

PARTIAL CHARACTERIZATION OF PROTEASES FROM *STREPTOMYCES CLAVULIGERUS* USING AN INEXPENSIVE MEDIUM

Keila Aparecida Moreira^{1,5}; Maria Taciana Holanda Cavalcanti^{1,5}; Helena Simões Duarte²;
Elias Basile Tambourgi³; Eduardo Henrique Magalhães de Melo¹; Valdinete Lins Silva⁴;
Ana Lúcia Figueiredo Porto^{2,5}; José Luiz de Lima Filho^{1,5*}

¹Departamento de Bioquímica, Universidade Federal de Pernambuco, Recife, PE Brasil; ²Departamento de Morfologia e Fisiologia Animal, Universidade Federal Rural de Pernambuco, Recife, PE, Brasil; ³Departamento de Separação Química, Faculdade de Engenharia Química, Universidade Estadual de Campinas, Campinas, SP, Brasil; ⁴Departamento de Engenharia Química and ⁵Laboratório de Imunopatologia Keizo Asami, Universidade Federal de Pernambuco, Recife, PE, Brasil

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ABSTRACT

The partial characterization of extracellular proteases from *Streptomyces clavuligerus* NRRL 3585 and 644 mutant was investigated. The enzyme production was carried out in batch fermentation using soy bean filtrate as nitrogen source. Maximum activity was obtained after 96h of fermentation with an initial pH of 7.0. The enzyme was partially purified by ammonium sulphate precipitation. Enzymes from the two strains retained 37% of their initial activities at pH 8.0 after 2 h incubation at 25°C. Enzyme half-life at pH 8.0 and 60°C was 40.30 and 53.32 min, respectively for both strains (partially purified extract). The optimum pH was obtained at pH 7.0-8.0 and 8.4 for enzymes produced for 3585 and 644 strains (crude extract), respectively, and 8.4 and 8.0 for enzymes from the partially purified extract 3585 and 644 strains, respectively. The optimum temperature for the crude extract was 21°C for both strains. However, for the partially preparation the optimum temperature was 50°C and 40°C for *S. clavuligerus* NRRL 3585 and 644 strains respectively.

Key words: *Streptomyces clavuligerus*, extracellular proteases, thermal stability, pH

INTRODUCTION

Proteases are the most important class of industrial enzymes and comprise about 25% of commercial enzymes on the world. Two thirds of the industrially produced proteases are from microbial source (5,10). The major applications of these enzymes are in the food, pharmaceutical and detergent industries. Alkaline proteases are mostly used in enzyme-containing detergent powders, and have minor uses in food processing, e.g. childproofing of beer and production of protein hydrolysate (13). Acid protease plays an important role in meat tenderization and in the production of fermented foods by moulds from soybean, rice and others cereals (12). They are also used in the baking industry for the modification of wheat proteins in the bread industry and in the dairy industry for clotting of milk to manufacture cheese (2). Porto *et al.* (15) have studied *Streptomyces clavuligerus* cultures for protease

production and reported that the amount of enzyme produced varies greatly with the culture media used. Proteases from *Streptomyces* origin offer an advantage as the mycelium can be easily removed by filtration (13). In order to assess the utility of the *Streptomyces clavuligerus* proteases for industrial detergents use, properties such as pH and temperature stability of the crude and partial purification extract were determined.

MATERIALS AND METHODS

Microorganisms

Streptomyces clavuligerus NRRL 3585 and mutant 644, isolated in medium with high concentration (600mg/mL) of clavulanic acid (4), were used in this study. The microorganisms were maintained at 28°C on ISP-2 agar slants (16), made up of malt extract (1.0% w/v), yeast extract (0.4% w/v), agar-agar (2.0% w/v).

* Corresponding author. Mailing address: Universidade Federal de Pernambuco, Laboratório de Imunopatologia Keizo Asami (LIKA), Setor de Biotecnologia, Av. Moraes Rego, S/N. 50670-420, Recife, PE, Brasil. E-mail: zeluz@hotlink.com.br

Production media and culture conditions

The inoculum was carried out using Erlenmeyers flasks (250 mL) containing 50 mL of the fermentation medium described by Porto *et al.* (15), which contained the following components: glycerol (1.0% w/v), soy bean filtrate (0.5% w/v), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.06% w/v), NH_4Cl (0.1% w/v), K_2HPO_4 (0.435% w/v) and 0.1 mL mineral solution (100 mg of the $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, $\text{ZnSO}_4 \cdot \text{H}_2\text{O}$, $\text{CaCl}_2 \cdot \text{H}_2\text{O}$ distilled water 100 mL, with initial 7.0). Culture was grown for 48 h at 28°C, in an orbital shaker (200 rpm). Growth curve experiments were carried out using Erlenmeyers flasks (500 mL) containing 125 mL of the culture medium started with 10% (v/v) inoculum, orbital shaking (200 rpm) over 96 h at 28°C. Samples were removed at time intervals (24 h) and supernatants were used to measure protease activity and protein concentration.

Biomass determination

The biomass concentration was determined as mycelia dry weight after centrifugation (5 000 g for 5min) of 10 mL of culture broth in duplicate, and dried at 105°C overnight until constant weight.

Protease assay

Total extracellular protease was assayed at 25°C as described by Ginther (6) in culture medium previously clarified by centrifugation (12 000 g for 5 min). Azocasein, 1.0% (w/v) (Sigma, ST. Louis, Mo USA) in 0.2 M Tris-HCl, pH 7.2, containing 1.0 mM CaCl_2 , was used as substrate. One unit of activity was defined as the amount of enzyme that produces an increase in the optical density of 1.0 in 1 h at 440 nm. Protein was determined using the method described by Bradford (3), with bovine serum albumin as the standard.

Effect of pH on protease activity

For determination of optimum pH of the enzyme, the reaction mixture buffer of the azocasein 1% w/v was varied over the pH range 5.0 - 9.0. The buffers used were citrate-phosphate (pH 5.0 - 5.7, 0.1M), phosphate (pH 6.0 - 8.0, 0.1M), and Tris-HCl (pH 7.2 - 9.0, 0.2M) Pimentel *et al.* (14).

Effect of temperature on protease activity

For the determination of optimum temperature, the reaction mixture containing of azocasein 1% (w/v) was incubated over a range of temperature from of 21°C to 70°C using 0.2M Tris-HCl pH 8.0.

Thermal stability of protease

For the determination of the thermal stability, the enzyme was pre-incubated over range of temperature from 21°C to 70°C. The time of incubation of the samples varied from 30 to 120 minutes. After incubation, the samples were submitted to determination of protease activity, using the azocasein 1% (w/v) at 25°C (11).

Stability at different pH values

The enzyme was incubated at 25°C in buffers of different pH values (pH 6.5 - 8.0, 0.1 M buffers as above). The time of incubation samples varied from 30 to 120 minutes. Protease activity of the samples was measured at 25°C, using 0.2M Tris-HCl buffer, pH 8.0 for samples 3585 and 644 pH 8.5.

Partial purification with ammonium sulphate

To remove mycelia, the 96 hours culture broth was centrifuged and ammonium sulphate was added to the supernatant to give a concentration of 20% saturation. The precipitate was removed by filtration. The ammonium sulphate concentration was increased stepwise to 100% saturation, where at every additional 20% of ammonium sulphate, precipitates were collected by filtration. The collected precipitates were dissolved in deionised water and dialysed against phosphate buffer 0.1M pH 7.0. After exclusion of ammonium sulphate, proteolytic activity in each dialysed solution was assayed by the method described Ginther (6).

RESULTS AND DISCUSSION

Protease production throughout this study was determined after 96 hours of growth, which corresponded to the optimum period for enzyme production (Fig. 1 and 2). Both figures show that the activity keeps going up, but the biomass decreases after 50 hours of growth. These results are in agreement with Bascaran *et al.* (1), who showed that synthesis of protease by *Streptomyces clavuligerus* starts in the post-exponential phase of growth. As we can see from Fig. 1 and 2, proteases activities was could be detected 10 hours of growth and increased after the exponential phase.

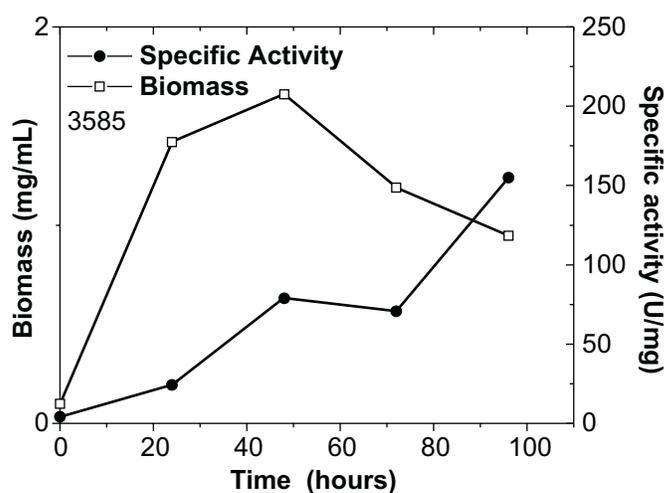


Figure 1. Biomass (□) and specific activity (●) of protease from *S. clavuligerus* 3585.

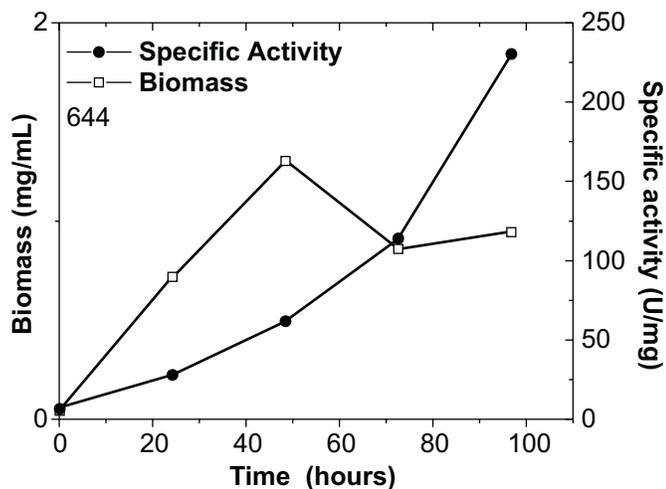


Figure 2. Biomass (□) and specific activity (●) curves of proteases from *S. clavuligerus* 644.

Experiments reported by Bascaran *et al.* (1), using *S. clavuligerus* for protease production after nutritional shift-down, indicated that initiation of protease formation is observed with decreased nutrients available. Good enzyme production was obtained using nitrogen-free medium or in presence of poorly utilized amino acids, but decreased with amino acids supporting higher growth rate.

The mechanisms by which control of protease production is achieved in many prokaryotes systems are not still known (1).

The effect of pH on activity and stability of protease

The effect of pH on protease activity is shown in Figs. 3 and 4. The optimum activity for both proteases was at pH 7.0-8.0 and 8.4, respectively for enzymes produced by 3585 and 644 strains (crude extract). These results are in agreement with those found by Sampath *et al.* (17) for another strain of *Streptomyces* (*Streptomyces* spp.). There the optimum pH was around 7.0-8.4 for almost several substrates used, and it was stable over pH range 6.5-8.0 and very unstable after pH 10.0.

However these results are compatible with t obtained with the *Conidiobolus coronatus* protease (13), which showed a high level of stability up to pH 8.0, which is the pH of most commercial detergent solutions. At present, there is considerable interest in the identification of alkaline proteases that act effectively as detergent enzymes when used at ambient temperature. This is essentially due to the energy cost involved in heating the water for washing. In countries like Brazil, detergents are commonly used at room temperatures (28°C) and hence, the enzyme could be promising for this application, in spite of being less stable to heat than the *Bacillus* enzymes. Fig. 5 and 6 also show the stability of proteases in various pH values. The enzymes were stable over a pH range 6.5 - 8.0 and

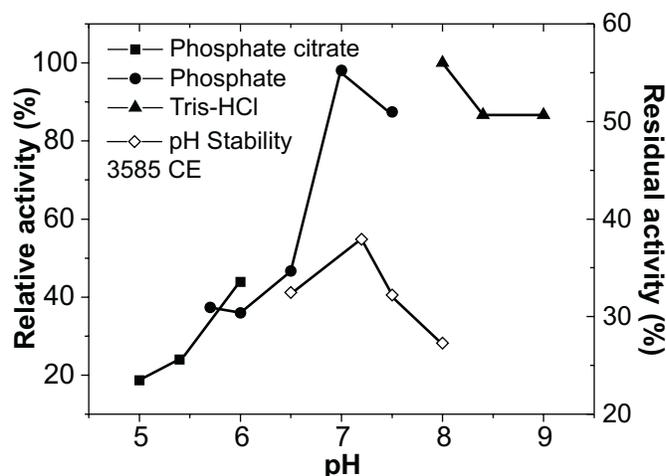


Figure 3. Effect of pH on activity (■, ● and ▲) and stability of protease (◇) from *S. clavuligerus* 3585 (crude extract - CE).

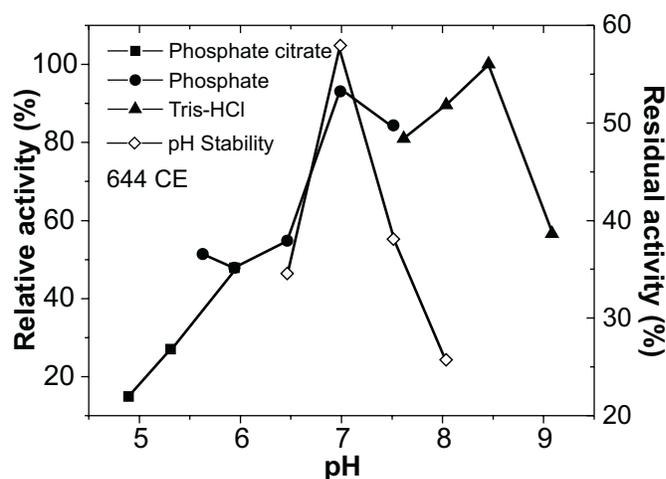


Figure 4. Effect of pH on activity (■, ● and ▲) and stability of protease (◇) from *S. clavuligerus* 644 (crude extract-CE).

6.5 - 7.5 for proteases produced from 3585 and 644 strains (PPE), respectively. Approximately 37% (3585 and 644) activity remained after 120 min.

The partially purified extract of protease from *S. clavuligerus* 644 PPE in pH 7.5 retained less than 62% of this activity in the presence of Cl⁻ ions, where HCl 0.2M pH 8.0 buffer solution was used.

Effect of temperature on activity and stability of protease

Thermostability of enzyme was investigated at 21°-70°C at pH 8.0. The enzyme was stable at 21°C for 120 minutes (100%), therefore the enzyme was stable a wide range 30°- 60°C, its retained about 28 and 40% activity during this time for crude extract 3585 and 644, respectively.

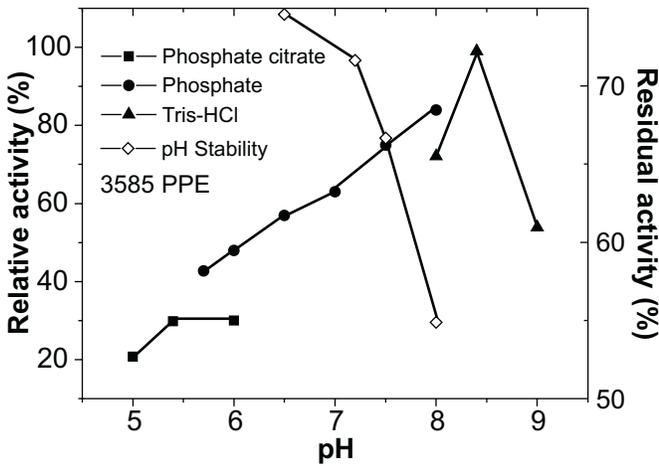


Figure 5. Effect of pH on activity (■, ● and ▲) and stability of protease (◇) from *S. clavuligerus* 3585 (partially purified extract - PPE – fraction 80 – 100%).

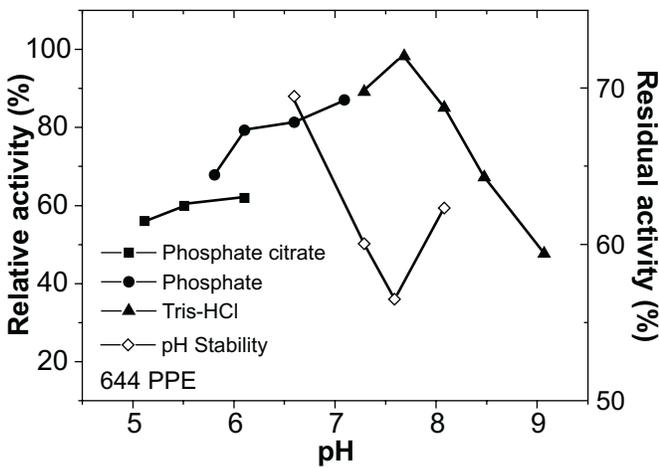


Figure 6. Effect of pH on activity (■, ● and ▲) and stability of protease (◇) from *S. clavuligerus* 644 (partially purified extract - PPE – fraction 80 – 100%).

After 2 hours at 70°C, the activity was completely lost for both enzymes (crude extract and partially purified) because of thermal inactivation (Fig. 7 and 8). In the Fig. 7, the decay constant at pH 8.0 at different temperatures of incubation is shown. Proteases from *Aspergillus oryzae* and *Aspergillus claudie* lost their activities beyond 40°C (7).

The protease from *Endothia parasitica* was found to be inactive within 5 min at 60°C (8). The present protease from *S. clavuligerus* exhibited greater thermal stability than the enzymes from other fungal sources, suggesting possible biotechnological and industrial importance.

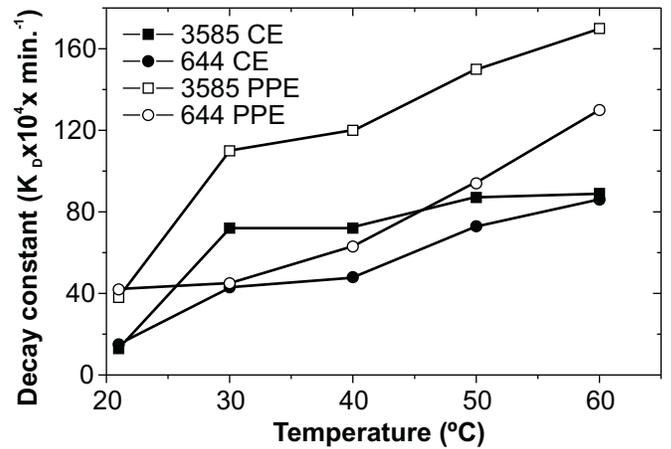


Figure 7. Decay constant for pH 8.0 at different temperatures of proteases from *S. clavuligerus* 3585 (■) and 644 (●) using crude extract - CE and 3585 (□) and 644 (○) partially purified - PPE (fraction 80 – 100%).

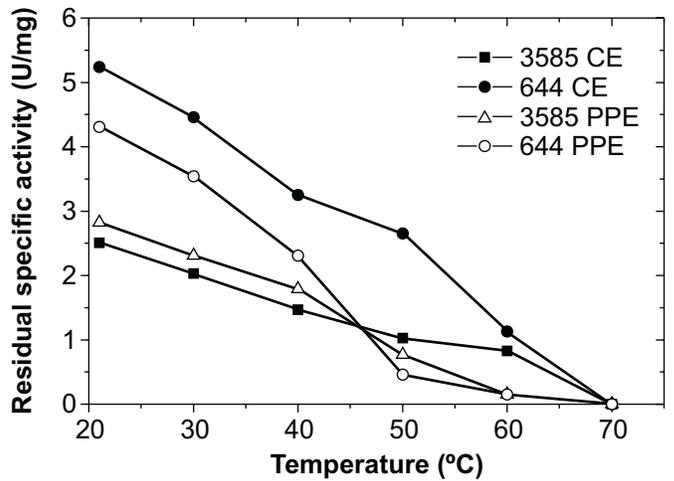


Figure 8. Thermal stability of proteases from *S. clavuligerus* 3585 (■) and 644 (●) and using crude extract- CE and 3585 (△) and 644 (○) partially purified – PPE (fraction 80 – 100%).

As shown in Fig. 9 and 10, the enzymes have an optimal activity at 21°C for crude extract, and 50°C and 40°C, respectively, for strains 3585 and 644 partially purified extract. This behaviour could be explained by the presence of an inhibitor, which is activated at temperatures above 20°C.

However, the purification procedures seem to eliminate the inhibitor from the medium. Comparing the curves for optimum temperature for activity and for thermal stability, it can be seen that they are not coincident. The curves obtained by Kang *et al.* (9).

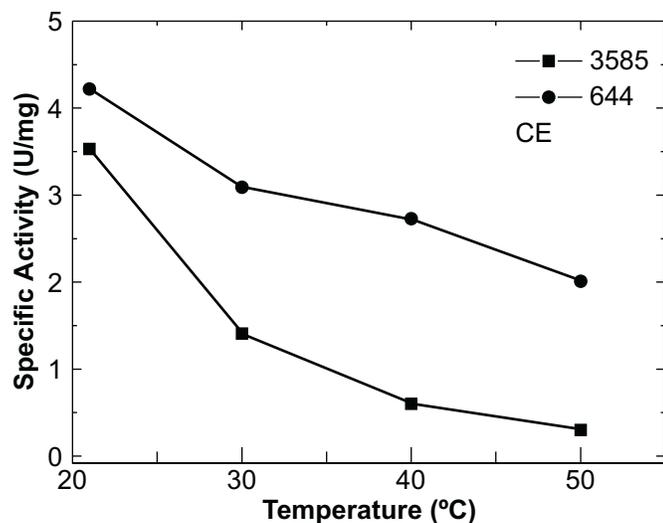


Figure 9. Temperature optimum of proteases from *S. clavuligerus* 3585 (■) and 644 (●) using crude extract – CE.

The phase for extracellular protease from *Streptomyces albidoflavus*, showed an optimum temperature at 40°C, and unstability at temperatures above 45°C during 90 minutes. Optimum temperature for thermostable proteases by *Aspergillus niger* and *Aspergillus saitoi* have been found to be 60° and 30°C, respectively (8,18).

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RESUMO

Caracterização parcial de proteases extracelulares de *Streptomyces clavuligerus* usando um meio de cultura econômico

A caracterização parcial de proteases extracelulares de *Streptomyces clavuligerus* foi investigada. A produção da enzima foi realizada pela fermentação em batelada utilizando farinha de soja como fonte de nitrogênio. O máximo rendimento foi obtido após 96 horas de fermentação com um pH inicial de 7,0. A enzima foi purificada parcialmente por precipitação com sulfato de amônia. Ambas enzimas reteram 37% de sua atividade inicial a pH 8,0, após 2 horas de incubação a 25°C. O tempo de meia-vida das enzimas foi de 40,30 e 53,32 minutos, respectivamente para ambos 3585 e 644 (extrato parcialmente purificado) a pH 8,0 e 60°C. Com relação ao pH, a atividade ótima foi alcançada a pH 7,0-8,0 e 8,4 para enzimas produzidas

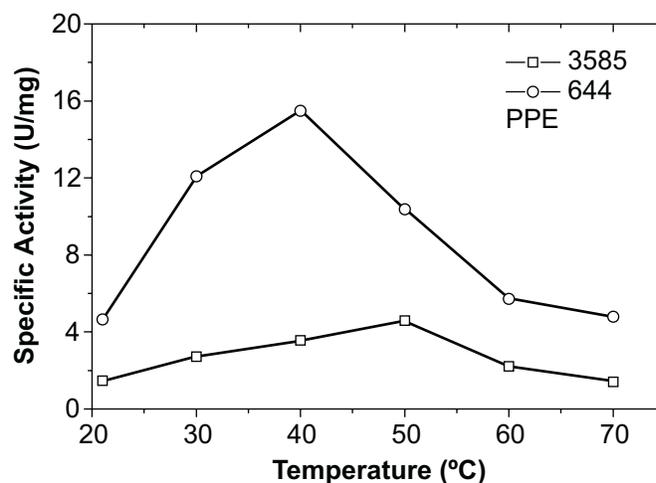


Figure 10. Temperature optimum of proteases from *S. clavuligerus* 3585 (□) and 644 (○) using extract partially purified - PPE – fraction 80–100%.

pelas amostras 3585 e 644 (extrato bruto) respectivamente e 8,4 e 8,0 para as enzimas obtidas a partir dos extratos parcialmente purificados das amostras 3585 e 644, respectivamente. A temperatura ótima para o extrato bruto foi de 21°C para *S. clavuligerus* NRRL 3585 e 644. Contudo, para a preparação parcialmente purificada, a temperatura ótima foi de 50 e 40°C para amostras *S. clavuligerus* NRRL 3585 e 644 respectivamente.

Palavras-chave: *Streptomyces clavuligerus*, proteases extracelulares, estabilidade térmica, pH

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