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CHARACTERIZATION OF A-AMYLASES COMPLEX PRODUCED BY A *BRADYRHIZOBIUM*SP. ISOLATED FROM AMAZONIAN SOIL

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ABSTRACT

The α-amylase (EC 3.2.1.1) production permits the conversion of starch in products which are afterwards converted to alcohol. This study evaluated some α-amylases complex characteristics from the*Bradyrhizobium*sp. strain INPA R001 isolated from Amazonian soils. Enzyme essays were conducted to verify the amylolitic activity under different temperatures, exposition time, pHs, and thermal stability. The bacteria grew well and produced amylolitic halo and was thermophilic on starch medium. The enzyme complex of *Bradyrhizobium* was more active at 100˚C, and more active at pH 4.5. The INPA R001 enzyme showed thermal stability at 100˚C. The highest amylolitic activity showed by the α-amylase complex of this bacteria was 1.6 U/mL.

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INTRODUCTION

Brazilian Pro-Alcohol program uses sugar-cane as its main raw material. Sugar-cane crop is annual so that the period of time between harvests is characterized by inactivity for rural workers. Several sugar and alcohol producing companies are starting to cultivate sorghum, corn and other crops whose starch content may be used for the production of alcohol to fill this inactivity period. In the Amazon region, this strategy will allow the production of alcohol from cassava, sweet potato, and "babaçu" (*Orbignyaphalerata*). The production of the α-amylase (EC 3.2.1.1) became important since this enzyme permits the conversion of starch in products which are afterwards converted to alcohol. They are widely used in the food, pharmaceutic and detergent industries. Most of this enzyme in use in Brazil is imported.Alpha amylases (endo-1, 4-α-D-glucan glucanohydrolase EC 3.2.1.1) are extracellular endoenzymes that randomly cleave α-1,4 linkages between adjacent glucose units in the linear amylose chain and ultimately generate glucose, maltose, and maltotriose units (Bano *et al*., 2011). Enzymes are vital proteins which catalyze highly specific chemical reactions. In addition to be the basis of the metabolic system of all microorganisms, these enzymes permit enormous opportunities to industrial companies to achieve fine bio catalytic conversions which are more efficient and more economic (Hankin and Anagnostakis, 1975). Under natural conditions, microorganisms, mainly those found in soils, are the main source of α-amylases.Research programs to select new microbe sources of α-amylases are growing all over the world. Among those are the bacteria capable of inducing nodule formation in leguminous plants. These rhizobacteria, usually known as rhizobia, present a set of enzymes of high industrial value such as the nitrogenases, hydrogenases, lipases, pectinases, proteases, α-amylases, phosphate solubilizers (Van Beilen, 2002; Oliveira *et al*., 2006; 2007 a, b; 2010a,b; Chagas Jr *et al*., 2010). Also, they are not pathogenic to plants and animals, being, therefore, of unrestricted use for bio industries. The chemical properties of each α-amylase such as thermostability, pH profile, pH stability, and Ca-

independency are important to its application. α-amylase used in starch industry for example, must be active and stable at low pH, but also must be at high pH values in the detergent industry (Souza and Magalhães, 2010).The present study was developed to characterize the α-amylasecomplex produced by the isolate of a rhizobacteria, mainly as to her stability at high temperatures, a very important characteristic for industrial purpose, since they can be used under temperatures with less contamination of microorganisms.

MATERIALS AND METHODS

Selection of α-amylase-producing bacteria: The bacteria INPA R001 (*Bradyrhizobium*sp.) is part of the Ecology and Biotechnology of Soil Microorganisms Laboratory collection, a unit of the Amazon National Research Institute (LEBMAM/INPA). This bacterium was tested as to the growth capability in a starch containing medium as a carbon source (Oliveira and Magalhães, 1999). In addition to that, the presence of α-amylasecomplex was detected using iodine vapor as an indicator. A slight-yellow zone around the colony in contrast with the blue medium was an indication of a positive result for amylolysis activity (Buzzini and Martini, 2002). This strain was selected because, among a total of 50 bacterium strains and species, it displayed the best growth at the temperature of 55˚C after 48 hours of incubation.

Bacteria amylolysis index, and iodine test: The bacteriawas tested as to halo formation in a culture medium containing 1% of starch, using the iodine vapor coloration method to determine the amylolysis index (IA) defined as the ratio between the colony diameter and the halo diameter (Hankin and Anagnostakis, 1975). The evaluations were taking place at the inoculation day, 1, 2, and 3 days after incubation. For the test, the bacterium was inoculated in a LB liquid medium (Sezonov*et al*., 2007) with 1% of starch. An aliquot of 1 mL was taken at each day of observation which was placed in a microtube of 1.5 mL of volume to which a drop of liquid iodine was added. Then the developed coloration was examined.

Activity of the amylolysis enzymes complex produced by the bacteria: The enzyme broth or supernatant complex used in these tests resulted from the inoculation of the bacterium in a liquid LB medium containing 1% of starch. After growth, the number of cells was counted with a Neubauer chamber, diluting the cell suspension with the LB medium with starch up to a concentration of 10^6 cells. The inoculum was then centrifuged, discarding the cells and using the supernatant with the enzymes complex produced by the bacterium. These supernatants were used for the enzyme tests. The enzyme essays were conducted according to the dextrinizing method (Fuwa, 1954), in which an enzyme unit was defined as the amount of enzyme needed to hydrolyze 0.1 mg of starch. All the tests related to temperature were conducted in a thermal mixer.

Enzyme activities at different temperatures and pH's.The tested temperatures were of 20, 30, 40, 50, 60, 70, 80, 90, and 100˚C for 5 minutes to verify which the most adequate temperatures for the activity ofbacterial enzyme complex. To evaluate the effect of acidity on the α-amylase, tests with the buffer sodium acetate at the pH's of 3.5, 4.5, 5.5 and 6.5were conducted under the temperatures which gave the best results in the previous test.

Incubation time of the bacteria enzymes: The INPA R001 bacterium enzyme was tested under 80, 90, and 100˚C. All tests were conducted for 5, 10, 15, 20, 30, and 45 minutes.

Thermal stability: The bacteria enzyme was tested as to thermal stability at the temperatures of 70, 80, 90, and 100˚C for 40, 50, 60, 70, 80, and 90 minutes.

RESULTS AND DISCUSSION

Growth in a starch medium and apparent α-amylase production: For the industrial production of ethanol, the best condition is that the bacteriumis capable of growth in high temperatures and of metabolizing thermophilic and thermostable enzymes (Arikan, 2008). The INPA R001 was placed in Petri dishes to grow in a temperature of 55˚C, showing to be capable of growth in that temperature, being considered thermophilic bacterium (Table 1), witha high growth rate being observed on the 6th day of evaluation, using the scores of Oliveira and Magalhães (1999). The bacteriumalso showed the ability todegrade starch using the iodine vapor method (Table 1) (Nwokocha and Ogunmola, 2014). Other studies (Oliveira *et al*., 2006; 2007 a, b; 2010) also reported amylolitic activity in other rhizobacteria isolated from Amazonian soils, thus showing the potential of these bacteria as suppliers of α-amylase. As they are not pathogenic to plants and animals, they may be considered as a safe source of α-amylases for the use of bio industries.

Table 1. Growth and amylase production in a medium with starch at 55°C

Bacterium	Days*				α-amylase
Bradyrhizobium sp.	3	6	9	12	+
INPA R001	2.67	3.50	3.58	3.92	

*Scores as Oliveira andMagalhães (1999). Scores higher than 3.0 are considered of high growth on the culture medium.

This thermophilic bacteria characteristic is important (Palma-Fernandez *et al*., 2002), since the thermostable enzymes, in general, are advantageous for industrial purposes, because biotechnological processes conducted under high temperatures are less likely to contamination by mesophyll microorganisms, which usually prevail in industrial environments.

Amylolisis index: This test showed that the INPA R001 α-amylase complex had an index of 2.8 (Table 2).The ratio between the hydrolysis halo diameter and that of the colony (expressed as enzyme index – EI) equal to or larger than 2.0 indicates a good production of extra-cellular enzymes(Lealem and Gashe, 1994). So, the INPA R001 had a high enzyme activity. These results were similar to those reported by other authors, such as Oliveira *et al*. (2007 a,b).

Table 2. Enzyme index for amylase

Bacterium	Diameter		EI (dh/dc)
INPA R001 (*Bradyrhizobium*sp.)	Halo (dh)	Cologne (dc)	
	----------cm----------		
	1.4	0.5	2.8

Rhizobia capability to degrade starch has been discussed in some works. Several studies suggest a possible participation of some glycosidases, such as the α-amylases, in the intracellular establishment of the leguminous-*Rhizobium*

symbiosis (Singh and Singh, 1985; Van Spronsen *et al*., 1994; Berthelot and Delmotte, 1999).

Iodine test in the presence of starch: During the experiment, the bacterium did not showamylolysis activity which resultedin the development of the yellow color. But, after three days of observation, it presented a grey color which is an indication of a high enzyme activity (Table 3). Considering the sequence of colors and numbers, blue (1) represents absence of amylolysis and yellow (5), a high enzyme activity. As pointed out by Nwokocha and Ogunmola (2014), the disappearance of the blue color of starch-iodine complex indicates the amylose transformation from the starch and may be useful as an index of retrogradability of starch pastes.

Table 3. The iodine test in the presence of starch

Bacterium	Days after inoculation			
INPA R001 (*Bradyrhizobium sp.*)	0	1	2	3
Iodine test	1	1	3	4

Iodine test: 1 - dark blue; 2 - bluish purple; 3 - light purple; 4 – grey; 5 – yellow.

***Tests at different temperatures*:** Sodium acetate at pH of 6.5 was used as the buffer solution. The INPA R001 (*Bradyrhizobium*sp.) enzyme complex was less active – it varied from 0.0U.mL^{-1}, at 70˚C to 1.54 U.mL^{-1}, at 100˚C. Its activity was constant between 20 and 50˚C when it started to decline up to 70º C, and after that it increased again until the temperature of 100˚C (Figure 1), indicating the action of more than one α-amylase in the complex. Future experiments must be done to find out how many α-amylases are in the complex, as well as to isolate, purify and to characterize each one of them. The commercial use of α-amylase usually does not require purification of the enzyme, but in pharmaceutical and clinical sectors they must be high purified (Souza and Magalhães, 2010). They also must be used in the purified form in studies of structure-function relationships and biochemical properties (Gupta *et al*., 2003). The presence of activities above 50 °C indicate that the α-amylase complex of INPA R001 may be useful for bio industries application, as pointed out by Souza and Magalhães (2010), once this thermophilic property may improve the industrial process.

Tests under different pH's and temperatures: The best combination of pH and temperature for the α-amylase complex activity was determined by conducting the tests at different pH's and three temperatures. The enzyme produced by the INPA R001 (*Bradyrhizobium*sp.) bacterium presented the best activity at pH of 4.5 and temperature of 100ºC, with 1.36 U.mL^{-1} (Figure 2), followed by the pH of 6.5 at the temperature of 90 °C, with 1.22 U.mL^{-1}.There was a significant increment in activity when the pH went from 3.5 to 6.5 at the temperature of 90˚C. At the temperature of 100˚C, a steep increment in activity was observed when the pH went from 3.5 to 4.5.

Tests at different incubation times: The enzymecomplex was tested during the periods of time of 5, 10, 20, 30, and 45 minutes of the enzyme reaction at three temperatures and at the pH's 6.5. The INPA R001 (*Bradyrhizobium*sp) enzyme complex showed a very different behavior (Figure 3). At 100˚C, its activity was far more intense during the initial 10 minutes than those observed at 80 and 90˚C, but there was a significant reduction at that temperature, much larger than the observed at the other two temperatures. At the temperatures of 80 and 90˚C, a sudden increment in activity was observed between 15 and 20 minutes, decreasing after that.

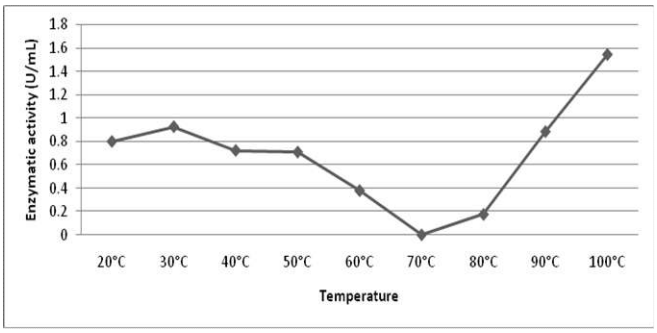

Figure 1. α-amylase enzyme complex activity of INPA R001 (*Bradyrhizobium sp.*) under different temperatures

At the temperature of 80˚C, the best activity was observed at 20 minutes with 0.8 U.mL^{-1}, followed by the time of 15 minutes with 0.6 U.mL^{-1}. At the temperature of 90˚C, though, the best activity took place at 15 minutes with 0.9 U.mL^{-1} followed by the time of 10 minutes with an activity of 0.5 U.mL^{-1}.

Thermal stability: The INPA R001 α-amylase complex was more stable at the temperature of 100˚C and was maintained at the levels of 0.15 U.mL^{-1}to 0.20 U.mL^{-1}during the 90 minutes of reaction (Figure 4). At 70˚C it was more active at 50 minutes (0.25 U.mL^{-1}) and decreased as the time of reaction increased.

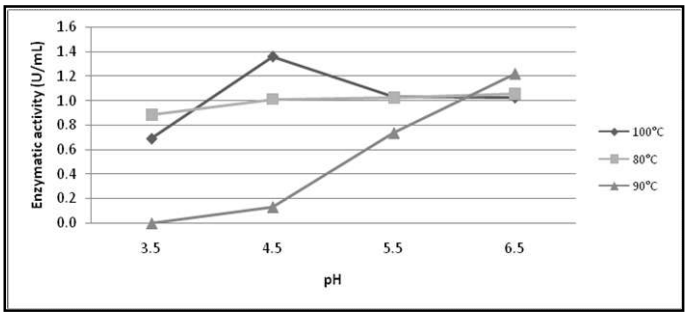

Figure 2. Amylolysis enzyme complex activity of INPA R001 (*Bradyrhizobium sp.*) under different pH 's and temperatures

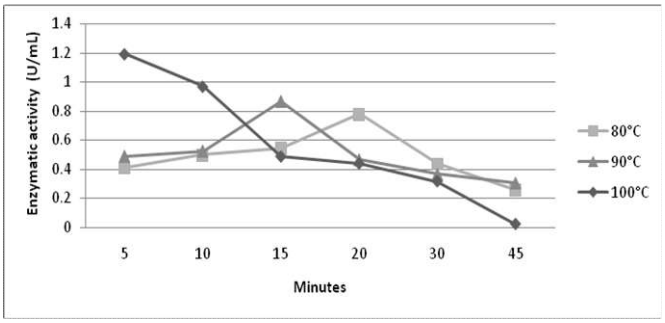

Figure 3. Amylolysis enzyme complex activity of INPA R001 (*Bradyrhizobium sp.*) at different reaction times and temperatures

At the temperature of 80˚C, the enzyme reached the highest level of activity at 50 minutes of the reaction, with a result of 0.27 U.mL^{-1}, this being the highest activity level, like that verified at the temperature of 90˚C.If the highest level of activity is necessary, the ideal would be the temperature of 80˚C for up to 70 minutes. From that point onwards, the

temperature should be raised to 100˚C.Oliveira *et al*. (2010b) also observed that α-amylase from two other indigenous Amazonian rhizobia are active under a large variation of pHs and temperatures. The crude enzymes of both rhizobia were active over a pH range from 4.5 to 8.5 and temperatures from 30 to 50 °C.

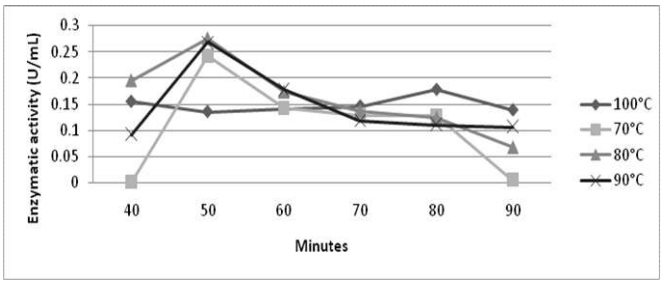

Figure 4. Thermostability of α-amylase complex activity of INPA R001 (*Bradyrhizobium sp*.) at different times and temperatures

These chemical enzymes characteristics may be useful for bio industries, since they can be used under different work conditions. The special characteristics of enzyme exploited for itscommercial interest and industrial applications include thermotolerance, thermophilic nature, tolerance to a varied range of pH, stability of enzyme activity over a range of temperature and pH, and other harsh reaction conditions (Nigam, 2013). Thus, the crude α-amylase found in INPA R001 present desirable characteristics for industrial applications.

Conclusions

- The INPA R001 α-amylase complex presented the highest activity at 100˚C and pH 4.5.
- The INPA R001 α-amylase complex was more thermal stable at 100˚C.
- The highest amylolysis activity shown by the α-amylasecomplex was 1.6 $U.mL^{-1}$.

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