Vol. 53, n. 1: pp.35-45, January-February 2010
ISSN 1516-8913 Printed in Brazil

BRAZILIAN ARCHIVES OF
BIOLOGY AND TECHNOLOGY
A N I N T E R N A T I O N A L J O U R N A L

# Partial Characterization of Amylases of two Indigenous Central Amazonian Rhizobia Strains

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## ABSTRACT

*Amylase production and partial characterization of crude enzyme preparations from two rhizobia strains (R-926 and R-991) were evaluated. For both the strains, maximal amylase activities were achieved during the early-to-mid-exponential growth phase; both were active over a pH range from 4.5 to 8.5 and temperature from 30 to 50 $^oC$. None of the ions studied ($K^+$, $Na^+$, $Ca^{2+}$, $Hg^{2+}$, $Mg^{2+}$, $Mn^{2+}$, $Cu^{2+}$ and $Zn^{2+}$) was required for the catalytic activity of strain R-926; amylase activity of strain R-991 was stimulated in the presence of $K^+$, $Hg^{2+}$ and $Zn^{2+}$. The surfactants SDS, Triton X-100 and Tween-80 did not have a pronounced inhibitory effect on enzyme activities; SDS and Tween-80 caused the highest stimulatory effects. Amylase activities from the rhizobia strains were reduced by up to 30% in the presence of EDTA; amylase activity of R-926 was also inhibited by $HgCl_2$, suggesting that $Ca^{2+}$and cysteine residues could be important for activity of this strain.*

**Key words:** *Rhizobium*, *Bradyrhizobium*, Amylase production, Enzymatic properties

## INTRODUCTION

Amylases are among the most important enzymes used in modern biotechnology, particularly in the processes involving starch hydrolysis. The extensive application of amylases in the food, starch liquefaction and saccharification, detergent, textile, paper, brewing and distilling industries has paved a way for their large-scale commercial production (Gupta et al., 2003). Although amylases originate from different sources (plants, animals and microorganisms), microbial amylases generally meet industrial demands best, due to their short growth period, productivity and thermostability (Burhan et al., 2003). Today, a large number of microbial amylases are available commercially and they have almost completely replaced chemical hydrolysis of starch in the starch processing industry (Pandey et al., 2000). Microbial amylases are produced mainly from cultures of *Aspergillus*, *Bacillus*, *Clostridium*, *Pseudomonas*, *Rhizopus* and *Streptomyces* species (Pandey et al., 2000). Because of the industrial importance of amylases, there is an increasing worldwide interest in the screening of new microorganisms producing amylases suitable for new industrial applications (Burhan et al., 2003; Gupta et al., 2003). Rhizobia are gram-negative soil bacteria belonging to the family Rhizobiaceae that are capable of infecting and nodulating the roots of their hosts, leguminous plants. Cellulolytic and pectinolytic (Jimenéz-Zurdo et al., 1996), proteolytic (Glenn and Dilworth, 1981) and ureolytic (Toffanin et al., 2002) enzyme activities

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have been detected in pure rhizobial culture. Nonetheless, studies on rhizobia amylases are still scarce. Recently, Oliveira et al. (2006) reported the occurrence of extracellular amylases in rhizobia strains in solid and liquid media (Oliveira et al., 2007). The aim of this study was the production and characterization of extracellular amylases from two indigenous Central Amazonian rhizobia strains.

## MATERIALS AND METHODS

### Indigenous rhizobia strains, culture conditions and inoculum preparation

The rhizobia strains used in this study were *Rhizobium* sp. INPA R-926 and *Bradyrhizobium* sp. INPA R-991 isolated from soils of the Central Amazonian floodplain. The rhizobial strains were identified by partial sequencing of the 16S rRNA gene (Oliveira et al., 2007). Stock cultures were maintained on extract-mannitol agar (YMA) slants (Vincent, 1970) at 4 $^oC$. The medium used for enzyme production was composed of (g $L^{-1}$): 10.0 maltose, 0.4 $K_2HPO_4$, 0.1 $K_2HPO_4$, 0.2 $MgSO_4.7 H_2O$, 0.1 NaCl and 0.4 yeast extract. The pH of the medium was adjusted to 6.8 with KOH and autoclaved at 120 $^oC$ for 20 min. Fifty milliliters of medium were taken in 150 mL Erlenmeyer flasks and inoculated with 1 mL of inoculum culture, grown in YM medium (Vincent, 1970) for 3 (R-926 = 1.4 x $10^9$ CFU) and 5 days (R-991 = 2.0 x $10^9$ CFU), and incubated at 28 $^oC$ with rotary shaking (65 cycles per min). Cultures were harvested and centrifuged (12000 rpm, 10 min) at 24-h intervals for 8 (R-926) and 12 days (R-991) of growth. At these intervals, the cell-free culture supernatant served as the enzyme source to evaluate the reducing sugars, amylase activity, total extracellular protein and final pH of the medium. The turbidity of the cultures was determined by measuring the increase in optical density at 560 nm in a Spectrum UV-Vis Spectrophotometer. Biomass production (g. $L^{-1}$) was also determined by drying the pellet at 105 $^oC$ overnight until constant weight.

For enzymatic characterization studies, the rhizobia strains were incubated for 48 h in the same experimental conditions mentioned above.

### Analytical methods

Amylase activity was determined by monitoring starch hydrolysis and production of reducing sugars from starch. The reaction mixture consisted of 300 mL of previously gelatinized corn starch solution (1%), 350 mL of 0.05 M phosphate buffer (pH 6.5) and 350 mL of crude enzyme preparation. After 30 min of incubation at 37 $^oC$ in a water bath, the reducing sugars (glucose equivalents liberated) were estimated by Nelson's modified Somogyi's method (Nelson, 1944). The colour was read at 511 nm using a Spectrum UV-Vis Spectrophotometer. Glucose was used as the standard. The blank contained 300 mL of corn starch solution and 700 mL of 0.05 M phosphate buffer (pH 6.5). One enzyme unit (U) was defined as the amount of enzyme that released 1 μmol of reducing sugar $mL^{-1}$ $min^{-1}$ under the standard assay conditions described above. Total extracellular protein was measured by the biuret method (Gornall et al., 1949), using bovine serum albumin (BSA) in the standard curve.

### Effect of pH on activity and stability

The effect of pH on enzyme activity was determined by measuring the activity at 37 $^oC$ using citrate – phosphate (0.05 M, pH 3.5 – 5.5), phosphate (0.05 M, pH 6.5 – 7.5) and Tris-HCl (0.05 M, pH 8.5 – 9.5) buffers for 30 min. Stability of enzyme without substrate was also studied by pre-incubating the crude enzyme at different pH values ranging from 3.5 to 9.5 for 24 h, at room temperature, with the residual activity determined under standard conditions.

### Effect of temperature on activity and stability

The effect of temperature on enzyme activity was determined by performing the standard assay procedure for 30 min within a temperature range of 30 – 60 $^oC$ in phosphate buffer (0.05 M, pH 7.5). Thermostability in the absence of substrate was investigated at pH 7.5 after pre-incubation of the crude enzyme at temperatures ranging from 30 to 60 $^oC$ for 2 h in a water bath, and then the samples were removed and the remaining activity assayed under standard conditions.

### Effect of cations on enzyme activity

The influence of cations ($K^+$, $Na^+$, $Ca^{2+}$, $Hg^{2+}$, $Mg^{2+}$, $Mn^{2+}$ and $Zn^{2+}$) on amylase activity was studied by adding them to the reaction solution to final concentrations of 1 and 5 mM. All metals used were in the chloride and sulphate forms. The activity was assayed under optimum conditions of

both pH and temperature as required by the rhizobia amylases. Activity in the absence of any additives (control) was taken as 100%.

### Effect of surfactants on enzyme activity

To investigate the effect of surfactants on enzyme activity, the detergents, Triton X-100, Sodium Dodecyl Sulfate (SDS) and Tween-80 were added to the enzyme at final concentrations of 1 and 2%. Amylase activity was then measured in the same way as mentioned earlier.

### Effect of inhibitors on enzyme activity

The assay procedure was the same as described above except that the reaction mixture included different inhibitors, such as Ethylene Diamine Tetraacetic Acid (EDTA), $HgCl_2$ and p-Methyl Sulphonyl Fluoride (PMSF), at final concentrations of 5 and 10 mM. Amylase activity was determined as described previously.

### Experimental design and statistical analyses

The enzymatic assays were organized using a complete randomized design with three replicates and repeated twice for reproducibility. Tukey's test was used to identify means that differed significantly ($p < 0.05$). Pearson's correlation coefficients were also considered significant at $p < 0.05$.

## RESULTS AND DISCUSSION

### Growth curve and amylase production

Maximum biomass production and optical density ($DO_{560}$) for both the strains were achieved after the 4th day of cultivation (Figs 1 and 2). *Bradyrhizobium* strain exhibited maximum total extracellular protein on the 2nd day of cultivation, whereas the *Rhizobium* strain exhibited its maximum on the 7th day of incubation. For both the strains, maximal amylase activities were achieved during the early-to-middle-exponential growth phase and then decreased gradually, as observed for other bacterial amylases (Castro et al., 1999; Aguilar et al., 2000).

Final pH of the culture medium after 72-h growth of *Rhizobium* strain increased from 6.8 to 7.4 (Fig. 1). The rise of pH could be due to the utilization of organic acids or production of alkaline compounds, as suggested by Cibis et al. (2004) and Rahman et al. (2005). On the other hand, the final pH of the culture medium of *Bradyrhizobium* strain decreased to 5.7 during this period (Fig. 2). This could be attributed to the production of acids during the bacterial growth.

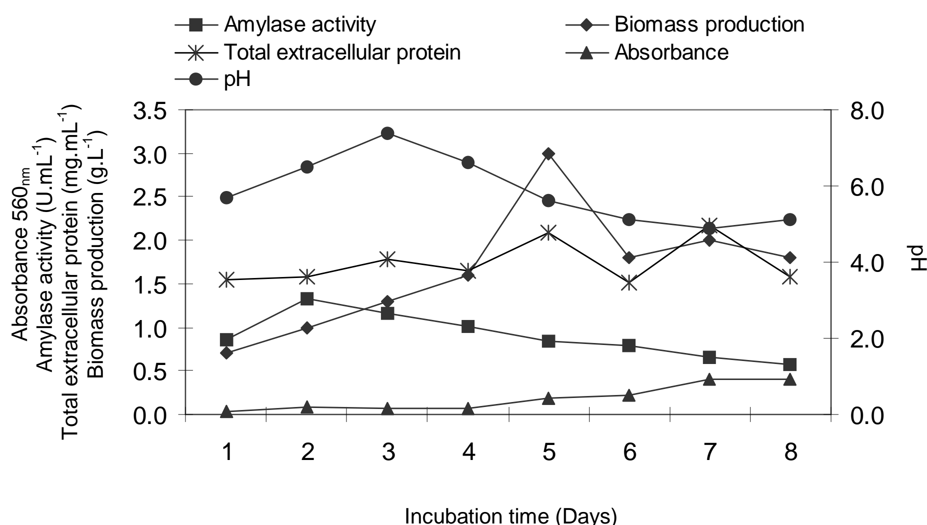


**Figure 1 -** Time course of amylase production by *Rhizobium* sp. strain INPA R-926 using maltose as sole carbon source. Amylase activity (■); Biomass production (◆); Total extracellular protein (∗); Absorbance (▲); pH (•).

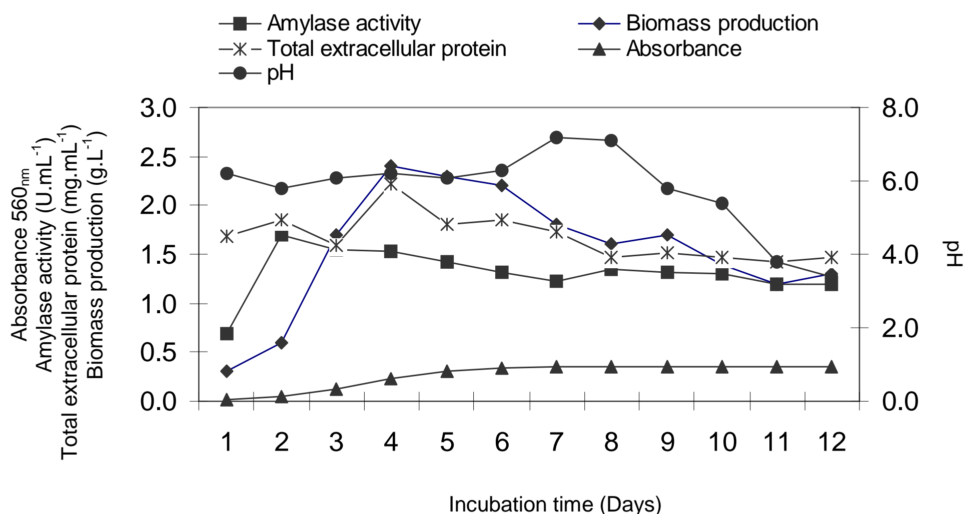


**Figure 2 -** Time course of amylase production by Bradyrhizobium sp. strain INPA R-991 using maltose as sole carbon source. Amylase activity (■); Biomass production (◆); Total extracellular protein (∗); Absorbance (▲); pH (•).

**Correlations among the parameters evaluated**

There was no significant correlation between amylase production and final pH of the medium of the *Bradyrhizobium* strain. On the other hand, amylase production and final pH of the medium were closely associated for the *Rhizobium* strain (Table 1). Similar findings have been previously documented by Sunna and Hashwa (1990), Wijbenga et al (1991) and Stamford et al. (2001) for other microbial amylases.

Amylase production from the *Rhizobium* strain presented significant negative correlations with biomass and optical density ($OD_{560}$). These negative correlations could be because of the higher amylase production that occurred during the early-to-mid-exponential growth phase, while during the late exponential and early stationary growth phase, rhizobial biomass production was higher. The negative correlation observed between amylase production and optical density ($OD_{560}$) supported this observation (Table 1). On the other hand, correlation between amylase production and bacterial biomass from *Bradyrhizobium* strain was significantly positive, confirming several authors who also found this type of linear correlation in *Bacillus stearothermophilus* (Davis et al., 1980), *B. amyloliquefaciens* (Castro et al., 1993), *Nocardiopsis* sp. (Stamford et al., 2001), *Bacillus* sp. (Cordeiro et al., 2002) and in *Rhodothermus marinus* (Gomes et al., 2003). There was no significant correlation between amylase production and total extracellular protein for either rhizobia strains (Table 1).

**Table 1 -** Correlation analysis between amylase production and final pH of the culture medium, optical density (DO), biomass production and total extracellular protein secretion in two indigenous Central Amazonian rhizobia strains.

| Rhizobia strains | Final pH | Biomass production | $OD_{560}$ | Total extracellular protein |
|---|---|---|---|---|
| INPA R-926 (n = 24) | 0.86[a] | -0.45 | -0.79 | -0.23 |
| | (0.000)[b] | (0.026) | (0.000) | (0.277) |
| INPA R-991 (n = 36) | 0.09 | 0.47 | 0.08 | 0.30 |
| | (0.702) | (0.019) | (0.703) | (0.075) |

[a] Pearson's coefficient of correlation; [b] Probability level

**Effect of pH on amylase activity and stability**

The pH profiles showed a broad range of activity at 37 °C for 30 min, exhibiting 70-88% of maximum activities in the pH range 4.5-8.5 (Figs 3 and 4). The pH for the optimum activity of amylase from the *Rhizobium* strain was 7.5 (Fig. 3). A similar result was reported by Cordeiro et al. (2002) for an amylase produced by *Bacillus* sp. The optimum pH for amylase activity from the *Bradyrhizobium* strain was also 7.5 (Fig. 4), but significant levels of activity (above 88%) were still detected between pH 5.5-8.5. The amylases produced by several bacterial sources, including *Bacillus* sp., have a variety of pH profiles. The maximum activity of most of the enzymes earlier reported has been in the pH range 5.0-12.0 (Kim et al., 1995; Leveque et al., 2000; Hagihara et al., 2001; Gomes et al., 2003; Goyal et al., 2005). In spite of earlier reports on the amylase activity at low pH for some amylases (Buonocore et al., 1976; Ohdan et al., 1999), there are few reports on amylases that have maximum activity at pH lower than 5.0 (Vihinen and Mäntsälä, 1989; Matzke et al., 1997; Jorgensen et al., 1997). In this study, the amylases produced by these rhizobia strains retained up to 78% of their maximum catalytic activities at pH 4.5 (Figs 3 and 4).

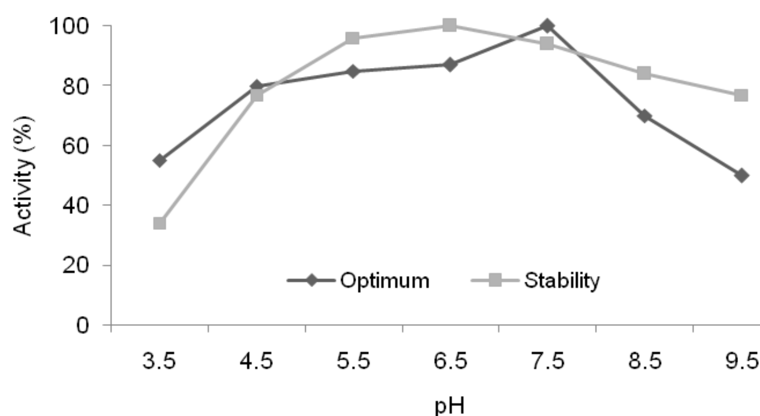


**Figure 3** - Optimum pH and stability pH of *Rhizobium* sp. strain INPA R-926 amylase. Relative activity is expressed as a percentage of the maximum (100% of enzyme activity = 1.9 U.mL$^{-1}$).

The rhizobia amylases were very stable, retaining more than 70% of their original activities in the range pH 4.5-9.5 (Figs 3 and 4). The stability of amylase from the *Rhizobium* strain increased up to pH 6.5 and then decreased gradually above this pH (Fig. 3). Above pH 5.5, the stability of amylase secreted by the *Bradyrhizobium* strain decreased significantly (Fig. 4). The amylase enzyme produced by the *Rhizobium* strain retained up to 94% of its original activity at pH 7.5, while the enzyme from the *Bradyrhizobium* strain had a loss of 16% (Figs 3 and 4). Similar results have been found in other studies (Cordeiro et al., 2002; Gupta et al., 2003; Sajedi et al., 2005).

**Effect of temperature on amylase activity and stability**

The amylase from the *Rhizobium* strain showed good catalytic activity from 30 to 50 °C, with an optimum temperature of 40 °C (Fig. 5). Above the optimum temperature, amylase activity decreased significantly. The relative activities at 50 and 60 °C were 65 and 55%, respectively. Optimal temperatures were between 30 and 50 °C for amylase from the *Bradyrhizobium* strain (Fig. 6). At 60 °C, however, a decrease of 37% in enzyme activity was registered. Several authors have reported that the majority of the bacterial amylases have an optimum temperature in the range of 30-100 °C, with pH values between 4.6 and 9.0 (Pandey et al., 2000; Cordeiro et al., 2002; Gupta et al., 2003). Based in these studies, it could be inferred that the optimal temperatures found in this study were similar or lower than those described for other bacterial amylases.

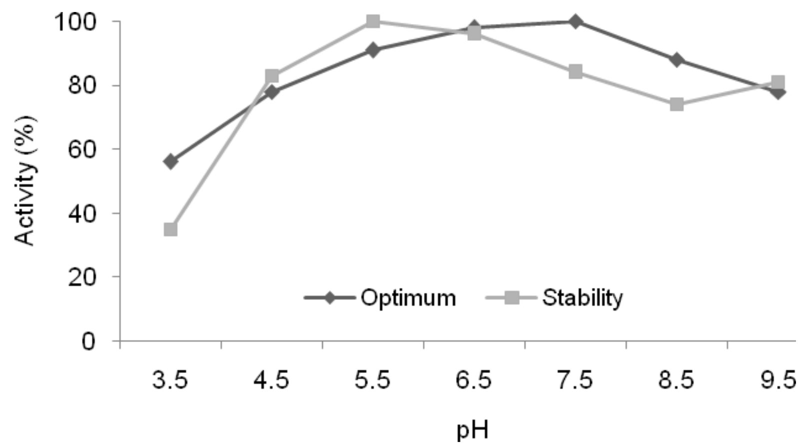


**Figure 4 -** Optimum pH and stability pH of Bradyrhizobium sp. strain INPA R-991 amylase. Relative activity is expressed as a percentage of the maximum (100% of enzyme activity = 1.7 U.mL-1).

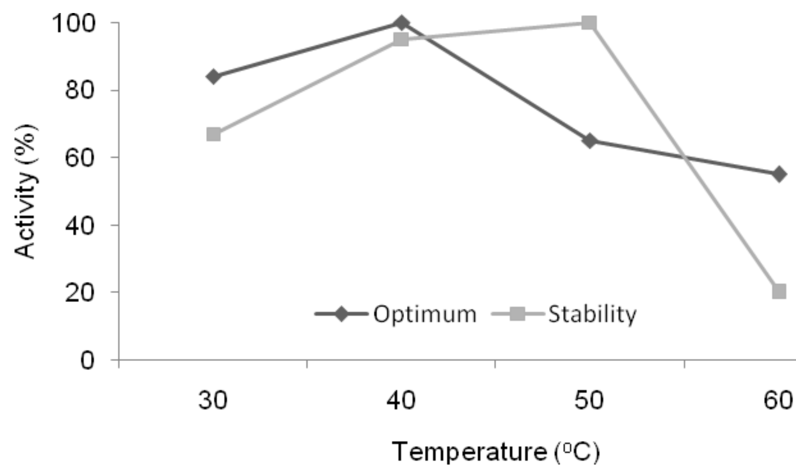


**Figure 5 -** Optimum temperature and stability temperature of Rhizobium sp. strain INPA R-926 amylase. Relative activity is expressed as a percentage of the maximum (100% of enzyme activity = 2.6 U.mL-1).

The amylase produced by the *Rhizobium* strain retained 95 and 100% of its original activity at 40 and 50 °C, respectively (Fig. 5). Above 50 °C, enzyme activity declined very sharply, possibly because of thermal inactivation. Similarly, amylase from the *Bradyrhizobium* strain was fully stable at 30 °C, but lost 26, 29 and 78% of its original activity at 40, 50 and 60 °C, respectively (Fig. 6). These data were comparable to the results documented by other researchers (Hamilton et al., 1999; Pandey et al., 2000; Cordeiro et al., 2002; Bernhardsdotter et al., 2005).

## Effect of metal ions on amylase activity

None of the metal ions evaluated was required for the activity of amylase from the *Rhizobium* strain (Table 2). The following metal ions had a slight inhibitory effect on enzyme activity: $Na^{+}$ (5 mM), $Ca^{2+}$ (5 mM), $Cu^{2+}$ (1 and 5 mM), $Mg^{2+}$ (1 and 5 mM) and $Mn^{2+}$ (1 and 5 mM). On the other hand,

stronger inhibitory effects were observed in the presence of $K^+$ (5 mM) and $Hg^{2+}$ ions (5 mM) after 30 min incubation. Inhibitor effects of 30, 27, 24, 23 and 20% were observed in the presence of 1 mM of $K^+$, $Hg^{2+}$, $Na^+$, $Ca^{2+}$ and $Zn^{2+}$, respectively. In addition, $Zn^{2+}$ (5 mM) also inhibited enzyme activity by 21%. Metal ions play a key role in protein folding or in catalysis.

Most amylases are known to be metal ion dependent enzymes (Pandey et al., 2000; Gupta et al., 2003; Ramachandran et al., 2004); in contrast, Chakraborty et al. (2000) reported an amylase from *Bacillus stearothermophilus* whose activity was inhibited by divalent metal ions. Inhibition or no effect in the presence of some mono and trivalent cations (1 mM, 5 mM, 10 mM or 50 mM) were also documented for amylases from *Lactobacillus manihotivorans* LMG 18010T (Aguilar et al., 2000), *Bacillus amyloliquefaciens* (Sarikaya and Gürgün, 2000), *Bacillus* sp. KSM-K39 (Hagihara et al., 2001) and *Bacillus* sp. (Cordeiro et al., 2002).

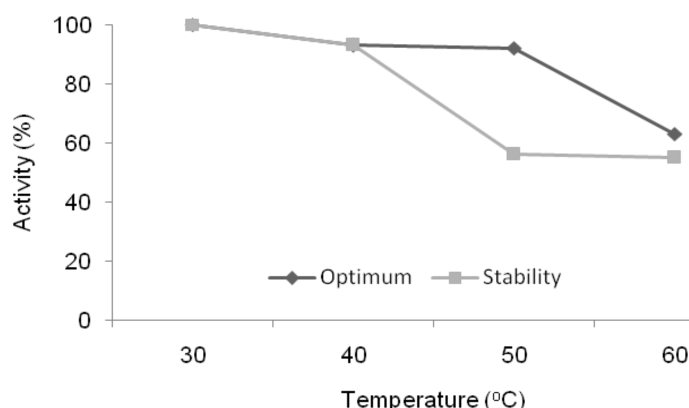


**Figure 6 -** Optimum temperature and stability temperature of Bradyrhizobium sp. strain INPA R-991 amylase. Relative activity is expressed as a percentage of the maximum (100% of enzyme activity = 2.5 U.mL-1)

**Table 2 -** Influence of metal ions on amylase activities in indigenous rhizobia strains of Central Amazonian.

| Cationic ions | Concentration (mM) | INPA R-926 (Relative activity, %) | INPA R-991 (Relative activity, %) |
|---|---|---|---|
| Control (No addition) | 0 | 100 | 100 |
| $K^+$ (KCl) | 1 | 70 | 137 |
| | 5 | 56 | 154 |
| $Na^+$ (NaCl) | 1 | 76 | 72 |
| | 5 | 90 | 58 |
| $Ca^{2+}$ ($CaCl_2$) | 1 | 77 | 84 |
| | 5 | 87 | 77 |
| $Hg^{2+}$ ($HgCl_2$) | 1 | 73 | 137 |
| | 5 | 51 | 119 |
| $Mg^{2+}$ ($MgCl_2 . 7H_2O$) | 1 | 87 | 88 |
| | 5 | 87 | 91 |
| $Mn^{2+}$ ($MnSO_4$) | 1 | 93 | 86 |
| | 5 | 87 | 77 |
| $Cu^{2+}$ ($CuSO_4 . 5H_2O$) | 1 | 85 | 88 |
| | 5 | 85 | 91 |
| $Zn^{2+}$ ($ZnSO_4 . 7H_2O$) | 1 | 80 | 84 |
| | 5 | 79 | 105 |

[a] Values are means of three triplicates. Relative activity is expressed as a percentage of the maximum (100% of enzyme activity = 2.9 $U.mL^{-1}$ for strain INPA R-929 and 2.7 $U.mL^{-1}$ for strain INPA R-991).

Activity of amylase from the *Bradyrhizobium* strain was strongly stimulated by $K^+$ and $Hg^{2+}$ (Table 2). The stimulatory effect of $K^+$ ions (5 and 10 mM) on amylase activity has been documented previously (Kekos and Macris, 1983; Pandey et al., 2000). In relation to $Hg^{2+}$, present data contradicted the results obtained by others (Shih and Labbé, 1995; Aguilar et al., 2000; Cordeiro et al., 2002; Najafi et al., 2005). However, 1 mM $Hg^{2+}$ had no inhibitory effect on amylase activity of *Bacillus* sp. strain KSM-K38 (Hagihara et al., 2001). These results suggested that the effect of $Hg^{2+}$ could vary among the microbial amylases, as observed for other heavy metal ions such as $Cd^{2+}$, $Co^{2+}$, $Fe^{2+}$ and $Ni^{2+}$ (Pandey et al., 2000; Lo et al., 2001; Dey et al., 2003). In addition, the presence of 5 mM $Zn^{2+}$ had a slight stimulatory effect on amylase activity (Table 2). On the other hand, activity was strongly or moderately reduced in the presence of $Na^+$ (1 and 5 mM), $Ca^{2+}$ (5 mM) and $Mn^{2+}$ (5 mM), while $Ca^{2+}$ (1 mM), $Mg^{2+}$ (1 and 2 mM), $Mn^{2+}$ (1 mM), $Cu^{2+}$ (1 and 5 mM) and $Zn^{2+}$ (1 mM) showed a slight inhibitory effect on enzyme activity. These results differed from those of previous studies which showed that $Ca^{2+}$ $Mg^{2+}$, $Mn^{2+}$ and $Na^+$ ions at a concentration of 5 mM enhanced amylase activity (Shih and Labbé, 1995; Sarikaya and Gürgün, 2000; Bernhanrdsdotter et al., 2005; Najafi et al., 2005). However, a similar inhibitory effect of heavy metal ions such as $Cu^{2+}$ and $Zn^{2+}$ on amylase activity of *Bacillus* sp. strain TS-23 (Lin et al., 1998), *B. subtilis* (Sarikaya and Gürgün, 2000), *B. thermooleovorans* NP54 (Malhotra et al., 2000) and *Bacillus* sp. strain L1711 (Bernhanrdsdotter et al, 2005) has been reported.

**Effect of surfactants on amylase activity**

None of the surfactants tested had a pronounced inhibitory effect on enzyme activities. Only Triton X-100 showed a moderate inhibitory effect (14%) on amylase activity of *Rhizobium* strain (Table 3). On the other hand, the addition of SDS (1%) and Tween-80 (2%) was found to stimulate activity by 9 and 21%, respectively. SDS had a pronounced effect on amylase activity (65%) of strain R-991, while Triton X-100 and Tween-80 at a concentration of 1mM increased activity by 6 and 22%, respectively (Table 3).

**Table 3 -** Influence of surfactantes on amylase activities in indigenous rhizobia strains of Central Amazonian[a].

| Surfactants | Concentration (%) | INPA R-926 (Relative activity, %) | INPA R-991 (Relative activity, %) |
|---|---|---|---|
| Control (No addition) | 0 | 100 | 100 |
| SDS | 1 | 109 | 176 |
| | 2 | 94 | 165 |
| Triton X-100 | 1 | 86 | 106 |
| | 2 | 86 | 97 |
| Tween-80 | 1 | 97 | 122 |
| | 2 | 121 | 100 |

[a] Values are means of three triplicates. Relative activity is expressed as a percentage of the maximum (100% of enzyme activity = 1.7 $U.mL^{-1}$ for the *Rhizobium* strain and 1.8 $U.mL^{-1}$ for the *Bradyrhizobium* strain).

The stimulatory effect of surfactants on amylase activity is controversial. Arnesen et al. (1998) reported a stimulatory effect of Tween-80 (1.3%) on amylase activity of *Thermomyces lanuginosus*. In the same study, Triton X-100 had no beneficial effect. The activity of amylase was stimulated by the presence of Tween-80 (1%) in *Bacillus* sp. (Oberoi et al., 2001). In another study, Triton X-100 (0.1 mM) and Tween-80 (0.05 mM) had weak stimulatory effects on amylase and pullulanase activities in *Clostridium thermosulfurogenes* (Reddy et al., 1999). Similar results were reported by Srisvastava and Baruah (1986) in *B. stearothermophilus*. On the other hand, addition of SDS (0.03%) and Tween-80 has pronounced effects on amylase activity in *Geobacillus thermoleovorans* (Rao and Satyanarayana, 2003). A stimulatory effect of SDS (6%) on amylase activity and stability in *Bacillus* sp. strain TS-23 was documented by Lo et al. (2001). From the previous reports and the present study, it was evident that the same surfactant could have different effect on the activity of the same enzyme in the same microorganism, or on the activity of different enzymes in the same microorganism, as previously discussed by Reddy et al. (1999).

**Effect of inhibitors on amylase activity**

When EDTA (5 mM) and $HgCl_2$ (5 mM) were included in the reaction mixture, the enzyme activity of *Rhizobium* strain was reduced by 30 and 47%, respectively (Table 4). EDTA (5 mM) was also found to reduce by 30% amylase activity of *Bradyrhizobium* strain (Table 4). In contrast and independently of the concentration tested, $HgCl_2$ increased amylase activity of *Bradyrhizobium* strain. PMSF, a modifier of serine residues, either had no effect or stimulated activity of both rhizobia strains. Similar findings were reported for amylases from *Bacillus* strains TS-23 (97%) (Lin et al., 1998), ANT-6 (103%) (Buhan et al., 2003) and L1711 (91%) (Bernhardsdotter et al., 2005). The results of the present study suggested, at least in part, that $Ca^{2+}$ ions and cysteine could be important for activity of the amylase from strain R-926. The importance of cysteine residues for catalysis was previously described for amylases independently of their origin (Pandey et al., 2000; Lo et al., 2001; Díaz et al., 2003).

From the results, it could concluded that the broad range of pH activity and stability profiles, optimum activity at lower pH and the high tolerance towards surfactants such as SDS, Triton X-100 and Tween-80 could make these rhizobial amylases attractive for both basic research and industrial biotechnological processes.

**Table 4 -** Influence of inhibitors on amylase activities in indigenous rhizobia strains of Central Amazonian[a].

| Inhibitors | Concentration (mM) | INPA R-926 (Relative activity, %) | INPA R-991 (Relative activity, %) |
|---|---|---|---|
| Control (No addition) | 0 | 100 | 100 |
| EDTA | 1 | 85 | 81 |
| | 5 | 70 | 70 |
| $HgCl_2$ | 1 | 79 | 137 |
| | 5 | 53 | 119 |
| PMSF | 1 | 111 | 94 |
| | 5 | 103 | 84 |

[a] Values are means of three triplicates. Relative activity is expressed as a percentage of the maximum (100% of enzyme activity = 1.4 $U.mL^{-1}$ for the *Rhizobium* strain and 1.5 $U.mL^{-1}$ for the *Bradyrhizobium* strain).

## RESUMO

A produção e parcial caracterização de extratos brutos de amilase de duas estirpes de rizóbio (R-926 e R-991) foram avaliadas. Para ambas as estirpes, as máximas atividades amilolíticas foram obtidas no início/meio da fase exponencial de crescimento. As amilases rizobiais foram ativas numa variação de pH de 4,5 a 8,5 e temperatura de 30 a 50 $^{o}C$. Nenhum dos íons testados ($K^+$, $Na^+$, $Ca^{2+}$, $Hg^{2+}$, $Mg^{2+}$, $Mn^{2+}$, $Cu^{2+}$ e $Zn^{2+}$) foi exigido para a atividade catalítica da estirpe R-926. A amilase produzida pelo R991 foi estimulada na presença de $K^+$, $Hg^{2+}$ e $Zn^{2+}$. Os surfactantes SDS, Triton X-100 e Tween-80 não exerceram um pronunciado efeito inibitório sobre as atividades enzimáticas, e SDS e Tween-80 causaram os maiores efeitos estimulatórios. A atividade amilolítica rizobial foi reduzida em até 30% na presença de EDTA; a amilase produzida pela estirpe R-926 também foi inibida pelo $HgCl_2$, sugerindo, ao menos em parte, a importância de $Ca^{2+}$ e resíduos de cisteína na atividade amilolítica dessa estirpe.

## ACKNOWLEDGEMENTS

The authors gratefully acknowledge the financial support from the Fundação de Amparo à Pesquisa do Estado do Amazonas (FAPEAM Process 624/04) and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq Process 474007/2003-0). LAO is a CNPq fellow. They thank Dr. Charles Roland Clement (INPA/CPCA) for reviewing the English text.

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Received: Janeiro 04, 2007;
Revised: September 03, 2007;
Accepted: April 14, 2009.