



## PRODUCTION AND CHARACTERIZATION OF PROTEASE VIA SUBMERGED FERMENTATION OF *Bacillus* isolated FROM AMAZONIAN SOIL

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### Resumo

Proteases catalisam ligações peptídicas em proteínas, possuem importante papel em processos fisiológicos. Estas enzimas constituem 60% do total de enzimas comercializadas industrialmente no mundo. As produzidas por *Bacillus* representam 20% desse montante. O presente trabalho tem como objetivos isolar e identificar as bactérias proteolíticas do ambiente Amazônico. O isolamento foi realizado com choque térmico, semeadas em Ágar gelatina-leite a 37 °C por 24 horas. As bactérias protease-positivas foram purificadas e identificadas. Para a determinação da atividade proteolítica os *Bacillus* foram inoculados em 4 mL de meio de fermentação, pH 7,0, e caseína (2% p/v) como substrato, o extrato bruto foi analisado a 280 nm. O pH ótimo foi determinado em diferentes valores de pH (4-9) incubados à temperatura ambiente por 1 hora. O efeito da temperatura na atividade proteolítica foi realizado em triplicata e incubado em diferentes temperaturas (25, 37, 40, 50, 60, 70 e 80 °C) por 1 hora e depois determinada a atividade enzimática. Das coletas realizadas, foram isoladas 37 bactérias e dentre estas 21 identificadas do gênero *Bacillus*. Foram selecionadas para as etapas posteriores 8 isolados que obtiveram seu pz menor que 0,4. Entre as selecionadas, o *B. firmus* (1) foi o que apresentou maior quantitativo de produção de protease (3322,2 U mL<sup>-1</sup>), seu pH ótimo foi o 7 e seu pH na estabilidade enzimática foi o 6, já sua temperatura ótima foi a de 37 °C e sua temperatura na estabilidade enzimática foi a de 80 °C.

**Palavras-Chave:** Ambiente amazônico, atividade enzimática, *Bacillus*, bactéria, protease.

### Abstract

Proteases catalyze peptide bonds in proteins and play important role in physiological processes. These enzymes account for 60% of the total industrial enzymes marketed worldwide. Those produced by *Bacillus* represent account for 20% of this amount. This study aims at isolating and identifying the proteolytic bacteria from Amazon environment. The isolation was performed with heat shock, plated on gelatin-milk agar at 37 °C for 24 hours. The protease-positive bacteria were purified and identified. To determine the proteolytic activity, *Bacillus* were inoculated into 4 mL of fermentation medium, pH 7.0, and casein (2% w/v) as substrate, the crude extract was analyzed at 280 nm. The optimum pH was determined at different pH values (4-9) incubated at room temperature for 1 hour. The temperature effect on the proteolytic activity were performed in triplicate and incubated at different temperatures (25, 37, 40, 50, 60, 70 and 80 °C) for 1 hour and then determined the enzymatic activity. From collections were isolated 37 bacteria, 21 having been identified as genus *Bacillus*. Eight isolates that got their pz less than 0.4 were selected for the subsequent steps. Among those selected, *B. firmus* (1) showed the greatest amount of production of protease (3322.2 U ml<sup>-1</sup>), its optimum pH was 7 and its pH on enzymatic stability was 6, while its optimum temperature was 37 °C and the temperature on enzymatic stability was 80 °C.

**Keywords:** Amazonian Environment, enzymatic activity, *Bacillus*, bacteria, protease.

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## 1. Introduction

The proteolytic activity is an essential process for all living organisms, either used for growth or cell death, blood clotting, immune defense, and/or maintenance of systemic homeostasis of individuals (VANDEPUTTE-RUTTEN & GROS, 2002; INÁCIO *et al.*, 2013)

These biocatalysts are found in various microorganisms such as viruses, bacteria, protozoa, yeasts and fungi (RAO *et al.*, 1998; POZA *et al.*, 2001; KUMAR & VATS, 2010). The inability to meet the global demand for proteases of animal and plant origin and the most desirable characteristics for biotechnology has led to an increasing interest in microbial proteases (RAO *et al.*, 1998; SILVA NEVES *et al.*, 2006; SEVINC & DEMIRKAN, 2011).

In the pharmaceutical industry, they are used in healing ointments and have potential for use with other medicines. They hydrolyze the proteins into peptides and amino acids facilitating their uptake by cells, due to their role in depolymerization with important action in nutrition. They are also involved in key biological processes such as blood clotting, cell death and tissue differentiation (RAO, *et al.*, 1998; MURI, 2013).

Additionally, microorganisms represent excellent source of enzymes due to ease genetic manipulation and wide biochemical diversity (SILVA NEVES *et al.*, 2006). (SILVA NEVES *et al.*, 2006; RAO *et al.*, 1998).

Proteases catalyze peptide bonds in proteins, play important role in physiological processes and constitute one of the most important groups of industrial enzymes accounting for approximately 60% of the enzymes marketed worldwide (RAO *et al.*, 1998; SAID & PIETRO, 2004; DELATORRE *et al.*, 2010).

In this context, this study aims isolating proteolytic bacteria from Amazon environment, identifying those proteolytic enzyme producers and characterizing the major quantitative enzymatic producer.

## 2. Material and Methods

### 2.1. Isolation of protease-producing bacteria from soil samples

Isolation and selection of protease-producing bacteria were 30 soil samples from the southern sector of Federal University of Amazonas.

One gram of the samples were placed in testing tube (16.5 mm x 150 mm) and submitted to heat shock 80 °C for 12 minutes, then placed on ice for 5 minutes. Subsequent to this treatment, 100 µL were seeded in Petri dishes (100 mm x 15 mm) containing agar gelatin-milk (1 % w/v) and incubated at 37 °C for 24 hours. (PATEL, 2005).

### 2.2. Purification and maintenance of isolates

Elapsed the aforementioned incubation period, the protease-positive bacteria (hyaline halo around the colony), the enzymatic activity (Pz) of the bacterium was determined, which is the ratio between the colony and the halo diameter (CRUZ FILHO *et al.*; 2013; SANTOS *et al.*; 2013). The isolated bacteria were those with  $Pz > 0.4$ . The isolates were also maintained in nutrient agar for identification according to the methodology of Barrow and Feltham (1999).

### 2.3. Production of proteases in liquid medium

The protease-positive *Bacillus* identified were reactivated in nutrient broth at 37 °C for 24 hours of which an aliquot 1000 µL was inoculated into 125 mL Erlenmeyer flask containing 40 mL of fermentation medium [ $KH_2PO_4$ , 2 g L<sup>-1</sup>;  $(NH_4)_2SO_4$ , 1 g L<sup>-1</sup>;  $MgSO_4 \cdot 7H_2O$ , 0.1 g L<sup>-1</sup>;  $Na_2HPO_4 \cdot 2H_2O$ , 0.9 g L<sup>-1</sup>; yeast extract, 1 g L<sup>-1</sup>], pH 7.0, added gelatin (1% w/v) MACIEL *et al.*, 2010; SILVA NEVES *et al.*, 2006). The fermentation was carried out in orbital shaker at 180 rpm at 37 °C for 24 hours. Crude extract was carried out by membrane filtration (0.22 µm), following the determination of proteolytic activity (SANTOS *et al.*, 2013a).

### 2.4. Determination of proteolytic activity

To determine the proteolytic activity 150 µL of crude extract was used in 1.5 ml casein 2.0 % (w/v) as substrate in 1.0 mL of 0.15 M phosphate buffer, pH 7.5 and 0.5 mL of enzymatic solution incubated at 30 °C for 30 minutes. The reaction was stopped by adding 3.0 mL of 0.4 M solution of trichloroacetic acid (TCA) followed by filtration through 0.22 µm membrane. The filtrate absorbance was determined at 280 nm (FLEURI; SATO, 2008).

One unit of protease activity was defined as the amount of enzyme capable of producing increased absorbance of 0.001/min. for one hour and expressed in U mL<sup>-1</sup> (DOSORETZ *et al.*

1990; PORTO *et al.*, 1996; SILVA NEVES *et al.*, 2006).

## 2.5. Characterization of protease from *Bacillus* selected with higher protease activity

### 2.5.1. Effect of pH on the activity and stability

The optimum pH of the protease was determined at different pH values using the following buffer solutions: 0.2 M citrate buffer solution (pH 4 and 6), 0.2 M phosphate buffer solution (pH 7 and 8) and 0.2 M Tris-HCl buffer solution (pH 9) incubated at room temperature for 1 hour. For testing pH stability, the enzyme solution was incubated in the different aforementioned buffers. The incubation time of samples was from 0 to 90 minutes for each pH range, determining enzymatic activity every 15 minutes (RAO *et al.*, 2009).

### 2.5.2. Effect of temperature on enzyme activity

To evaluate the effect of temperature on the proteolytic activity, the reaction system and the blank were performed in triplicate and incubated at 25, 37, 40, 50, 60, 70 and 80 °C for 1 hour and then determined enzymatic activity. For stability testing the enzymatic extract was incubated at different temperatures (25, 37, 40, 50, 60, 70 and 80 °C) and the time of incubation of samples varied from 0 to 90 minutes at each temperature, determining enzymatic activity every 15 min. (RAO *et al.*, 2009).

## 3. Results and Discussion

Table 1 shows 21 strains of the genus *Bacillus* isolated from Amazonian soil and their enzymatic index (Pz). Eight isolates that showed Pz less than 0.4 were selected for subsequent steps, which according to Santos *et al.* (2013) have strongly positive activity for protease. The isolated bacteria included two colonies of *Bacillus firmus* and *B. pumilus*, and one colony of *B. anthracis*; *B. megaterium*; *B. thuringiense* and *B. brevis*.

Among the previously selected bacteria, *B. firmus* (1) had the highest quantity of protease activity, 3322.2 U ml<sup>-1</sup> (Table 2), which is the microorganism used to determine the optimal pH and temperature and also the effect of pH and temperature on the activity and enzymatic stability. The results presented in this study were higher than those obtained by Joshi (2010), who obtained 390 U ml<sup>-1</sup> using *Bacillus firmus* Tap5 and azocasein as substrate, and Rao and Narasu

(2007) using *Bacillus firmus* MTCC 7728 and casein as inductor obtained 215 U ml<sup>-1</sup>.

Table 1: Values of enzymatic activity (Pz) presented by different isolates

Species	Pz
<i>B. amyloliquefaciens</i> (1)	0.50
<i>B. amyloliquefaciens</i> (2)	0.43
<b><i>B. anthracis</i></b>	<b>0.23</b>
<b><i>B. brevis</i></b>	<b>0.33</b>
<i>B. circulans</i>	0.42
<b><i>B. firmus</i> (1)</b>	<b>0.36</b>
<b><i>B. firmus</i> (2)</b>	<b>0.22</b>
<i>B. firmus</i> (3)	0.77
<i>B. licheniformis</i> (1)	0.83
<i>B. licheniformis</i> (2)	0.60
<b><i>B. megaterium</i></b>	<b>0.28</b>
<i>B. mycoides</i> (1)	0.41
<i>B. mycoides</i> (2)	0.40
<i>B. pantothenius</i>	0.43
<b><i>B. pumilus</i> (1)</b>	<b>0.35</b>
<b><i>B. pumilus</i> (2)</b>	<b>0.23</b>
<i>B. pumilus</i> (3)	0.40
<i>B. pumilus</i> (4)	0.82
<i>B. pumilus</i> (5)	0.42
<i>B. shpaericus</i>	0.41
<b><i>B. thuringiensis</i></b>	<b>0.30</b>

Pz = Enzymatic index

Table 2: Quantitative results for protease production in U/ml and standard deviation

Species	N	U mL <sup>-1</sup>
<i>B. firmus</i> (1)	3	28500 <sup>a*</sup>
<i>B. brevis</i>	3	16900 <sup>b*</sup>
<i>B. thuringiensis</i>	3	5960 <sup>c*</sup>
<i>B. pumilus</i> (2)	3	2960 <sup>d*</sup>
<i>B. megaterium</i>	3	2820 <sup>d*</sup>
<i>B. firmus</i> (2)	3	2700 <sup>d*</sup>
<i>B. anthracis</i>	3	77,8 <sup>e*</sup>
<i>B. pumilus</i> (1)	3	4,4 <sup>f*</sup>

\*GroupingTukeyMethod

The optimum pH presented by the enzyme produced by *Bacillus firmus* (1) was pH 7.0 as demonstrated in Figure 1, diverging from Joshi (2010) and Rao and Narasu (2007) found that pH 8 and 9, respectively. As regards the effect of pH on the stability, *Bacillus firmus* (1) has demonstrated stable enzyme at pH 6 (Figure 2). For Joshi (2010) stability occurs in the pH range 6-10.

For the effect of temperature on the enzymatic activity produced by *Bacillus firmus* (1) activity

was 37 °C (Figure 3), diverging from Joshi (2010) and Rao and Narasu (2007), who found optimum temperature of 60 °C and 40 °C respectively. As regards the effect of temperature on the stability, *Bacillus firmus* (1) has demonstrated stable enzyme in the range of 80 °C (Figure 4). However, Joshi (2010) found stability at 60 °C.

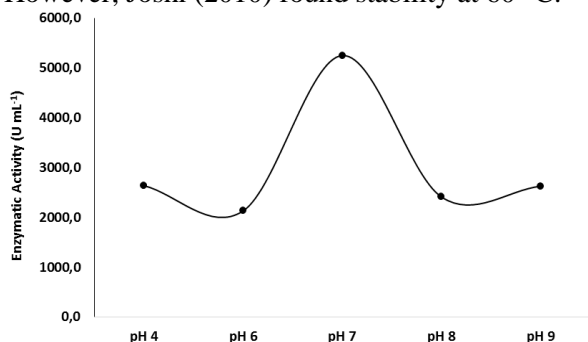


Figure 1: Effect of pH on enzymatic activity (optimum pH)

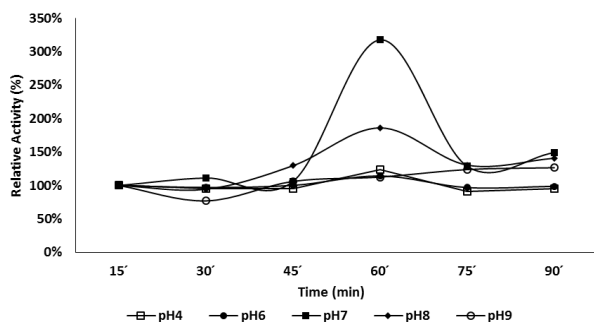


Figure 2: Effect of pH on enzyme stability

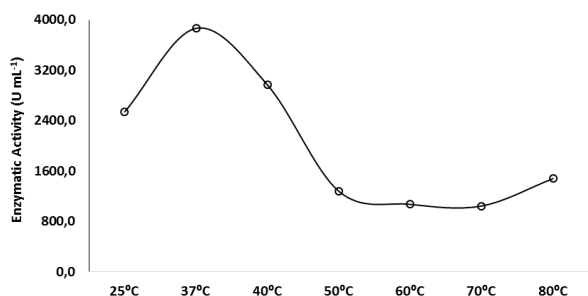


Figure 3: Effect of temperature on enzymatic activity (optimum temperature).

#### 4. Conclusion

All bacteria analyzed are producing protease, out of the 37 bacterial isolates, 21 were from the genus *Bacillus*; *B. firmus* (1) showed higher production of protease, 3322.2 U ml<sup>-1</sup>. The optimum pH of the enzyme was pH 7 with stability at pH 6; the optimum temperature of the enzyme was 37 °C with stability at 80 °C. The results find that the Amazonian soil is rich in

proteolytic bacteria, therefore being feasible for biotechnological prospecting surveys.

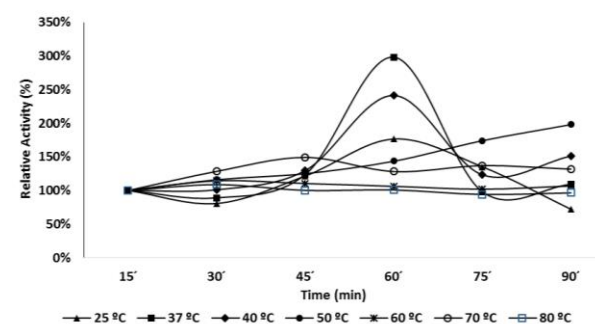


Figure 4: Effect of temperature on enzymatic stability.

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#### Disclosure

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