

## Protease with collagenolytic activity produced by *Bacillus* sp. DPUA 1728 from Amazonian soil

Lorena A. Lima<sup>1</sup>, Raimundo F. Cruz Filho<sup>2</sup>, Januário G. dos Santos<sup>2</sup>, Wilson C. Silva<sup>1</sup>

<sup>1</sup>Programa de Pós-Graduação em Biotecnologia e Recursos Naturais da Amazônia, Universidade do Estado do Amazonas, Manaus, AM, Brazil.

<sup>2</sup>Laboratório de Microbiologia, Universidade Federal do Amazonas, Manaus, AM, Brazil.

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### Abstract

Qualitative analyses were carried out on solid medium with insoluble collagen 0.25% (w/v) to detect proteases with collagenolytic activity produced by *Bacillus* sp. In cultures incubated for 24 h, a 2<sup>3</sup> full factorial design with four repetitions at the center point was developed to analyze the effects and interactions between initial pH, temperature and the concentration of gelatin. Based on the results of the first 2<sup>3</sup> full factorial design, a successive 2<sup>3</sup> full factorial design was performed. The most favorable production conditions were found to be 1.5% (w/v) gelatin, pH 9.0 and 37 °C with enzymatic activity of 86.27 U/mL. The enzyme showed optimal activity at 50 °C and pH 9.0, and it was stable over wide pH (7.2-10.0) and temperature (45 °C-60 °C) ranges. These results indicate that *Bacillus* sp DPUA 1728 is a potential source for producing collagenolytic protease with possible biotechnological applications, such as in the food, cosmetics and leather industries.

**Key words:** collagenolytic protease, *Bacillus* sp, factorial design, Amazonian soil.

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### Introduction

Collagen is the major class of insoluble fibrous protein found in the extracellular matrix in connective tissues. Its main characteristics are its triple-helical conformation and its amino acid content, in which the amount of hydroxyproline residues present is much higher than the amount found in other proteins existing in nature due to their rigid structure, and only a limited number of proteases may cleave collagen, such as collagenolytic proteases (Harrington, 1996). Collagenolytic proteases are of considerable importance in medicine and in industrial activities, though the most several applications of these proteases are in therapeutic and biotechnological scenarios (Parks, 1999). These proteases have been used experimentally in the food industry, and oral administration of collagen peptides has been demonstrated to prevent osteoporosis, protect against gastric ulcer, promote relaxation, combat hypertension, and stimulate metabolism in the skin (Kim *et al.*, 2007; Watanabe, 2004; Vargas *et al.*, 1997; Díez *et al.*, 1995). The use of collagen peptides can be extended to the cosmetics in-

dustry, collagen polypeptides are isolated as raw material for the cosmetics industry (Langmaier *et al.*, 2002), and they can also be used as nontoxic and eco-friendly biocatalysts facilitating the dyeing of leather (Kanth *et al.*, 2008). Furthermore, collagenolytic protease may also be of great agricultural importance in the degradation of the outer cuticle of nematodes that infest plants (Galper *et al.*, 1991; Labadie and Hebraud, 1997; Sela *et al.*, 1998). Among various sources of collagenolytic proteases, microbial proteases such as those derived from *Clostridium histolyticum*, *Vibrio alginolyticus* and *Porphyromonas gingivalis* (Petrova *et al.*, 2006) play an important role in controlling biotechnological processes, accounting for approximately 59% of the enzymes used (Chu, 1987). These microorganisms, however, are pathogens that potentially produce toxins, in which case they may jeopardize the application of collagenolytic proteases. In recent years, microorganisms that are generally considered safe for the production of collagenases have been selected from soil, water and caviar, including *Bacillus pumilus*, *Bacillus subtilis*,

*Geobacillus collagenovorans*, and *Bacillus* sp. strain MO-1 (Okamoto *et al.*, 2001; Tran and Nagano, 2002; Itoi *et al.*, 2006; Wu *et al.*, 2010). Nevertheless, commercially available collagenase is produced by *Clostridium histolyticum*, but due to the high cost involved in the commercial use of collagenolytic proteases, it has become necessary to search for new microorganisms producing this protease (Watanabe, 2004).

The genus *Bacillus* is the most important source of various commercial microbial enzymes, and it can be grown under different conditions of temperature and pH, resulting in more stable products in a wide range of environments (Gençkal, 2004). Bacteria of the genus *Bacillus* are commonly found in soil. *Bacillus* strains may produce and secrete large quantities (20-25 g/L) of extracellular enzymes, characteristics that place it among the most important microorganismal producers of industrial enzymes (Petrova *et al.*, 2006). The aims of this work were to develop a statistical approach to study the influence of gelatin concentration, pH and temperature on collagenase production by *Bacillus* sp. DPUA 1728 and to characterize the enzyme obtained under the most favorable conditions.

## Materials and Methods

### Microorganism and culture medium

The *Bacillus* sp. DPUA1728 strain was obtained from the Culture Collection of the Department of Parasitology of the Federal University of Amazonas (DPUA/UFAM). The sample was activated in Mueller-Hinton broth and incubated for 24 h at 37 °C. For quantitative analysis, the strain was inoculated into gelatin/milk agar 1% (w/v) and incubated at 37 °C for 24 h. The enzymatic activity was visualized with a clear zone around colonies after the strain was inoculated in collagen agar 0.25% (w/v) according to Wu *et al.* (2010) with modifications. The medium was composed of 0.5 g of KH<sub>2</sub>PO<sub>4</sub>, 0.2 g MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.1 g ZnSO<sub>4</sub>·H<sub>2</sub>O; 1 g NaCl, 0.25% insoluble collagen (w/v) and 1.5% agar (w/v), pH 7.2. After 24 h of incubation at 37 °C, the digested collagen was revealed by adding 1% Coomassie brilliant blue G (w/v) (Lupan and Nziramasanga, 1986; Doust and Mobarez, 2004).

### Factorial design: determination of the best conditions for collagenase production by *Bacillus* sp. DPUA 1728

Two 2<sup>3</sup> full factorial designs with four repetitions at the central point (Table 1 and 2) were developed for the analysis of different pH values, temperatures and substrate concentrations. The factorial designs and Pareto charts were carried out using the Minitab® 16 Statistical Software. The results were statistically analyzed by variance analysis (ANOVA) at a significance level of  $p \leq 0.05$ . The fermentation medium was prepared with 2.0 g KH<sub>2</sub>PO<sub>4</sub>, 1.0

**Table 1** - Factor levels used in the first 2<sup>3</sup> design for studying collagenase production by *Bacillus* sp. DPUA 1728.

Factor	Level		
	-1	0	+1
pH	5.0	6.0	7.2
Temperature (°C)	25	37	45
Concentration of gelatin (w/v)	0.25	0.5	1.0

g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.1 g MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.9 g Na<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O; 1.0 g yeast extract (Santos *et al.*, 2009; Wu *et al.*, 2010), 0.1 g CaCl<sub>2</sub> and varying concentrations of gelatin.

### Enzymatic assay for protease activity determination

A McFarland scale 1.0 cell suspension was prepared and 200 µL thereof was transferred to 125 mL Erlenmeyer flasks containing 25 mL of fermentation medium. Each bacterial culture was shaken at 160 rpm for 24 h at temperatures as defined in the factorial design. After the stirring period, the supernatant was separated by centrifugation at 10,000 g for 10 min and filtered through a 0.22 µm membrane.

The enzyme assay was performed according to the method developed by Moore and Stein (1948) with slight modifications. The mixture consisted of 0.2 mL of the supernatant enzyme, 0.8 mL of 50 mM Tris-HCl containing 4 mM CaCl<sub>2</sub> and 1% (w/v) of soluble collagen in pH ranges established by the factorial design. It was subsequently incubated for 10 h at decreasing temperatures according to the factorial design. After this period, the reaction was stopped by adding 0.2 mL of 0.1 M of 10% (w/v) trichloroacetic acid. Tubes containing the mixture were centrifuged at 10,000 g for 10 min and the enzyme extract was filtered through a 0.22 µm membrane.

One unit of the free amino group of collagen was measured according to the ninhydrin method, in which 0.5 mL of 2% Ninhydrin solution and 0.2 mL of the supernatant are heated for 10 min and then cooled in ice water for 5 min. Next, the mixture was diluted with 5 mL of 50% (v/v) 1-propanol. The mixture was centrifuged at 12,000 g for 10 min and its absorbance was measured at 570 nm. The collagenolytic activity was expressed as µmol of glycine equivalents released per minute.

**Table 2** - Factor levels used in the second 2<sup>3</sup> design for studying collagenase production by *Bacillus* sp. DPUA 1728.

Factor	Level		
	-1	0	+1
pH	7.2	8.0	9.0
Temperature (°C)	37	45	60
Concentration of gelatin (w/v)	0.5	1.0	1.5

**Protein determination**

Protein concentration was determined according to the Bradford Method (1976) using bovine serum albumin as the standard. Specific activity was calculated as the ratio between the enzymatic activity (U) and the total protein in the sample (mg/mL) and expressed as U/mg.

**Proteolytic activity**

Proteolytic activity was determined using 150 µL of the enzyme extract and 250 µL of azocasein 1% (w/v) as substrate (Sigma, St. Louis, MO USA) in Tris-HCl 0.05 M pH 7.2. The reaction was kept in a dark chamber, and after 60 min, 1.2 mL of 10% (w/v) trichloroacetic acid was added to stop the reaction. The residue was removed by centrifugation (8,000 g) at 4 °C for 10 min. Next, 0.8 mL of supernatant was removed and added to 1.4 mL of 1 M sodium hydroxide, whereupon reading at 440 nm ensued. One unit of protease activity was defined as the amount of enzyme required to produce a change in absorbance equal to 0.1 in 60 min, and it is expressed in relative activity as in Leighton *et al.* (1973) with modifications.

**Effects of pH and temperature on proteolytic activity and stability**

To evaluate the effect of pH on enzyme activity, the pH of a reaction mixture containing 1% (w/v) azocasein was varied over the range 5.0-10.0. The buffers used to adjust the pH were 50 mM citrate (5.0-6.0), 50 mM Tris-HCl (pH 7.2-9.0) and 50 mM carbonate bicarbonate (pH 10.0). To determine the optimum temperature for enzyme activity, the reaction mixture was incubated at the selected temperature (from 25 to 80 °C) for 60 min. The stability of the temperature and pH was measured for 105 min, and en-

zyme activity was expressed as percent relative activity. All experiments were performed in triplicate.

**Results and Discussion**

**Bacteria as protease-producers**

To qualitatively determine the activity of protease producers, halos (or precipitation zones) in the medium were first observed in gelatin/milk agar 1% (w/v) after 24 h. Clear zones around colonies were observed in collagen agar 0.25% (w/v), demonstrating the production of collagenolytic enzyme.

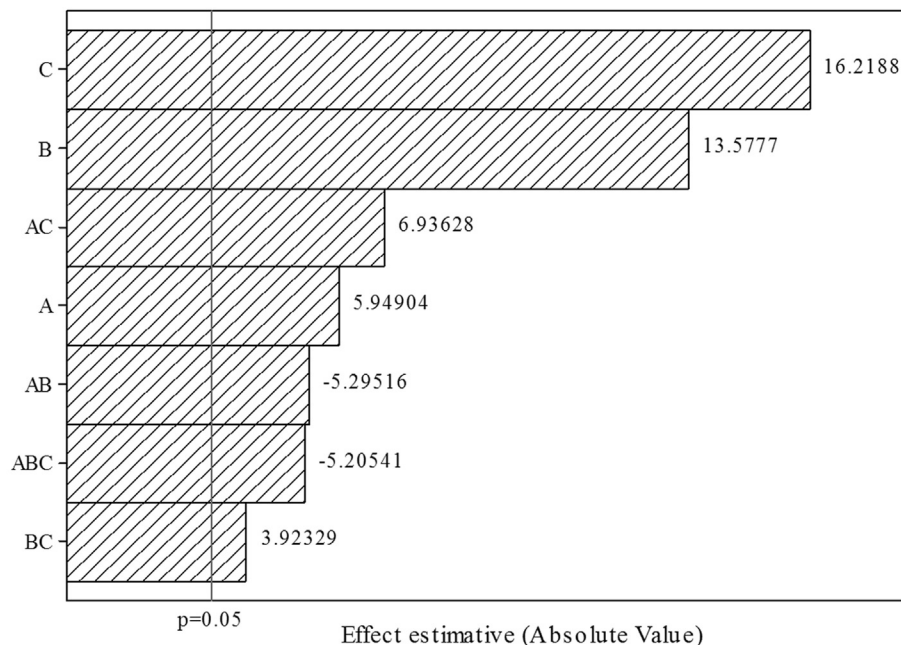
Table 3 shows the data for the first 2<sup>3</sup> factorial design for enzyme activity with *Bacillus* sp. DPUA1728. The highest value obtained for collagenolytic activity of *Bacillus* sp. DPUA1728 after 24 h of fermentation was 64.29 U/mL, with specific activity of 110.89 U/mg at pH 7.2 at 45 °C with 1% (w/v) substrate. For each factorial design a Pareto chart was constructed, which revealed the relationship between the factors and allowed the best conditions for enzymatic production to be determined. The values of significant effects of the variables were analyzed to verify the production of enzyme extract from *Bacillus* sp. DPUA1728, and they are illustrated in a Pareto chart (Figure 1).

The vertical line is used to evaluate which effects were significant, and bars that extend beyond this line represent effects at the 5% level of statistical significance. The values of the significant effects indicate that, on average, higher activities were obtained when factors A (initial medium pH), B (temperature) and C (concentration of substrate) were set at their highest levels. Furthermore, several interactions were significant: AC, BC and ABC; this means that a simultaneous increase in the levels of these variables favors collagenase production even more (Figure 1). Al-

**Table 3** - Collagenolytic protease production of *Bacillus* sp. DPUA1728 in the first test matrix factorial design (2<sup>3</sup>).

	pH	T (°C)	S (%)	Ac (U/mL)	Tp (mg/mL)	As (U/mg)
1	5.0	25	0.25	21.16	0.42	50.34
2	7.2	25	0.25	19.89	0.46	42.88
3	5.0	45	0.25	34.92	0.38	92.66
4	7.2	45	0.25	33.40	0.72	46.09
5	5.0	25	1.00	21.37	0.75	28.36
6	7.2	25	1.00	54.40	0.52	104.27
7	5.0	45	1.00	60.92	0.84	72.47
8	7.2	45	1.00	64.29	0.58	110.89
9*	6.0	37	0.50	25.97	0.70	37.34
10*	6.0	37	0.50	23.44	0.71	33.00
11*	6.0	37	0.50	27.60	0.71	38.87
12*	6.0	37	0.50	23.62	0.71	33.26

pH = initial pH, S = substrate concentration T = temperature, Ac = collagenolytic activity, Tp = total protein; As = specific collagenolytic activity.  
\*central points.



**Figure 1** - Pareto chart for the effects of variables pH (A), temperature (B) and substrate concentration (C) on collagenolytic activity from first full factorial design.

though the interaction AB has a negative effect, the relationship between the three factors increased enzyme activity when at their highest levels - a positive influence (5.20541). In practical terms, this implies that the effect of a given factor depends on the levels of the others.

Pareto charts (Figure 1) representing the interrelationships between the independent variables affecting collagenolytic activity showed the need to perform a second factorial design trial (Table 2), and to explore in more detail the area around the previously selected experimental conditions for protease production, two successive full fac-

torials were performed. Table 4 shows the values of enzyme activity during the second test of *Bacillus* sp. DPUA1728. The highest value obtained for collagenolytic activity for 24 h was 86.27 U/mL with specific activity of 145.18 U/mg at pH 9.0, 37 °C and 1.5% (w/v) substrate, verifying activity higher than the values obtained during the first factorial design. Similar results were reported by Patel *et al.* (2005) using gelatin in the fermentation of *Bacillus* sp. The authors observed that a gradual increase in substrate concentration influences the maximum production of protease. In a study by Lima *et al.* (2009) using

**Table 4** - Collagenolytic protease production by *Bacillus* sp. DPUA1728 in the second test matrix factorial design (2<sup>3</sup>).

	pH	T (°C)	S (%)	Ac (U/mL)	Tp (mg/mL)	As (U/mg)
1	7.2	37	0.50	35.22	0.59	71.13
2	9.0	37	0.50	53.31	0.48	111.47
3	7.2	50	0.50	19.02	0.30	62.49
4	9.0	50	0.50	16.38	0.33	49.13
5	7.2	37	1.50	70.12	1.06	66.27
6	9.0	37	1.50	86.27	0.66	145.18
7	7.2	50	1.50	22.31	0.55	40.52
8	9.0	50	1.50	17.28	0.35	49.68
9*	8.0	45	1.00	33.76	0.70	43.14
10*	8.0	45	1.00	36.47	0.70	52.43
11*	8.0	45	1.00	35.32	0.65	54.15
12*	8.0	45	1.00	32.85	0.65	50.37

pH = initial pH, S = substrate concentration T = temperature, Ac = collagenolytic activity, Tp = total protein; As = specific collagenolytic activity.  
\*central points.

*Candida albicans* URM3622, it was reported that the pH of the fermentation medium influences the increase in protease production, showing the importance of initial pH to production optimization.

Our results here were different from those obtained by Lima *et al.* (2011) using *Penicillium aurantiogriseum* URM 4622, however the level used were similar (Table 2). Maximal production took place under conditions where the temperature was at its highest level and substrate concentration and pH were the lowest, with 28 °C, 0.75% (w/v) and pH 8.0, respectively. Divergent results were obtained by Suphatharaprateep *et al.* (2011) using *Bacillus cereus* CNA1 for the production of collagenolytic protease in a study where various carbon sources at different pH values were tested. The best conditions for maximum production of collagenolytic protease were pH 7.5 and 1% (w/v) gelatin, the lowest values tested.

The values of the significant effects in the second factorial design indicate that, on average, higher activities were obtained when factor B (temperature) was set at its lowest level and A (initial medium pH) and C (concentration of substrate) were set at their highest levels. It was observed that increasing the concentration of the substrate is the aspect that promotes the highest collagenolytic protease production. However, interactions between temperature, pH and substrate concentration had no significant effect (Figure 2).

**Effects of pH on proteolytic activity and stability**

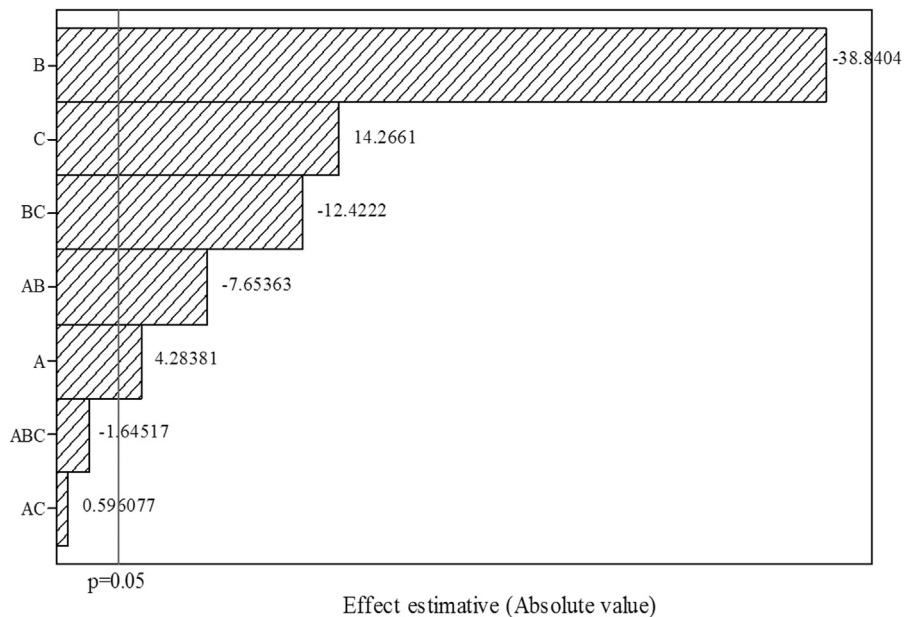
The effect of pH on the proteolytic activity of the enzyme extract from *Bacillus* sp. DPUA 1728 showed activity in the pH range 5.0 to 10.0 for 60 min. However, the

maximum activity of the enzyme extract of *Bacillus* sp. DPUA 1728 was at pH 9.0 (Figure 3A), with 80% protease activity occurring in the pH range 7.2 to 9.0 and over 60% of this activity occurring from pH 6.0 to 10.0. Similar protease production results were observed in the study by Lima *et al.* (2011), although the authors used *Penicillium aurantiogriseum* URM 4622, which showed the highest activity at pH 9. In the work of Liu *et al.* (2010), who used *B. cereus* MBL13, and of Zambare *et al.* (2011), who fermented *Pseudomonas aeruginosa* MCM-327, maximum production of protease was obtained at pH 8.0, which corresponds to over 80% of the activity observed in the present study. However, Kawahara *et al.* (1993) used *Bacillus alvei* DC-1 to produce collagenolytic protease and observed maximum activity at pH 8.0.

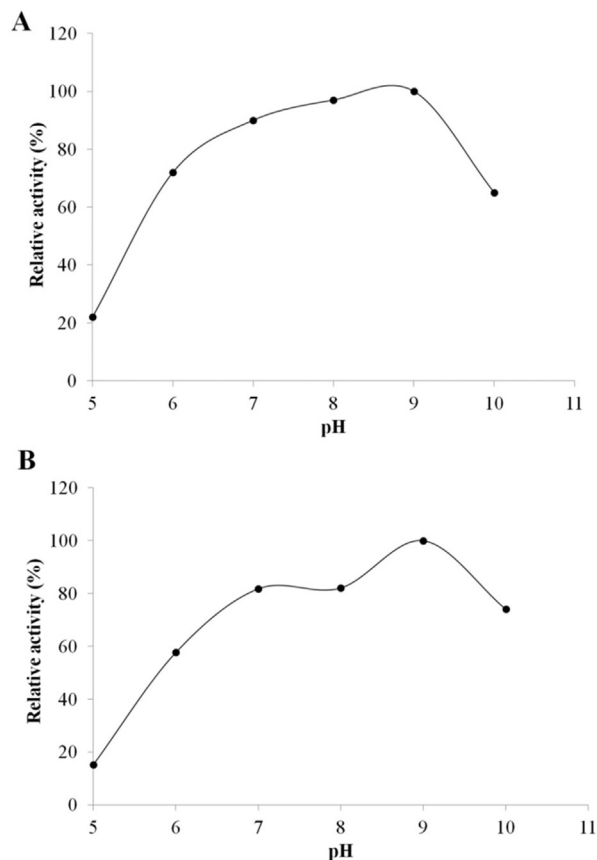
The proteolytic activity of the enzyme extract of *Bacillus* sp. DPUA 1728 was above 74% of its maximum in the alkaline pH ranges (7.2 to 10.0) (Figure 3B). However, these results differ from those obtained by Lima *et al.* (2009), in which the proteolytic activity of *Candida albicans* URM 3622 remained stable above 90% in the pH range from 6.0 to 8.2 for 90 min, reducing to less than 60% at pH 10.0.

**Effects of temperature on proteolytic activity and stability**

The maximum proteolytic activity of the enzyme extract of *Bacillus* sp. DPUA 1728 was at 50 °C (Figure 4A). The temperature profile of *Bacillus* sp. DPUA 1728 showed more than 50% enzymatic activity between 45 °C and 60 °C. A similar result was obtained in the study by Nagano and To (1999) using *Bacillus subtilis* FS-2, con-



**Figure 2** - Pareto chart for the effects of variables pH (A), temperature (B) and substrate concentration (C) on collagenolytic activity from second full factorial design.



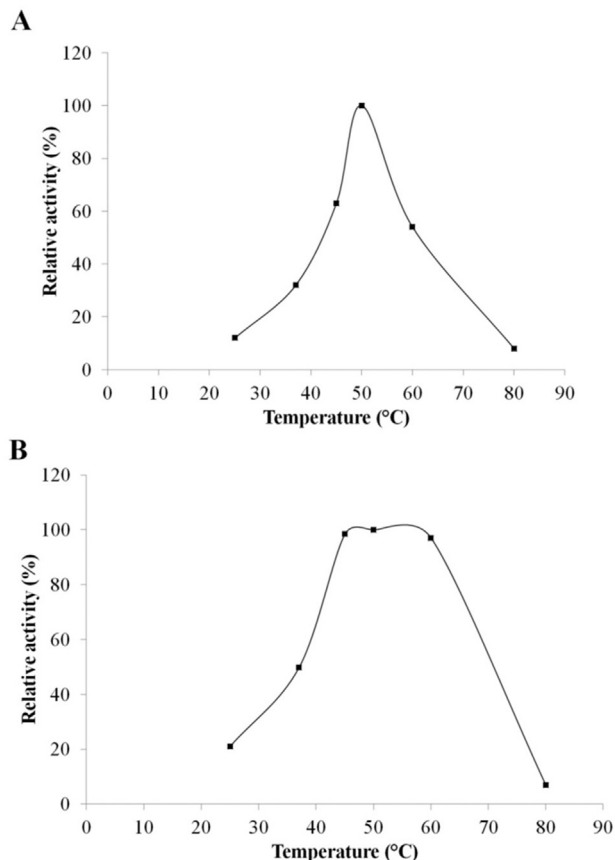
**Figure 3** - (A) Effect of pH on activity of extracellular protease and (B) Effect of pH on stability of extracellular protease produced by *Bacillus* sp. expressed as a percentage of the maximum obtained at 50°C.

firming 50 °C as the optimum temperature for protease production. In contrast, Suphatharaprateep *et al.* (2011) and Wu *et al.* (2010), who used, *B. cereus* CNA1 and *Bacillus pumilus* Col-J, respectively, obtained optimum protease activity production at 45 °C, within the range of 60% of the optimum activity obtained in this study.

Regarding the effect of temperature on the stability of proteolytic activity produced by *Bacillus* sp. DPUA 1728 (Figure 4B), it was observed that the enzymatic extract showed activity at all temperatures tested (25 °C-80 °C) for 105 min. The stability of the extract from *Bacillus* sp. DPUA 1728 remained above 97% in the 45 °C to 60 °C temperature range. Our results are different from those obtained by Gupta *et al.* (2005) who used protease produced by *Bacillus* sp. and reported activity above 80% at 50 °C and above 92% at 45 °C, using a shorter time (40 min) with activity reduced to less than 20% at temperatures above 55 °C.

## Conclusion

Use of collagenolytic proteases for collagen degradation and collagen derivatives is critical in medicine, in agriculture, in food supplements, in cosmetics and in the leather



**Figure 4** - (A) Effect of temperature on the activity of extracellular protease and (B) Effect of temperature on stability of extracellular protease produced by *Bacillus* sp. expressed as a percentage of the maximum obtained at pH 7.2.

industry. *Bacillus* sp. DPUA 1728 evaluated in this research showed collagenolytic activity, and the application of factorial design optimized the selection of conditions necessary for enzymatic production. Future research with these micro-organisms should be conducted to purify this protease and submit it to complete characterization for possible biotechnological applications.

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