugation 80g for 10 min at 4 °C (CIENTEC CT – 6000R Piracicaba, SP – Brazil).

2.5 DETERMINATION OF CARBOXYMETHYL CELLULASE ACTIVITY

Enzyme activity assays were conducted based on standard procedures recommended by IUPAC (Ghose & Bisaria, 1987). The carboxymethyl cellulase activity was determined by the amount of reducing sugars released from the incubation of 0.1 mL of carboxymethylcellulose (CMC) at a concentration of 1% (w v^{-1}), with 0.5 mL of the crude enzyme extract in a water bath at 50 °C for 15 min. To control the reaction, 0.1 mL and sodium acetate buffer were incubated with 0.5 mL crude (previously diluted) enzyme extract. After the incubation period, the reducing sugars were dosed using the method of Miller (1959), adding 0.6 mL of 3,5-dinitrosalicylic acid (DNS) to the reaction media, followed by incubation in boiling water for 5 minutes and subsequent cooling. After dilution with 6 mL of distilled water, the absorbance of the samples was read in a spectrophotometer at wavelength at 540 nm and the amount of reducing sugar released was quantified using standard glucose curve. An international unit of enzyme activity (IU) was defined as the amount of enzyme capable of releasing 1 µmol of reducing sugars per minute at 50°C.

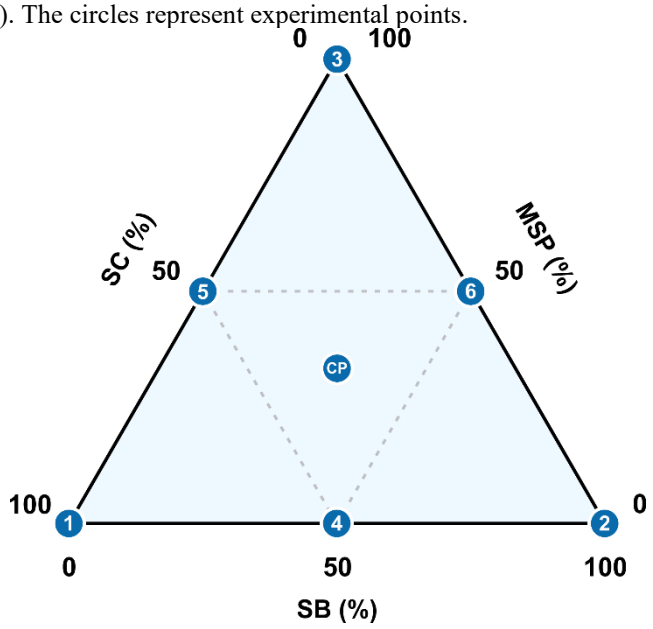
2.6 STABILIZATION OF CARBOXYMETHYL CELLULASE USING CHEMICAL PRESERVATIVES

Each tests tubes containing 1 mL of enzyme extract, 4mL of water and solutions of three preservatives (sodium chloride, sodium benzoate and monosodium phosphate) at a concentration of 1.0 mol / L the according to the planning presented in Table 1.

Aliquots of the mixture, containing preservatives and crude enzyme extract, were stored in microtubes protected from light at 4 ºC for 72 hours.

A simplex-centroid mixture design was applied to optimize the stabilization medium composed of sodium chloride (SC), sodium benzoate (SB), and monosodium phosphate (MSP). The preservatives were modeled at a concentration of 1 mol L^{-1} and varying from 0 to 100% v v^{-1} in the mixture. The tests were performed using the enzymatic extract in an aqueous medium in the ratio 1:4 (enzymatic extract: water, v v^{-1}). A total of 8 experiments (Fig. 1) were performed at random, of which two were repetitions of the central point (CP).

Fig. 1 Schematic view of the mixture design used in this study using sodium chloride (SC), sodium benzoate (SB), and monosodium phosphate (MSP). The circles represent experimental points.



In this optimization, the activity (U mL^{-1}) of CMCase from *Aspergillus niger* was evaluated in enzymatic extracts produced by solid-state fermentation from agro-industrial residues of passion fruit (A), acerola (B) and guava (C). Multiple response (MR) calculated according to Equation 1 (Bezerra et al. 2019; Felix et al. 2018), was used to obtain the simultaneous optimal condition for the three residues employed.

$$MR = \frac{Act(ResidueA)}{MaxAct(ResidueA)} + \frac{Act(ResidueB)}{MaxAct(ResidueB)} + \frac{Act(ResidueC)}{MaxAct(ResidueC)} \quad (1)$$

In this equation, Act corresponds to the activity of CMCase *A. niger* in an individual experiment and MaxAct to the maximum value of activity obtained in the set of experiments for each residue. The sum provided the multiple responses used in modeling the mixture and obtaining the mathematical model, which was evaluated based on the analysis of variance (ANOVA), including the criteria of statistical significance of the regression and coefficient of determination (R^2) (Bezerra et al. 2020).

The experimental data were processed using the Statistica software, version 12 (StatSoft, Tulsa, USA) at a 95% confidence level.

3 RESULTS AND DISCUSSION

3.1 OPTIMIZATION OF THE MIXTURE FOR ENZYMATIC STABILIZATION

It was investigated the composition of a mixture formed by three different chemical preservatives, sodium chloride (SC), sodium benzoate (SB), and monosodium phosphate (MSP) using simplex-centroid mixture design. Table 1 presents the percentage combinations of each preservative in the experiments carried out and the experimental responses (Activity) for each evaluated residue, in addition to the calculated multiple responses.

Table 1 Experimental matrix of the simplex-centroid mixture design, experimental responses, and multiple responses (72 h)

Experiment	SC (mL)	SB (mL)	MSP (mL)	Activity (U mL^{-1})			MR
				Passion fruit	Acerola	Guava	
1	1.00	0.00	0.00	0.350	0.125	0.117	2.622
2	0.00	1.00	0.00	0.304	0.147	0.105	2.556
3	0.00	0.00	1.00	0.094	0.117	0.114	1.840
4	0.50	0.50	0.00	0.363	0.112	0.077	2.293
5	0.50	0.00	0.50	0.286	0.097	0.082	2.007
6	0.00	0.50	0.50	0.226	0.075	0.127	2.003
7 (CP)	0.33	0.33	0.33	0.314	0.081	0.141	2.379
8 (CP)	0.33	0.33	0.33	0.303	0.081	0.146	2.387

Central point (CP), sodium chloride (SC), sodium benzoate (SB), monosodium phosphate (MSP), multiple response (MP).

Multiple response was used to obtain a second-order polynomial model that was evaluated based on ANOVA (Table 2). The regression for the special cubic model was able to adequately describe the investigated system and presented high statistical significance ($p = 0.014$) since the F calculated value was about 12x higher than the F tabulated (234). Also, R^2 was 0.9999, indicating that the regression satisfactorily describes the response behavior as a function of the levels of the investigated factors and also that random errors do not compromise the predictive capacity of the model.

Table 2 Analysis of variance for the special cubic model obtained using the multiple response

Source of variation	SS	Df	MS	F calculated	p-value
Regression	0.5565	6	0.0927	2898.2	0.014
Pure error	0.0001	1	0.0001		
Total	0.5566	7	0.0795		

Sum of squares (SS), degrees of freedom (Df), mean squares (MS).

Finally, after verifying that the special cubic model was adequate, the investigated factors were assessed for importance to the experimental response, through the standardized effects shown in the Pareto Chart (Fig. 2a).

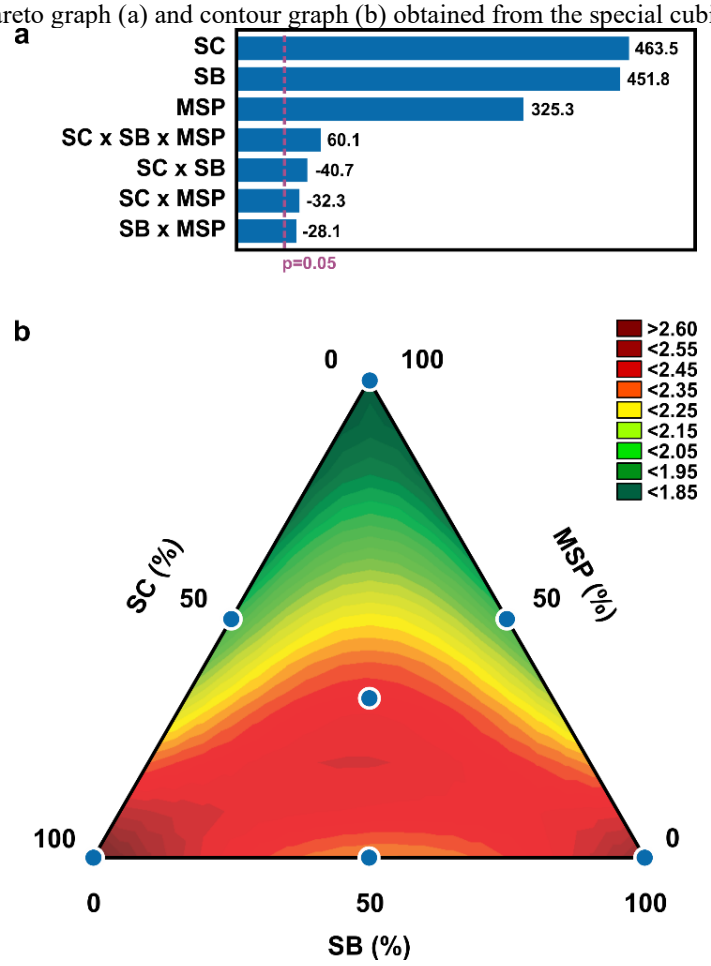
All factors and interactions were significant ($p>0.05$). SC and SB have the greatest effects and of the same magnitude, suggesting that an increase in the percentage of these components in the mixture favors an increase in response. Although the standardized effect for MSP is high and positive, the increase in the proportion of this component leads to a reduction in the response, suggesting that the interaction effects between the components affect the system. This fact is demonstrated by the effects of interaction between the negative components.

The relationship of the response and the variation in the proportion of the components is described by Equation 2.

$$MR_{predicted} = 2.62SC + 2.56SB + 1.84MSP - 1.18SC \times SB - 0.89SC \times MSP - 0.78\,SB \times MSP + 9.76\,SC \times SB \times MSP \qquad (2)$$

The contour graph produced from this equation is shown in Fig. 2b. An increase in response is observed with the increase of SC and SB in the mixture, while MSP induces a reduction. Thus, the best results were obtained in mixtures with higher proportions of SC and SB and lower ones of MSP. Finally, the mixture that favored the optimum condition simultaneous response was composed of 42% sodium chloride, 38% sodium benzoate, and 20% monosodium phosphate.

Fig. 2 Pareto graph (a) and contour graph (b) obtained from the special cubic model



The effect of preservatives was tested for 72 hours on the activity of the CMCase enzyme by incubating the enzyme with the salts of sodium chloride, monosodium phosphate and sodium benzoate at various concentrations, in citrate-phosphate buffer pH 5.0 at 50 mM, at a temperature of 50 ° C.

This experiment was developed with the objective of increasing the time in which enzymes remain active. For, enzymes of interest for technological and industrial applications should remain stable under established operating conditions for a long period of time.

The most relevant factors that can influence the stability of the enzyme in industrial processes are pH, salt concentration, ion concentrations, temperature, etc. According to Bailey and Ollis (1986) it is important to know these conditions, in order to try to ensure not only optimal stability in the storage of the product, but also to minimize loss during the process.

According to Carneiro (2003) most enzymes lose stability when they are used on an industrial scale, and to overcome this limitation, new sources of enzymes have been sought, especially those obtained from microorganisms living in extreme pH and temperature conditions, or the addition of stabilizers and/or preservatives that maintain enzymatic activity for longer is used.

The choice of preservatives was based on existing data in the literature of the fish that can contribute to increase the enzymatic stability.

In relation to ions, the three preservatives chosen have Na^{+} as cation, because in the work developed by Lima et al (2005), this ion stimulated the enzymatic activity of fungal endogluconases, which may be related to the fact of this ion acting as a cofactor.

Wang et al. (2003) evaluated the effects of monovalent anions, such as F, Cl, Br, I, and NO_3 for cellulase activity, and concluded that these ions are essential for exoglucanase activity, and Cl and Br have a better effect on the activity of endoglucanases.

Triverdi et al. (2010) verified that purified cellulase produced by *Bacillus flexus* NT strains were induced by Cd^{2+} and Li^{1+} metal ions, while Cr^{2+}, Co^{2+}, Zn^{2+} and EDTA chelating metal significantly inhibited enzyme activity. Ko et al. (2010) characterized purified cellulases of *Paenibacillus campinasensis* BL11 (Cel-BL11), and showed that Hg^{2+}, Cu^{2+} and Zn^{2+} ions strongly inhibited enzymatic activity, while Mn^{2+} and Co^{2+} strongly induced enzyme activity. Olunkuke et al studied the carboxymethyl cellulase (CMCase) production under submerged fermentation using a cost-effective plantain stalk–based medium and observed that Cellulase activity was enhanced by Mn^{2+}, Na^{+}, Mg^{2+} and K^{+} but inhibited by Pb^{2+}, Hg^{2+} and Cu^{2+} at 5 mM (Olunkuke et al., 2021).

Salt is also useful in inhibiting some undesirable enzymes and stabilizing some desirable enzymes used in the food industry (Ravihankar and Juneja et al 2021).

Margesin and Shinner, (2001) state that knowledge regarding the interference of sodium chloride NaCl is important as part of the enzymatic characterization stage, both for its stability or for activity. Several authors (Balsan, 2011; Voget et al., 2006; Triverdi et al., 2010; Wang et al., 2009; Gao et al., 2008; Hirasawa et al., 2006) evaluated the influence of NaCl on the enzymatic activity of cellulase. In some studies, it was found that sodium chloride induced the enzymatic activity of enzymes when added at concentration up to 5%. In commercial enzymatic solutions sodium chloride appears as stabilizing agents, helping to protect the enzyme against autolysis and inactivation.

Sodium benzoate is widely used in industries as a preservative, especially in the food industry. According to "Food Ingredients" Brazil (2012) was the first food preservative allowed by the Food and Drug Administration (FDA) and due to its low cost appears as one of the most used preservatives. It is a salt derived from benzoic acid that occurs naturally in many types of berries, plums and some spices. Although undissociated benzoic acid is the most effective antimicrobial agent, sodium benzoate is preferably used because benzoic acid is low soluble in water. Sodium benzoate acts by inhibiting the growth of microorganisms, being used to avoid contamination of the reaction medium in several studies that evaluate the activity of cellulase enzymes and fungal xylanases using agro-industrial residues (Dionisio et al, 2009; Camera; Moraes, 2012; Aquino, 2008; Zarpellon; Moraes; Zanin, 2010).

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. According to Passinate (2011) phosphates cover a wide pH range, and because phosphoric acid/phosphate solutions

are generally non-toxic, mixtures of these solutions are commonly used to produce buffering agents, where the desired pH depends on the proportions of phosphates in the mixtures.

Due to the above-mentioned characteristics, 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 (Paris, 2008, Aguiar, 2010; Santana, 2010; Pereira, 2013; Rocha, 2010).

4 CONCLUSIONS

The preservation of enzyme activity is a fundamental for provide commercial enzymes with good price and high enzyme activity. The results showed mixture that favored the optimum condition simultaneous response was composed of 42% sodium chloride, 38% sodium benzoate, and 20% monosodium phosphate. Then, the substances studied shows that are good option to preserve the CMCase enzyme produced fungus *Aspergillus niger* ATCC 1004.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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