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Production and Some Properties of Crude Alkaline Proteases of Indigenous Central Amazonian Rhizobia Strains

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ABSTRACT

Two rhizobia strains isolated from soils of the Central Amazonian floodplain produced appreciable quantities of crude alkaline protease extracts with inexpensive carbon and nitrogen sources. These protease crude extracts were optimally active at pH 9.0-11.0. The optimum temperatures were 35 ^{o}C for Rhizobium sp. *strain R-986 and 55 ^{o}C for* Bradyrhizobium sp. *strain R-993. Protease activities in the crude extracts were enhanced in the presence of 5 mM metal ions, such as Na^{+}, Ca^{2+}, Mg^{2+} and Mn^{2+}. Rhizobia proteases were strongly inhibited by PMSF, a serine-protease inhibitor. The enzymes were active in the presence of surfactants (SDS and Triton X-100) and stable in oxidizing (H_2O_2) and reducing agents (β-mercaptoethanol), and organic solvents (acetone, hexane, methanol, 1-propanol and toluene).*

Key words: *Rhizobium*, *Bradyrhizobium*, enzymatic activities, organic surfactants

INTRODUCTION

Proteases [serine protease (EC. 3.4.21), cysteine (thiol) protease (EC. 3.4.22), aspartic protease (EC. 3.4.23) and metalo-protease (EC. 3.4.24)] are the most important class of enzymes that catalyze total hydrolysis of protein, and have been studied extensively since the advent of enzymology. Proteases from the microbial sources are preferred to enzymes from plant and animal sources, since they possess almost all the characteristics desired for the biotechnological applications (Gupta et al., 2002). Bacterial alkaline proteases are of particular interest due to their numerous applications in the detergent, tanning, chemical and dairy industries. Furthermore, they are used in pharmaceuticals and medical diagnosis, recovery of silver from X-ray films, etc. (Gupta et al., 2002; Joo et al., 2003).

At present, *Bacillus* (Gupta et al., 2002; Nilegaonkar et al., 2007), *Pseudomonas* (Ogino et al., 2001; Rahman et al., 2005), *Aspergillus* (Tunga et al., 2003), *Streptomyces* (Azeredo et al., 2004) and *Penicillium* (Agrawal et al., 2004) species are considered to be the most important sources of industrial proteases. Nevertheless, various other sources of microbial proteases are being investigated (Elibol and Moreira, 2005; Seeta Laxman et al., 2005; Vidyasagar et al., 2006).

While alkaline proteases from several bacteria

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have been extensively characterized, similar attention has not been paid to rhizobia bacteria. Rhizobia are widespread soil bacteria able to induce the formation of root nodules and to fix the atmospheric nitrogen in the cultivated and wild legumes. To date, symbiotic biological fixation is the most important topic of investigation involving these soil bacteria. However, the ability to produce a variety of other enzymes may be an attractive characteristic in these beneficial microorganisms (Oliveira, 2006). In previous studies (Oliveira et al., 2006a,b), several indigenous strains of rhizobia from Central Amazonia as promising sources of industrially relevant enzymes for biotechnological uses have been reported. On solid medium, some of these stains were able to grow and produce appreciable amounts of extracellular proteases at pH 8.0 (Oliveira et al., 2006a), indicating the probable alkaline nature of these rhizobia proteases. However, these extracellular enzymes have not been characterized yet. This paper reports the production of crude alkaline protease extracts from two rhizobia strains and provides some information on their physiochemical properties.

MATERIALS AND METHODS

Bacteria strains and culture conditions

The rhizobia used in this study were isolated from the soils of the Central Amazonian floodplain naturally rich in nutrients and low in acidity. The two rhizobia strains were previously identified at the Center for Nuclear Energy and Agriculture (CENA, Piracicaba, SP, Brazil). Based on 16S rRNA sequence comparison, strain INPA R-986 was identified as *Rhizobium* sp. (99% homology), and INPA R-993 as *Bradyrhizobium* sp. (99% homology) (Oliveira, 2006). The R-986 and R-993 strains are deposited in the culture collection of the National Institute for Amazonian Research – INPA, Manaus, AM, Brazil.

Stock cultures were maintained on yeast extract-mannitol agar (YMA) slants (Vincent, 1970) at 4 ^{o}C. Based on a previous study (Oliveira, 2006), two simple and inexpensive media for protease production were used. For strain INPA R-986, the medium was composed of (g L^{-1}): 10.0 gelatine, 0.4 K_2HPO_4, 0.1 K_2HPO_4, 0.2 $MgSO_4$.7 H_2O and 0.1 NaCl. For strain INPA R-993, the gelatine was replaced by soybean flour. The pH of the media were adjusted to 6.8 with KOH and autoclaved at 120 ^{o}C for 20 min. Fifty milliliters medium was placed in 150 mL Erlenmeyer flasks and inoculated with 1 mL of standardized inoculum culture (2.4 x 10^9 CFU), grown in liquid YM medium (Vincent, 1970) and incubated at 28 ^{o}C with rotary shaking of 65 cycles min^{-1}. Cultures were harvested and centrifuged (12000 rpm, 10 min) at 24 h intervals during 8 (R-986) and 11 (R-993) days of growth. At these intervals, the cell-free culture supernatant thus obtained served as the crude enzyme source to evaluate the protease activity, total extracellular protein and pH of the medium. Biomass production (g L^{-1}) was determined by drying the cell mass at 105 ^{o}C until constant weight.

Assay of protease activity

Protease activity was determined according to the modified method of Olajuyigbe and Ajele (2005). The reaction mixture in a total volume of 600 µL was composed of 1% (w/v) azocasein (Sigma-Aldrich, St. Louis, Mo., U.S.A) solution in Tris-HCl buffer (50 mM, pH 7.0) and appropriately diluted enzyme crude extract. After 60 min incubation at 37 ^{o}C in a water bath, the reaction was terminated by the addition of 500 µL of 15% (w/v) trichloroacetic acid (TCA). After the separation of the un-reacted azocasein precipitate by centrifugation, 1000 µL of clear supernatant was mixed with equal volume of 1.0 M NaOH. Absorbance was read at 440 nm using a Spectrum UV-Vis Spectrophotometer and appropriate blanks were included. One unit (U) enzyme activity was defined as the amount of crude enzyme required to produce an increase in absorbance equal to 1.0 in 60 min under experimental conditions.

Protein assay

Total extracellular protein secretion was estimated by the biuret method (Gornall et al., 1949), using bovine serum albumin as the standard.

Properties of the crude enzymes

The protease crude extracts obtained after cell growth for seven (INPA R-986) and eight (INPA R-993) days were used for characterization assays.

Influence of pH on enzyme activity and stability

The protease activity in the crude extract was measured at different pH values. The pH was adjusted using the following buffers (50 mM): citrate phosphate (pH 5.0-6.0), phosphate (pH 7.0-8.0), Tris-HCl (9.0-10.0) and glycine NaOH (11.0-

12.0). Reaction mixtures were incubated for 30 min at 37 °C and the activity was measured as in the standard assay above. The pH stability was determined by pre-incubating the crude enzyme extracts in different buffers (pH 5.0-12.0) for 24 h at 37 °C.

Influence of temperature on enzyme activity and stability

The activity of the crude protease extract was determined by incubating the reaction mixture at different temperatures, ranging from 25 to 80 °C, for 30 min in a 50 mM Tris-HCl (pH 10.0). To determine the enzyme stability with changes in temperature, crude protease extract was pre-incubated for 2 h at 25-80 °C. After incubation, residual enzymatic activity was determined under the standard assay conditions.

Influence of metal ions on enzyme activity

The effects of K^+, Na^+, Ca^{2+}, Hg^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+} ions on crude protease extract were investigated by adding these cations to the reaction mixture to final concentrations of 1 and 5 mM. Relative enzyme activities were measured under the optimum conditions of both pH and temperature as required by each rhizobia protease.

Influence of surfactants on enzyme activity

The enzyme activity against different surfactants (Triton X-100, Sodium Dodecyl Sulfate (SDS) and Tween 80) in reaction mixture was determined by assaying the crude enzyme in the presence of 1 and 2% of each surfactant at 37 ºC for 60 min. Protease activity was determined as described previously.

Influence of inhibitors on enzyme activity

The assay procedure was the same as described above, except that the reaction mixture included β-mercaptoethanol and $HgCl_2$ (cysteine protease inhibitors), Ethylene Diamine Tetraacetic Acid (EDTA, metalloprotease inhibitor), and p-Methyl Sulphonyl Fluoride (PMSF, serine protease inhibitor) at final concentrations of 5, 10 and 100 mM. Protease activity was determined as described previously.

Influence of oxidizing and reducing agents on enzyme activity

The crude protease extracts were incubated with H_2O_2 (oxidizing agent) and β-mercaptoethanol (reducing agent) at concentrations of 5 and 10 mM for 30 min at 37 °C. After incubation, protease activity was determined as described previously.

Influence of organic solvent on enzyme activity

The crude enzymes were incubated at 37 °C for 30 min with two different concentrations (10 and 20%) of acetone, n-hexane, methanol, 1-propanol and toluene. Proteolytic activity without organic solvent was considered to be 100%.

Experimental design and statistical analyses

All the experiments were organized using a completely randomized design with three replicates, repeated twice for reproducibility. The analysis of variance was carried out using the Statistica 7.0 software package (StaSoft, Inc., Tulsa, OK). Pearson's correlation coefficients also were considered significant if $p < 0.05$. The standard error in results of all assays was lower than 5%.

RESULTS AND DISCUSSION

Growth curve and protease production

Rhizobium sp. strain INPA R-986 and *Bradyrhizobium* sp. strain INPA R-993 showed maximal biomass production on the 4th and 6th days of cultivation, respectively (Figs 1, A and B). This result could be explained by the fact that the isolate of *Rhizobium* present a fast growth rate, while *Bradyrhizobium* a slow growth rate as referenced in Oliveira (2006). Strain INPA R-986 exhibited maximum total extracellular protein on the 4th day of incubation, whereas the strain INPA R-993 reached its maximum on the 3nd day of cultivation (data not shown).

Both rhizobia strains exhibited maximal protease activities when the cell growth reached the stationary phase (Figs 1, A and B). Similar results have been documented for other bacterial proteases (Sánchez-Porro et al., 2003; Patel et al., 2005; Nilegaonkar et al., 2007). A comparison among the strains showed that the enzyme activity of strain INPA R-993 (88 U mL^{-1}) was about six-fold higher than the activity of strain INPA R-986 (15 U mL^{-1}). There was a slight drop in pH from 6.8 to 6.3 during the first 24 h growth of the strain INPA R-986. This could be attributed to the production of acids during the bacterial growth. Then, the pH of the culture medium increased to 7.3 after 8 days incubation (Fig. 1A). Similarly,

the pH of the culture medium of *Bradyrhizobium* strain increased from 6.8 to 8.4 after 10 days growth (Fig. 1B). The rise of pH could be due to the utilization of organic acids or production of alkaline compounds, as suggested by Rahman et al. (2005).

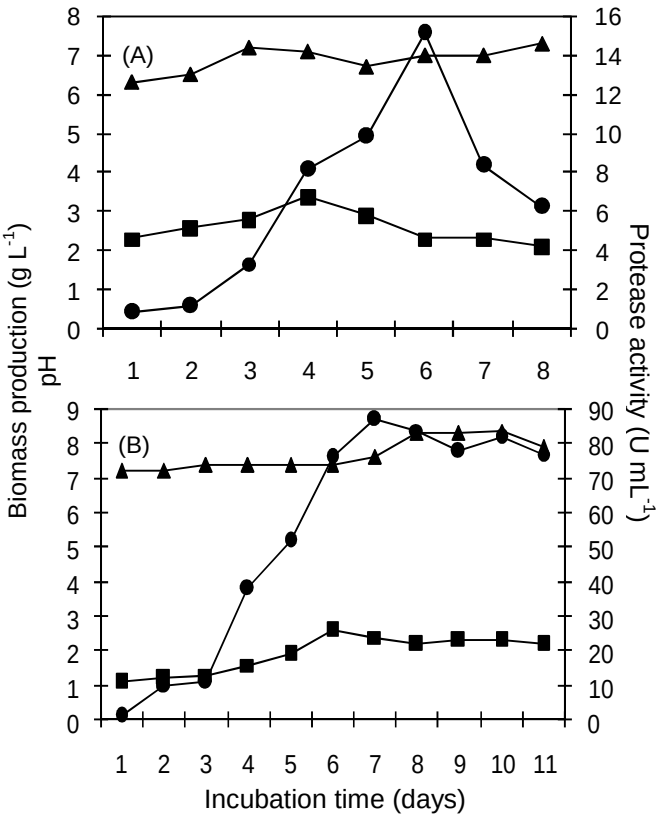

Figure 1 - Biomass production (■), pH variation (▲) and enzyme activities (●) of strains R-986 (A) e R-993 (B) at different incubation times.

Correlations among the parameters evaluated

For both rhizobia strains, there were significant and positive correlations between the proteolytic activity and pH of the culture medium. This interdependence was more prominent for the strain R-993 ($r = 0.75$; $p < 0.001$; $n = 33$) than for the strain R-986 ($r = 0.47$; $p < 0.021$; $n = 24$), suggesting that the protease produced by the *Bradyrhizobium* was relatively more tolerant to an alkaline environment. Similar relationships have been registered for proteases from *Nocardiopsis* sp. (Moreira et al., 2003), *Bacillus licheniformis* (Al-Shehri et al., 2004), *Teredinobacter turnirae* (Elibol and Moreira, 2005), *Conidiobolus coronatus* (Seeta Laxman et al., 2005) and *Bacillus* sp. (Genckal and Tari, 2006). Protease activity and biomass production were also closely associated for both rhizobia strains (for the R-993, $r = 0.97$; $p < 0.001$; $n = 33$, and for the R-986, $r = 0.62$; $p < 0.002$; $n = 24$), confirming other studies with *Pseudomonas fluorescens* 22 F (Schokker and van Boekel, 1997), *Bacillus* sp. (Nascimento et al., 2004) and *P. aeruginosa strain* K (Rahman et al., 2005). Protease production from the *Bradyrhizobium* strain was negatively correlated ($r = -0.52$; $p < 0.003$; $n = 33$) to the total extracellular protein, indicating the presence of more than one type of protease in the crude enzymatic extract. Similar findings were reported for crude enzyme preparations from *Nocardiopsis* sp. (Moreira et al., 2003) and *Streptomyces* sp. (Azeredo, 2004).

Influence of pH on enzyme activity and stability

The crude protease of the *Rhizobium* strain R-986 was active between pH 6.0 and 11.0, with an

optimum between pH 10.0-11.0 (Fig. 2). The optimum pH for protease activity from strain R-993 was between 9.0-10.0, but significant levels of activity (above 70%) were still detected at pH 8.0 and 11.0 (Fig. 2).

Optimum pH between 9.0 and 11.0 has been reported for alkaline proteases from *Bacillus* spp. (Al-Shehri et al., 2004; Patel et al., 2005; Genckal and Tari, 2006) and *Pseudomonas* spp. (Schokker and van Boekel, 1997; Najafi et al., 2005; Rahman et al., 2005). The rhizobia protease was very stable in a broad pH range, maintaining over 78% of activity between pH 8.0 and 11.0 (Fig. 3). The protease produced by strain R-986 retained only 53% of their original activity at pH 6.0, while the enzymes from strain R-993 had a loss of 58%. This suggested instability of these enzymes in an acid environment.

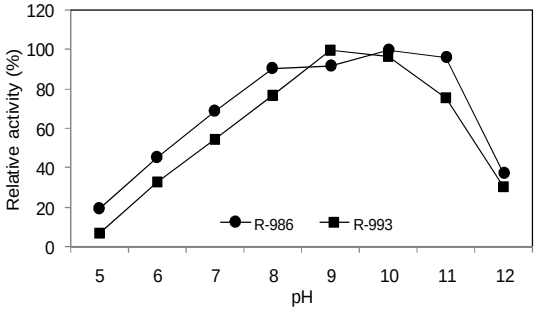

Figure 2 - Effect of pH on the proteolytic activities of the crude extracts from R-986 (13.2 U mL^{-1}) and R-993 (87.0 U mL^{-1}) strains. The enzyme activities of the strains are expressed as percentages of maximum.

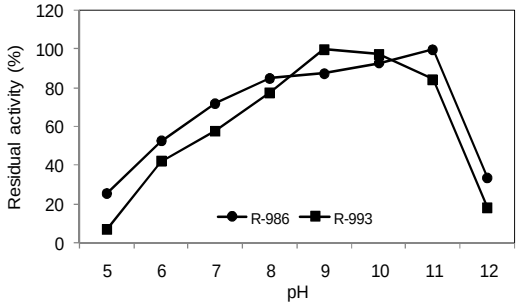

Figure 3 - Effect of pH on the stabilities (24 h) of the crude protease extracts from R-986 (12.8 U mL^{-}) and R-993 (86.2 U mL^{-1}) strains. The enzyme activities of the strains are expressed as percentages of maximum.

Influence of temperature on enzyme activity and stability

The optimum temperature for protease activity from strain R-986 was 35 °C and activity decreased gradually above this temperature (Fig. 4). The temperature range for optimum catalytic activity of numerous proteases was between 30 and 47 °C (Schokker and van Boekel, 1997; Singh et al., 2001; Dutta and Banerjee, 2006; Genckal and Tari, 2006). At temperatures from 25 to 55 °C, significant increases in enzyme activity of strain R-993 were observed (Fig. 4). Above the optimum temperature of 55 °C, protease activity decreased significantly. Similar results have been documented for other microbial proteases (Yang et al., 2000; Kumar, 2002; Moreira et al., 2003; Nascimento and Martins, 2004; Gupta et al., 2005).

The thermostability profile showed that at 2 h, the crude protease extract from strain R-986 retained more than 90% of its catalytic activity at 25 to 35 ºC (Fig. 5). However, above these temperatures, activity decreased significantly over the time. At 35 °C, the crude enzyme produced by the strain R-993 was fully heat stable (Fig. 5). The crude protease extract of this strain also retained more than 80% of its maximum activity at 45 °C. Above 45 °C, the activity declined sharply,

possibly because of thermal inactivation. These crude protease extracts can be classified as mesophilic. In this study, the thermostabilities observed for both the rhizobia proteases were comparable to those mentioned for *Bacillus* proteases (Singh et al., 2001; Adinarayana et al., 2003; Genckal and Tari, 2006).

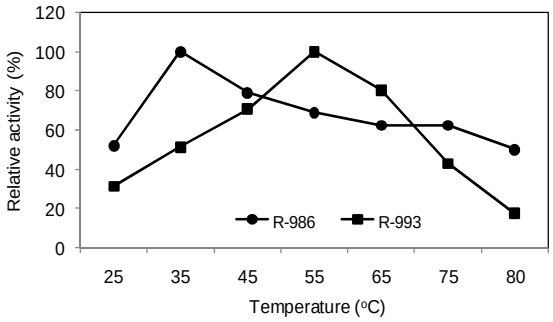

Figure 4 - Effect of temperature on the proteolytic activities of the crude extracts from R-986 (12.2 U mL^{-1}) and R-993 (85.4 U mL^{-1}) strains. The enzyme activities of the strains are expressed as percentages of maximum.

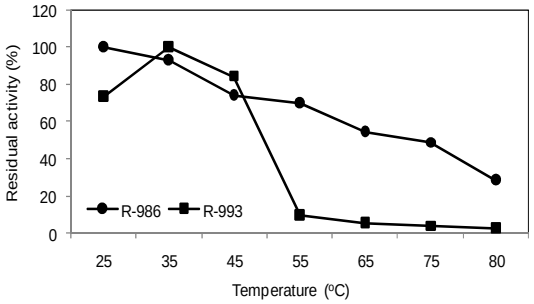

Figure 5 - Effect of temperature on the stabilities (2 h) of the crude protease extracts from R-986 (12.0 U mL^{-1}) and R-993 (84.8 U mL^{-1}) strains. The enzyme activities of the strains are expressed as percentages of maximum.

Influence of various metal ions on enzyme activity

The influence of metal ions on enzyme activities ranged from a moderate inhibitory effect to a strong stimulatory effect (Table 1). Moderate inhibitions were observed in the presence of Cu^{2+} and Zn^{2+}, which reduced the activity to between 15 and 27% of full activity. Heavy metal ions such as Cu^{2+} (Yang et al., 2000; Nascimento and Martins, 2004; Gupta et al., 2005) and Zn^{2+} (Nascimento and Martins, 2004; Gupta et al., 2005) have been reported to be inhibitory to alkaline proteases. Na^{+} (5 mM), Mg^{2+} (5 mM) and Mn^{2+} (1 and 5 mM) ions enhanced the proteolytic activities between 107 and 220%. Of these metal ions, Mn^{2+} enhanced the enzyme activities by a minimum of 75% (Table 1). This showed that the proteolytic enzyme from these strains required Mn^{2+} for optimal activity. Strong stimulatory effects (69-300%) of Mn^{2+} ions on microbial proteases also have been observed by other authors (Yang et al., 2000; Suntornsuk et al., 2004; Sumantha et al. 2006).

In relation to Hg^{2+}, present data contradict the results documented by other authors (Yang et al., 2000; Kumar, 2002; Nascimento and Martins, 2004; Yossan et al., 2006). On the other hand, Hg^{2+} ions had no significant inhibitory effect on protease activity of *B. subtilis* PE-11 (Adinarayana et al., 2003), *A. parasiticus* (Tunga et al., 2003) and *Halogeometricum borinquense* TSS101 (Vidyasagar et al., 2006). These results suggested that the effect of Hg^{2+} could vary among the microbial proteases, as observed for other heavy metal ions, such as Co^{2+} (Beg and Gupta, 2003; Karbalaei-Heidari et al., 2007), Ni^{2+} (Beg and

Gupta, 2003; Yossan et al., 2006) and Sr^{2+} (Rahman et al., 2006).

Influence of inhibitors on enzyme activity

Proteases can be classified by their sensitivity to various inhibitors. The effect of a variety of protease inhibitors, such as β-mercaptoethanol, ethylene diamine tetraacetic acid (EDTA), $HgCl_2$ and phenyl methyl sulphonyl fluoride (PMSF) on enzyme activity was investigated (Table 1). The crude enzymes secreted by the *Rhizobium* strain were slightly to moderately inhibited by β-mercaptoethanol (cysteine protease inhibitor), EDTA (metalloprotease inhibitor) and $HgCl_2$ (cysteine protease inhibitor). The β-mercaptoethanol (10 and 100 mM) also caused a moderate inhibition (20-36%) on the activity of the *Bradyrhizobium* strain (Table 1). In the presence of EDTA, there was up to 68% inhibition, which suggested the presence of metalloproteases in the crude enzyme extract of this strain. The PMSF caused strong inhibition (77-82%) of enzyme activities of both rhizobia strains (Table 1). These results suggested that the enzyme secreted by strain INPA R-986 could belong to the class of serine proteases, whereas that produced by strain INPA R-993 was possibly a mixture of serine protease with metalloprotease. This type of inhibition profile also was reported for proteases from *Pseudoperkinsus tapetis* (Ordás et al., 2001), *Vibrio fluvialis* (Venugopal and Saramma, 2006), *B. circulans* (Jaswal and Kocher, 2007).

Table 1 - Effect of metal ions, inhibitors, and oxidizing and reducing agents on activity of crude extracellular proteases of two indigenous Central Amazonian rhizobia strains.

Ions, inhibitors and oxidizing and reducing agents	**Concentration (mM)**	***Rhizobium* strain R-986 (%)**	***Bradyrhizobium* strain R-993 (%)**
Control	0	100	100
K^+ (KCl)	1	99	98
	5	95	99
Na^+ (NaCl)	1	99	103
	5	107	114
Ca^{2+} ($CaCl_2 . 2H_2O$)	1	94	99
	5	105	97
Hg^{2+} (HgCl)	1	106	105
	5	94	97
Mg^{2+} ($MgCl_2 . 7H_2O$)	1	114	107
	5	110	115
Mn^{2+} ($MnSO_4$)	1	208	175
	5	220	200
Cu^{2+} ($CuSO_4 . 5H_2O$)	1	85	82
	5	82	80
Zn^{2+} ($ZnSO_4 . 7H_2O$)	1	73	74
	5	74	75
β-Mercaptoethanol	5	92	108
	10	82	80
	100	71	74
EDTA	5	79	48
	10	79	48
	100	71	32
$HgCl_2$	5	102	104
	10	94	97
	100	75	81
PMSF	5	37	32
	10	34	28
	100	23	18
H_2O_2	5	87	115
	10	87	100
β-Mercaptoethanol	5	92	108
	10	82	80

The enzyme activities of strains R-986 (12.4 U mL^{-1}) and R-993 (84.5 U mL^{-1}) are expressed as percentages of the activity levels in the absence of metal ions, inhibitors, and oxidizing and reducing agents (control).

Influence of oxidizing and reducing agents on enzyme activity

The crude protease extract secreted by INPA R-986 was slightly inhibited by H_2O_2 (13%) and β-mercaptoethanol (8-18%) (Table 1). At the concentration of 10 mM, the β-mercaptoethanol also caused a slight reduction (19%) in the activity of INPA R-993. Slight inhibition by β-mercaptoethanol (Singh et al., 2001; Yang et al.,2000; Vidyasagar et al., 2006) and H_2O_2 (Oberoi et al., 2001; Genckal and Tari, 2006; Hadj-Ali et al., 2007) has been observed for other bacterial proteases.

Protease activity from strain R-993 was enhanced (15%) in the presence of 5 mM H_2O_2. This result was similar to those of Johnvesly and Naik (2001), Beg and Gupta (2003), Joo et al. (2003) and Nilegaonkar et al. (2007), where serine proteases were stimulated by H_2O_2 (1 and 5%). The tolerance to H_2O_2 is of great interest, especially in the detergent industry (Gupta et al., 2002; Maurer, 2004), in which this chemical agent is a fundamental component of many formulations.

Influence of surfactants on enzyme activity

Among the surfactants tested, only Tween-80 showed a stronger inhibitory effect (50-60%) on protease activity of the *Bradyrhizobium* strain (Table 2). In contrast, SDS (1%) and Triton X-100 (1%) enhanced the activity by 12 and 16%, respectively. The crude protease extract from the *Rhizobium* strain was stable and retained 100% activity in the presence of these two surfactants. However, SDS and Triton X-100 at 2% caused a moderate inhibition of 10 and 30%, respectively (Table 2).

The influence of surfactants on protease activity is extremely variable in the literature. Olivera et al. (2006) observed inhibitory effects (27%) of SDS (1%) on protease activity from *B. patagoniensis*. In the presence of Triton X-100 (1%), the study showed a stimulatory effect. At a concentration of 1%, SDS and Tween-80 caused strong inhibitory effects (50%) on protease activity from *Pseudomonas aeruginosa* PD100 (Najafi et al., 2005). SDS (0.1%) reduced by 39% the activity of the protease enzyme produced by *P. aeruginosa* PseA (Gupta et al., 2005). In the same study, Triton X-100 (0.1%) and Tween-80 (0.1%) showed stimulatory effects (6-12%). Similarly, SDS (0.1%) and Triton X-100 (0.2%) increased by up to 22% protease activity from *Streptomyces albidoflavus* (Bressolier et al., 1999).

Table 2 - Effect of surfactants and organic solvents on activity of crude extracellular proteases of two indigenous Central Amazonian rhizobia strains.

Surfactants and solvents	**Concentration (%)**	***Rhizobium* strain R-986 (%)**	***Bradyrhizobium* strain R-993 (%)**
Control	0	100	100
SDS	1	100	112
	2	90	99
Triton X-100	1	100	116
	2	70	108
Tween-80	1	87	60
	2	79	50
Acetone	10	108	72
	20	103	64
n-Hexane	10	74	52
	20	68	44
Methanol	10	82	80
	20	53	32
1-Propanol	10	68	44
	20	55	24
Toluene	10	92	34
	20	68	32

The enzyme activities of strains R-986 (11.0 U mL^{-1}) and R-993 (81.4 U mL^{-1}) are expressed as percentages of the activity levels in the absence of surfactants and organic solvents (control).

Influence of organic solvents on enzyme activity

The rhizobia proteases had the ability to act in the presence of different concentrations of solvents in the reaction system (Table 2). Protease activity from *Rhizobium* sp. was enhanced by up to 8% in the presence of acetone. Similar observations were reported for proteases isolated from *Pseudomonas aeruginosa* strains (Ogino et al., 1999; Gupta et al., 2005). In contrast, the addition of acetone caused a decrease of 28-36% in the activity of the *Bradyrhizobium* strain. Similar reductions of protease activity have been reported for *P. aeruginosa* PD100 (Najafi et al., 2005).

At a concentration of 20%, n-hexane, methanol, 1-propanol and toluene caused decreases of 32 to 76% on proteolytic activities from the rhizobia strains. However, at a concentration of 10%, more than 40% of full activity was observed in the presence of n-hexane, methanol and 1-propanol (Table 2). According to Najafi et al. (2005), this capability could be due to disulfide bonds. There are a number of reports on the importance of disulfide bonds for the stability of proteins in the presence of solvents (Ogino et al., 2001; Najafi et al., 2005; Rahman et al., 2005). The proteases studied here could be very useful for fermentation and other reactions in the presence of solvents. One of the most important advantages of this property is to reduce or abolish the microbial contamination during the degradation reactions (Najafi et al., 2005). Enzyme-catalysed reactions in organic solvents have found numerous applications, some of which have been commercialized (Klibanov, 2001).

CONCLUSIONS

Based on the biochemical properties evaluated in this study, the crude rhizobia protease extracts appeared to be useful sources of enzymes for the detergent formulations. Furthermore, they have the ability to act in the presence of several organic solvents and could, therefore, be used for the reactions in media containing organic solvents.

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RESUMO

Duas estirpes de rizóbia isoladas de solos de várzea da Amazônia Central produziram grandes quantidades de proteases alcalinas extracelulares, usando fontes baratas de carbono e nitrogênio. Os extratos brutos de proteases foram ativos em pH 9,0-11,0. As temperaturas ótimas foram de 35 ^{o}C para a enzima do *Rhizobium* R-986 e de 55 ^{o}C para a do *Bradyrhizobium* R-993. As atividades proteolíticas aumentaram na presença de 5 mM dos íons Na^{+}, Ca^{2+}, Mg^{2+} e Mn^{2+}. As proteases secretadas pelos rizóbios foram fortemente inibidas por PMSF, um inibidor de serina protease. As enzimas foram ativas na presença de surfactantes (SDS e Triton X-100), e estáveis na presença de agentes oxidantes (H_2O_2) e redutores (β-mercaptoetanol) e solventes orgânicos (acetona, hexano, metanol, 1-propanol e tolueno).

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