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# Statistical approach of $Ca^{2+}$ effect on the amylases production by *Saccharomyces diastaticus* ATCC 13007 using corn and cassava starch as inducers

## *Abordagem estatística do efeito de $Ca^{2+}$ na produção de amilases por* Saccharomyces diastaticus *ATCC 13007 usando amido de milho e mandioca como indutores*

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**ABSTRACT**
Amylase production from starch sources is associated with the nature of this carbon source, particle size and crystalline architecture of natural starch granules. The aim of this work was to evaluated the amylase production by *Saccharomyces diastaticus* ATCC 13007, using different $CaCO_3$ concentration and corn starch (*Zea mays*) and cassava starch (*Manihot esculenta*) as inducers. A $2^2$ Central Composite Rotatable Design with three replications at the center point was used to determine the influence of calcium and starch concentrations on the production process. Besides, the partial characterization of the crude enzymatic extracts obtained was investigated. $Ca^{2+}$ influenced the α-amylase production by both evaluated carbon sources. The production of glucoamylase was not influenced by the variables under study. Partial characterization of crude extracts indicated that, for corn substrate, the enzyme showed maximum activity at pH 5.0, and no influence of temperature was observed. Regarding cassava starch, the results showed that conditions at central point (pH 6.5 and 50ºC) should be avoided in order to maximize enzyme activity.
**Keywords:** carbono source; culture parameters; statistical design; submerged culture; yeasts.

**RESUMO**
A produção de amilase a partir de fontes amiláceas está associada à natureza dessa fonte de carbono, ao tamanho de partícula e à arquitetura cristalina dos grânulos de amido natural. O objetivo do presente trabalho foi avaliar a produção de amilase por *Saccharomyces diastaticus* ATCC 13007, utilizando diferentes concentrações de $CaCO_3$ e amido de milho (*Zea mays*) e amido de mandioca (*Manihot esculenta*) como indutores. Delineamento central composto rotacional, $2^2$, com três repetições no ponto central, foi utilizado para determinar a influência das concentrações de cálcio e amido no processo de produção. Além disso, investigou-se a caracterização parcial dos extratos enzimáticos brutos obtidos. O $Ca^{2+}$ influenciou a produção de α-amilase pelas duas fontes de carbono avaliadas. A produção de glucoamilase não foi afetada pelas variáveis estudadas. A caracterização parcial dos extratos brutos indicou que, para o substrato de milho, a enzima apresentou atividade máxima a pH 5,0, e não foi observada influência da temperatura. Em relação ao amido de mandioca, os resultados mostraram que as condições no ponto central (pH 6,5 e 50ºC) devem ser evitadas para maximizar a atividade enzimática.
**Palavras-chave:** cultivo submerso; fontes de carbono; levedura; parâmetros de cultivo; planejamento estatístico.

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## INTRODUCTION

Amylases are a group of enzymes that exhibits the specific capacity to hydrolyze glycosidic linkage in starch molecules. These molecules are reported as essential in the global market, with great applicability in industrial processes, ranging from food products, pharmaceuticals, cosmetic, textile and paper industries, etc. (LEDESMA-AMARO *et al*., 2015; SELVAM *et al*., 2016; RANA *et al*., 2017). In recent years, the emergence of the starch biorefinery industry has expanded the application of amylases, mostly in the field of food, beverage and ethanol, and secondly, in the production of organic acids and bioplastics (LÄUFER, 2017).

However, according to Xu *et al*. (2016), approximately 10% of amylolytic enzymes can digest raw starch, due to the differences in the particle size and crystalline architecture of natural starch granules. Indeed, Benavent-Gil & Rosell (2017) state that tuber starch is more resistant than cereal starch, regarding enzymatic hydrolysis, which can be attributed to the high quantity of branch points in non-crystalline regions, resulting in an increase in the density of amorphous regions and stable crystallites.

Hashemi *et al*. (2015) emphasize that $Ca^{+2}$ ions are important cofactors for some amylase activities, although such feature is variable and related to the starch source in the culture medium. Besides, calcium does not only play a significant role in the activity of the enzyme, but it also increases its stability against changes in temperature and pH (LI *et al*., 2015).

Despite the existence of several available yeast species, *Saccharomyces diastaticus* is reported as a good producer of amylases (α-amylase and glucoamylase), since it presents the potential to hydrolyze different types of starch in fermentable sugars (STEWART, 2016; MARYAM *et al*., 2017).

Hence, the present study aimed to evaluate the production of amylase by *Sacharomyces diastaticus* ATCC 13007 strain, using corn starch (*Zea mays*) and cassava starch (*Manihot esculenta*) as inducers. Besides, the effect of $Ca^{2+}$ concentration over different starch matrix was checked and the partial characterization of the crude enzymatic extracts obtained was investigated.

## MATERIAL AND METHODS

### MICROORGANISM AND INOCULUM PREPARATION

*Saccharomyces diastaticus* (ATCC 13007) was used in the study. The commercial culture was acquired from American Type Culture Collection (ATCC, USA). The culture was rehydrated into 200 mL of Sabouraud-glucose liquid medium (3% glucose ($w.v^{-1}$), 1% peptone ($w.v^{-1}$), 0.5% yeast extract ($w.v^{-1}$), 0.02% chloramphenicol ($w.v^{-1}$)) and incubated in rotatory shaker condition (200 rpm) at 25°C for 48 h.

At the end of the incubation period, the liquid medium and biomass were separated by centrifugation at 1,000 g for 15 min. The supernatant was discarded, and the biomass was re-suspended in 10 mL sterile acetate buffer solution (pH 5.0, 0.5 $mol.L^{-1}$), which was used in assays for amylases production. The biomass was standardized (($5\ g.L^{-1}$) wet weight) for each enzyme production test.

### ANALYTICAL METHODS AND EXPERIMENTAL DESIGN FOR ENZYMATIC HYDROLYSIS

The tests for amylases production were assessed using the submerged fermentation technique. A 2-factor-5-level Central Composite Rotatable Design (CCRD) was used, resulting in 11 assays. Table 1 shows the levels and variables analyzed in the study.

**Table 1** – CCRD for amylase production by Sacharomyces diastaticus ATCC 13007.

| | Levels | | | | |
|---|---|---|---|---|---|
| Variables | -α (-1.=41) | -1 | 0 | +1 | +α (+1.41) |
| $CaCO_3$ (%) | 0 | 0.18 | 0.25 | 0.43 | 0.5 |
| starch (%) | 0.5 | 0.67 | 0.75 | 0.93 | 1.0 |

All CCRD assays were randomly performed, and data were analyzed through Analysis of Variance (ANOVA, $p < 0.1$) and F-test, using statistical software R Project Statistical package for computing version R-2.15.2 (R Development Core Team, 2011).

Production experiments were carried out in Erlenmeyer flasks (250 mL) containing 90 mL of medium supplemented with fixed concentration of yeast extract (0.5%) and different concentrations of $CaCO_3$ and carbon source - either corn starch (*Zea mays)* or cassava starch (*Manihot esculenta*) - as specified in the experimental design. The culture medium was homogenized with acetate buffer (pH 5.0, 0.5 mol.$L^{-1}$), and then autoclaved at 121°C and 15 psi for 20 min. Then, the flasks containing medium were allowed to attain room temperature.

It was used 10 mL of the inoculum, obtained as previously described. The inoculum was aseptically added to the medium in the flasks, kept under shaking condition (200 rpm) at 30°C. Next, aliquots of the suspension were collected at regular time intervals (24, 48, 72 and 96 h), and centrifuged at 10,000 rpm for 20 min. The supernatant, named enzymatic extract, was submitted to the enzymatic activity test.

The amylase activity was determined according to the following procedure: 40 µL of sterile sodium acetate buffer (pH 5.5, 0.5 mol.$L^{-1}$), dispersed in 60 µL of the enzymatic extract, resulting a mixture, 100 µL of starch solution 0.5 % (w. $v^{-1}$) – using corn or cassava starch as substrate - was added. A blank experiment was also performed, in which 60 µL of distilled water was added, instead of enzymatic extract. The mixture was placed in a water bath at 40°C for 60 min.

Upon cooling, the enzyme activity was estimated. The alpha-amylase activity was determined through color reduction of the iodometric method described by Fuwa (1954), while glucoamylase activity was carried out using the commercial glucose oxidase kit (Labtest), with absorbance measured at 505 nm.

The amylase activity was expressed in International Units (U). One international unit was defined as the amount of enzyme required to hydrolyze 0.1 mg of substrate per mL per minute (mg. $min^{-1}$.$mL^{-1}$) of reaction, under tests conditions. In both cases, the enzyme activity is expressed in units per mL (U.$mL^{-1}$).

## PARTIAL CHARACTERIZATION OF CRUDE ENZYMATIC EXTRACT

Optimal pH and temperature were determined using the best results obtained from production assays.

The optimum pH for crude α-amylase extracts was obtained using sodium phosphate buffer (100 mM), at different pH values, according to Central Composite Rotatable Design (CCRD) (table 2).

Temperature optimal condition was evaluated through the pre-incubation of the enzymatic extract at different ranges of temperature (table 2) for 30 minutes in a thermostatic bath.

The test conditions were carried out in sealed vials, in order to prevent changes in the sample volume, therefore, in the enzyme concentration due to evaporation, based on the study reported by Loureiro *et al*. (2016).

**Table 2** – CCRD values and levels of partial characterization of crude enzymatic extract.

| | Levels | | | | |
|---|---|---|---|---|---|
| Variables | -α (-1.41) | -1 | 0 | +1 | +α (+1.41) |
| pH | 5.5 | 5.8 | 6.5 | 7.2 | 7.5 |
| Temperature (°C) | 40 | 43 | 50 | 57 | 60 |

Data were analyzed by ANOVA, at a significance level of 0.1, using the software R Project for Statistical Computing version R-2.15.2 (R Development Core Team, 2011).

## RESULTS AND DISCUSSION

EXPERIMENTAL DESIGN FOR AMYLASES PRODUCTION

Table 3 depicts the results of the α-amylase ($U.mL^{-1}$) activity at 24, 48, 72 and 96 hours of culture. The data indicate that *S. diastaticus* was able to grow in all the evaluated conditions. The highest amylase activity (2.91 $U.mL^{-1}$) was obtained using corn as inducer (assay 6: 0.5% $CaCO_3$ and 0.75% starch) at 72-hour of incubation. For cassava assays, the minimum enzyme production (0.09 $U.mL^{-1}$) was observed for run number 6 with 48-hour incubation time, while the maximum α-amylase activity (0.71 $U.mL^{-1}$) was achieved at 96-hour (0.71 $U.mL^{-1}$).

Our results are in a good agreement with findings of Oliveira *et al*. (2016), which reported that, among several starch vegetal sources, corn starch was more susceptible to hydrolysis by amylases produced from *Lichtheimia ramosa* and *Thermoascus aurantiacus*. This performance can be justified based on amylose and amylopectin content of each starch source, which provides specific features for the starch source, such as granule architecture, pore-size present on the granule surface and textural properties (OLIVEIRA *et al*., 2016).

Besides, enzymatic hydrolysis of starch contained in grains begins on the granule surface, and then spreads towards the inner side of the grains, resulting in deeper holes and channels, which promote favorable conditions for amylases performance. The rigid and smooth surface of tuber starches, on the other hand, acts as a barrier, which makes the hydrolysis process difficult (BENAVENT-GIL & ROSELL 2017).

The statistical analysis showed that when corn was used as a carbon source, none of the parameters demonstrated a significant effect on the α-amylase activity at 24, 48, and 72 hours. On the other hand, at 96 hours (table 4), enzyme production was positively affected by linear corn starch (t-value = 3.56) and calcium (t-value = 2.72) concentrations. Regarding the medium containing cassava (table 4), at 96-h incubation time, the enzyme production was negatively influenced by starch (t-value = - 8.063) and calcium (t-value = - 9.763).

**Table 3** – ANOVA results of $CaCO_3$ and corn/cassava starch concentrations effect on the α-amylase production (96 h) by S. diastaticus ATCC 13007.

| Variables | DF | Sum Square | Mean Square | F value | Pr (>F) | Estimate | Std.Error | t-value |
|---|---|---|---|---|---|---|---|---|
| **Corn** | | | | | | | | |
| Intercept | - | - | - | - | - | 2.065 | 0.206 | 10.00 |
| A ($CaCO_3$) | 1 | 0.948 | 0.948 | 7.41 | 0.041* | 0.344 | 0.126 | 2.72 |
| B (Starch) | 1 | 1.628 | 1.628 | 12.73 | 0.016* | 0.452 | 0.126 | 3.56 |
| $A^2$ | 1 | 0.0004 | 0.0004 | 0.003 | 0.956 | -0.014 | 0.151 | -0.09 |
| $B^2$ | 1 | 0.002 | 0.0019 | 0.015 | 0.905 | -0.018 | 0.151 | -0.12 |
| A:B | 1 | 0.931 | 0.9312 | 7.28 | 0.043* | -0.482 | 0.178 | -2.69 |
| Residuals | 5 | 0.639 | 0.1279 | - | - | - | - | - |
| Shapiro Wilk test: W = 0.95856; p-value= 0.6536 | | | | | | | | |
| R squared = 0.8459 | | | | | | | | |
| **Cassava** | | | | | | | | |
| Intercept | 1 | - | - | - | - | -3.3569 | 0.4032 | -8.325 |
| A ($CaCO_3$) | 1 | 0.0005 | 0.00047 | 0.2702 | 0.6253 | 3.8505 | 0.9219 | 4.1777 |
| B (Starch) | 1 | 0.000 | 0.000 | 0.0002 | 0.9880 | 9.2075 | 0.9175 | 10.035 |
| $A^2$ | 1 | 0.0428 | 0.0428 | 24.5389 | 0.0042* | -4.6815 | 0.5806 | -8.063 |
| $B^2$ | 1 | 0.1754 | 0.1755 | 100.434 | 0.0001* | -5.7034 | 0.5842 | -9.763 |
| A:B | 1 | 0.036 | 0.0036 | 2.0609 | 0.2106 | -1.6863 | 1.1746 | -1.436 |
| Residuals | 5 | 0.0087 | 0.0017 | - | - | - | - | - |
| Shapiro Wilk test: W= 0.90061; p-value = 0.9622 | | | | | | | | |
| R squared = 0.9622 | | | | | | | | |

DF: degree freedom; Std. Error: Standard Error; * Statistically significant variables ($p < 0.1$).

The positive linear terms and their corresponding t-values suggest an increase in enzyme production and/or activity as the concentrations of the variables increase, while the quadric term of cassava starch and $CaCO_3$ indicated that the evaluated parameters could perform as limiting components and any modification in their concentrations will change the α-amylase performance (ABDELWAHED *et al*., 2017). Furthermore, the results obtained in the experiments (table 4) corroborate the studies of Hashemi *et al*. (2015) and Paludo *et al*. (2018), which suggest that the larger the magnitude of F-test value and the smaller the p-value (Pr(>F)), the more significant the corresponding parameter.

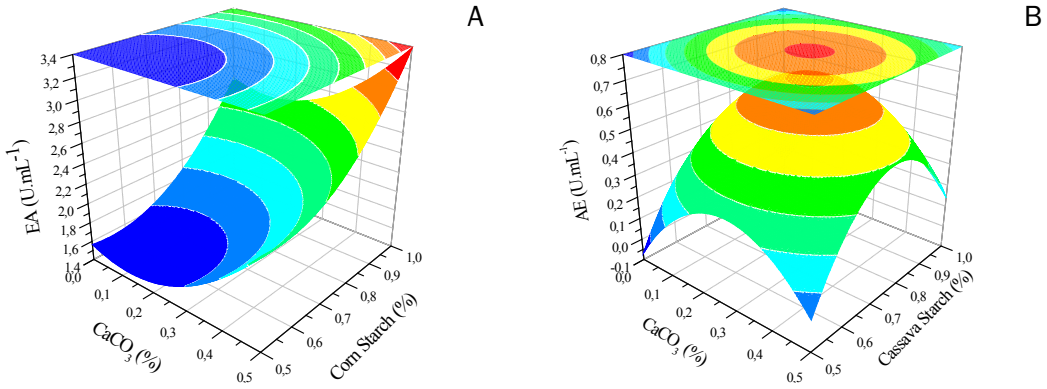


**Figure 1** – Effect of $Ca^{2+}$ on the α-amylase production by *S. diastaticus* ATCC 13007 at 96 hours of incubation time: (A) corn and (B) cassava starch. EA: enzymatic activity.

A good correlation between the experimental and the predicted values of α-amylase production at 96h, is shown in figure 2. The points around the sloping line were adequately adjusted to the model and represent an acceptable variation between the data for the range of assessed assays conditions (HASHEMI *et al*., 2015).

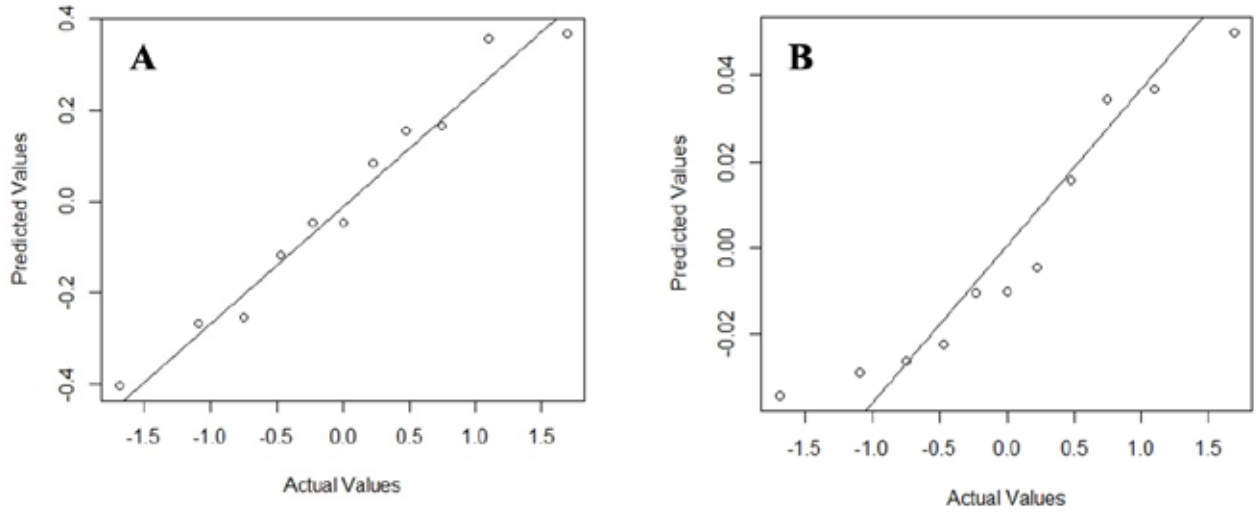


**Figure 2** – Predicted and observed responses for α-amylase production at 96 h: (A) corn starch; (B) cassava starch.

Additionally, the Shapiro test revealed that data assumes normal distribution (p-value > 0.05), suggesting that the predicted model values were in agreement with the values measured in experiments, which confirms the validity of the presented regression coefficients observed in table 4 (MONTGOMERY & RUNGER, 2011; SELVAN *et al*., 2016).

Deb *et al*. (2013) evaluated the effect of several divalent metal ions, $Ca^{2+}$, $Mg^{2+}$, $Mn^{2+}$, $Zn^{2+}$, $Fe^{2+}$, on the extracellular amylase production by *Bacillus amyloliquefaciens* P-001. The authors concluded that only $Ca^{2+}$ was required for the catalytic activity of the assessed enzyme, and reported that

α-amylase contains at least one $Ca^{2+}$ ion. In addition, the affinity of calcium is much stronger than that of other metal ions.

Furthermore, Li *et al*. (2015) emphasized that, when cassava is used as carbon inductor for amylase production, calcium ($Ca^{2+}$) or carbonate ($CO_3^{2-}$) ions act as cofactors and play specific physiological/ cellular roles, such as conversion of cassava starch to a reduction sugar and its buffering capacity.

Thus, as amylase is an inducible enzyme, it is generally induced in the presence of starch or its hydrolytic product (ARUNA *et al*., 2015). In addition, Deb *et al.* (2013) reported that an efficient induction might not occur until reaching the stationary phase and the reduction of the available carbon, which could justify the influence of $Ca^{2+}$ and starch source at 96 h, obtained in our study.

Regarding glucoamylase, it was observed that the production and the activity of this enzyme, by ATCC 13007 strain, did not exceed 0.19 $U.mL^{-1}$ and 0.03 $U.mL^{-1}$, for corn and cassava starch, respectively.

According to the literature (HASHEMI *et al*., 2015), this fact could be associated with the nitrogen source employed in the culture medium, since not only the type of carbon and its concentration, as well as chemical characteristics of the nitrogen source – organic or inorganic – can determine the extent and the variation of amylase synthesis. Besides, in the presence of an adequate amount of carbon and nitrogen in the culture medium, yeasts synthesize all L-amino acids, which are used in protein synthesis (DEB *et al*., 2013; DLAMINI, 2015). A study conducted by Deb *et al*. (2013) showed that both the lack and the excess of nitrogen are equally detrimental, resulting in enzyme inhibition. Escaramboni *et al*. (2018), for example, observed that urea was the best nitrogen source for glucoamylase production by *Aspergillus* sp, using wheat bran as a carbon source.

Previous studies (ARUNA *et al*., 2015) have identified that among several nitrogen sources, steep corn liquor demonstrated maximum effect on the increase of glucoamylases production, when maltose was used as carbon source.

The antagonistic behavior observed for the amylases studied in this work can have been caused by enzyme adsorbability onto different starch sources. A strong correlation between the adsorption rate and several raw starch hydrolysis was observed for α-amylases produced from *Bacillus amyloliquefaciens* (DEB *et al*., 2013). In contrast, although glucoamylases produced by *Aureobasidium pullulans* was able to adsorb potato, corn, and sweet potato starches, only raw potato starch was hydrolyzed by the enzymes (XU *et al*., 2016).

## PARTIAL CHARACTERIZATION OF CRUDE ENZYMATIC EXTRACT

The effect of pH and temperature over crude enzymatic extract can be observed in Table 5. Amylase activity was greatly influenced (Pr(>F) = 0.06) by pH when corn was used as an inductor, with maximum enzyme activity (0,17 $U.mL^{-1}$) achieved at pH 5.8 and 57°C. Data suggest that enzyme activity could increase as pH (t-value = 2.434) increases. On the other hand, for cassava inductor, both quadratic terms of pH (Pr(>F) = 0.02; t-value = 3.395) and temperature (Pr(>F) = 0.02; t-value = 3.363) were significant. However, it is interesting to observe that, for such a specific substrate, the central point values (pH 6.5 and 50°C) should be avoided, in order to maximize the process response. Besides, the 3D surfaces indicate (figure 3) that the optimal conditions were located within the design boundary.

**Table 4** – ANOVA results of pH and temperature effect over crude enzymatic extract.

| Coefficients variables | t value | Pr(>\|t\|) | t value | Pr(>\|t\|) |
|---|---|---|---|---|
| | Corn | | Cassava | |
| Intercept | 1.465 | 0.2028 | -0.008 | 0.994 |
| T (L) | 0.936 | 0.3920 | 0.247 | 0.815 |
| pH (L) | 0.164 | 0.8764 | 1.637 | 0.163 |
| T (Q) | 2.313 | 0.0687* | 1.411 | 0.217 |
| pH (Q) | 3.069 | 0.0278* | 1.411 | 0.217 |
| T*pH | -0.889 | 0.4146 | -0.887 | 0.416 |

* Statistically significant variables ($p < 0.1$); L-linear term; Q-quadratic term.

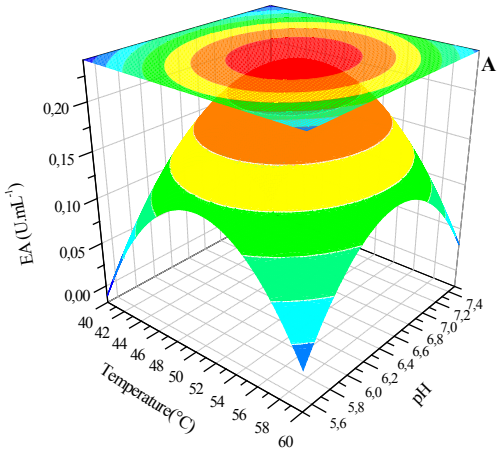


**Figure 3** – 3D response surface for pH and temperature effect over crude enzymatic extract.

According to Amid *et al*. (2014), the pH value of a liquid culture affects the charge distribution structure of enzymes active sites, which do not remain in a stable form, changing the reaction rate. The results achieved in this study are concurrent with earlier findings. Amid *et al*. (2014) claimed that the highest activity of amylase, isolated from Dragon (*Hylocereus polyrhizus*) peel, was obtained at pH 5.0. Selvam *et al*. (2016) reported that the optimal amylase activity produced by *Bacillus sp*. was observed at pH 7. Similarly, Gandhi *et al*. (2015) observed that the optimum pH for *Geobacillus stearothermophilus* SR74 recombinant alpha-amylase SR74 in *Pichia pastoris* was 7.0.

Data present in Figure 3 show that, at alkaline pH, the enzyme was in its stable form and the highest reaction rate between the active site of the biocatalyst and the substrate was achieved within this pH range. Moreover, in an earlier study (GANDHI *et al*., 2015), it has been reported that phosphate buffer provided the ideal pH, compared to other buffers such as acetate and tris-acetate, for α-amylase activity.

It was noticed (figure 3) that, at alkaline conditions, the enzyme shows maximum activity within all the ranges of the evaluated temperature. The results also indicate the thermophilic nature of the crude alpha-amylase preparation. Besides, such data are in line with the findings of many researchers (KHANNOUS *et al*., 2014; GANDHI *et al*., 2015) who found the highest amylase activity at approximately 60°C.

## CONCLUSION

The activity of α-amylase produced by strain ATCC 13007 was 2.91 $U.mL^{-1}$ and 0.71 $U.mL^{-1}$ for corn and cassava inductors, respectively. The production of glucoamylase was minimal (< 0.2 $U.mL^{-1}$) or non-existent for both starches. The results suggest that $CaCO_3$ was the main factor influencing the production/ activity of α-amylases when starched-based materials were used as carbon source, where corn was more susceptible to enzymatic hydrolysis, compared to cassava starch. Regarding crude enzymatic extract partial characterization, the pH was the only parameter affecting enzyme activity when corn was used as carbon source. On the other hand, in relation to cassava, both variables pH and temperature demonstrated influence over alpha-amylase activity. Besides, the region of central point (pH 6.5 at 50°C) should be avoided in order to enhance the activity of the enzymes under study.

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