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BIOLOGY

Bioconversion of poultry residues for the production of proteases by *Aspergillus* sp. isolated from Amazonian forest soil

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ABSTRACT: Feathers are by-products that are generated in significant quantities by the poultry industry. Microbial bioconversion has been investigated as a promising strategy for the processing of feathers, since, along with the degradation of these keratinous materials, bioprocessing can result in value-added products. Thus, from the perspective of industrial microbiology, chicken feathers can be considered a raw material for obtaining microbial proteases. Within this context, this research investigated and characterized the production of extracellular proteases by *Aspergillus* sp., isolated from soil of the Amazon Rainforest. The enzymatic production was evaluated using several growth substrates (whole feathers, feather meal, human hair, casein, gelatin, peptone and chicken beaks). With highest enzyme production was obtained the feather meal (FM) and peptone. After 48 h of fermentation, FM degradation was 15.82%. The crude protease showed optimal activity at pH 5.0 and 37 °C and enzymatic activity was enhanced with the addition of 1 and 5 mM of $CaCl_2$, $MnSO_4$, KCl, $MgSO_4$ and $CuSO_4$. The detergents Tween 20 and Triton x-100, at concentrations 0.5 and 1% (v/v), tended to stimulate activity. The presence of 0.5 and 1% (v/v) of organic solvents (methanol, acetone, butanol, acetonitrile, isopropanol and DMSO), maintained the enzymatic activity. β–mercaptoethanol stimulated proteolytic activity in the enzymatic assays. This study suggested new direction for waste management with industrial applications giving rise to green technology for sustainable development.
Key words: agro-industrial by-products, *Aspergillus* sp., feather residue, proteases.

Bioconversão de resíduos de aves para produção de proteases por *Aspergillus* sp. isolado do solo da floresta Amazônica

RESUMO: As penas são subprodutos que são gerados em quantidades significativas pela indústria avícola. A bioconversão microbiana tem sido investigada como uma estratégia promissora para o processamento de penas, uma vez que, juntamente com a degradação desses materiais queratinosos, o bioprocessamento pode resultar em produtos de valor agregado. Assim, do ponto de vista da microbiologia industrial, as penas de frango podem ser consideradas matéria-prima para a obtenção de proteases microbianas. Dentro deste contexto, o objetivo deste trabalho foi investigar e caracterizar a produção de proteases extracelulares por *Aspergillus* sp., isolados de solo da floresta Amazônica. A produção enzimática foi avaliada utilizando diversos substratos de crescimento (penas inteiras, farinha de penas, cabelo humano, caseína, gelatina, peptona e bicos de frango). Com maior produção de enzima foi obtida a farinha de penas (FP) e peptona. Após 48 h de fermentação, a degradação da FP foi de 15.82%. A protease bruta mostrou atividade ótima em pH 5.0 e 37 ^{0}C e a atividade enzimática foi aumentada com a adição de 1 e 5 mM de $CaCl_2$, $MnSO_4$, KCl, $MgSO_4$ e $CuSO_4$. Os detergentes Tween 20 e Triton x-100, nas concentrações 0.5 e 1% (v/v), tenderam a estimular a atividade. A presença de 0.5 e 1% (v/v) dos solventes orgânicos (metanol, acetona, butanol, acetonitrila, isopropanol e DMSO), mantiveram a atividade enzimática. O β–mercaptoetanol estimulou a atividade proteolítica nos ensaios enzimáticos. Este estudo sugere uma nova direção para a gestão de resíduos com aplicações industriais dando origem à tecnologia verde para o desenvolvimento sustentável.
Palavras-chave: subprodutos agroindustriais, *Aspergillus* sp., resíduo de pena, proteases.

INTRODUCTION

The meat industry generates enormous amounts of organic waste and by-products, such as viscera, bones, blood, skin, and meat trimmings that need to be adequately managed (LEMES et al., 2016). The consumption of chicken meat has increased in recent years due to its nutritional quality, availability and cost. According to the Food and Agriculture Organization (FAO), worldwide around 24 billion chickens were produced in 2018. Assuming that a chicken weighs about 2 kg and that the average percentage of feathers is approximately 5 % of the total weight, the total amount of chicken feathers produced in 2018 can be estimated at 2.4 million tonnes (FAO, 2019). Most of the feathers produced by the poultry industry end up in dumps, landfills and incinerators. Unfortunately, these methods can cause contamination of the environment due to the generation of greenhouse gases (ACDA, 2010).

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Nevertheless, feathers are considered a natural source of protein and can be used as fertilizers, in the formulation of animal feed, and also in other applications in industry (DONNER et al., 2019; FAKHFAKH et al., 2011).

Keratinolytic enzymes are highly active in the keratin substrate that is available, act on the peptide bonds, and convert them into more simplified forms (GOPINATH et al., 2015). Bacteria and fungi that produce keratinolytic enzymes have already been studied by several authors (BOHACZ, 2017; KOTHARI et al., 2017).

Fungal keratinases are of interest due to their high diversity, broad substrate specificity and stability in extreme conditions. (JISHA et al., 2013). With this, it is important to identify new keratinolytic microorganisms, since keratinases can be produced that can be used in industries and also in the production of keratin hydrolysates (GHAFFAR et al., 2018).

Therefore, the use of the keratinolytic potential of microorganisms emerges as an economically and environmentally-appropriate approach to the processing of feathers, with the aim of obtaining protein hydrolysates and increasing the value of these underutilized materials (LASEKAN et al., 2013). In this context, the objective of this research was to evaluate the production of proteolytic enzymes by *Aspergillus* sp. during submerged fermentations, as well as to characterize these enzymes for their potential utility in biotechnological processes (PLEISSNER & VENUS, 2016). In this context, the objective of this research was to evaluate the production of proteolytic enzymes by *Aspergillus* sp. during submerged fermentations, as well as to characterize these enzymes for their potential utility in biotechnological processes.

MATERIALS AND METHODS

Microorganism and qualitative evaluation of protease production

The fungus was originally isolated from soil samples from the Virua National Park, Roraima, in the extreme north of the Amazon Rainforest, Brazil. Fungus were obtained from the collection of the microbiology laboratory of the Federal University of Roraima and quantitatively evaluated in culture medium, which contained, per liter 0.025 g of $CaCl_2$, 0.005 g of $ZnSO_4$, 0.015 g of $FeSO_4$, 0.05 g of $MgSO_4$ and 0.5 g feather meal (FM). The pH was adjusted to 5.0 before autoclaving, according to the methodology described by ANBU et al. (2007), with modifications. Fungal spore suspensions with a final concentration of 10^5 spores/mL (ALVES & PEREIRA, 1998) were used, and incubation was performed at 27 °C with shaking at 120 x g for up to 10 days.

Protease production was qualitatively detected by inoculating *Aspergillus* sp. on skim milk agar (SMA) plates (RIFFEL & BRANDELLI, 2006). This medium was composed of peptone (5 g/L), yeast extract (3 g/L), UHT skim milk (100 ml/L) and agar (12 g/L). After incubation at 27 °C for 4 days, the presence of clear halos around the colonies of *Aspergillus* sp. was evaluated, since these indicated the production of proteolytic enzymes. The ability of the microorganism to grow in feather meal agar (FMA) was tested as described by RIFFEL & BRANDELLI (2002). The isolate was streaked on FMA plates and incubated at 27 °C for up to 5 days. The production of keratinases was observed through the formation of a degradation halo.

Preliminary fungal identification

The isolate was transferred to Czapek yeast agar (CYA: sucrose 30 g, yeast extract 5 g, $NaNO_3$ 3 g, KCl 0.5 g, $MgSO_4.7H_2O$ 0.5 g, $FeSO_4$. $7H_2O$ 0.01 g, K_2HPO_4 1 g, agar 20 g, water 1 L) or malt extract agar (MEA) and incubated at 25 and 37 ˚C for further identification at genus level. Preliminary identification of the isolate was performed through macroscopic and microscopic morphological observations using appropriate keys (PITT & HOCKING, 2009).

Preparation of chicken feather waste and inoculum

The feathers were supplied by a local chicken processing plant. To remove impurities, the feathers were washed with water at 50 °C, and then placed in a circulating air oven, at 60 °C for 48 h for drying, according to the methodology of TESFAYE et al. (2017), with modifications. After drying, the feathers were ground in a Willey knife mill to produce the feather meal. The microorganism was grown in 250 mL Erlenmeyer tubes with 100 mL of the liquid medium to produce the enzyme (autoclave sterilization, 15 min, 121 ºC). The assays with a concentration of 10^5 spores/mL were incubated at 27 ºC, with different concentrations of chicken feather meal (0.5, 1.0, 3.0 and 5% w/v) to determine the best proteolytic activity.

Time-course of protease production with 0.5% FM

Feather meal was selected as growth substrates (0.5 %) to produce proteases in medium (0.025 g of $CaCl_2$, 0.005 g of $ZnSO_4$, 0.015 g of $FeSO_4$ and 0.05 g of $MgSO_4$). The initial pH of the medium was adjusted to 5.0. Erlenmeyer flasks (250

mL) containing 100 mL of medium were inoculated with 1 mL of a spore suspension of *Aspergillus sp*. (10^5 spores/mL) and incubated at 27 °C in a rotary shaker (120 rpm) for 48 h. Experiments were performed in triplicate.

Azokeratin synthesis

Azokeratin was produced in the laboratory according to the methodology described by TOMARELLI et al. (1949). The feathers were ground (15 g) and added to 680 mL of distilled water, and 100 mL of $NaHCO_3$ (1 N) was added under continuous stirring. Simultaneously, a solution was prepared with 8.65 g of sulfanilic acid dissolved in 200 mL of NaOH (0.12 M), which was added to the feather meal mixture. Sequentially, the initial mixture was combined with 1.7 g of $NaNO_2$ and 10 mL of (5.0 M) HCl and stirred for another 2 min. Then, 10 mL of 5 M NaOH was added, and the mixture stirred for another 5 min and then dialyzed against distilled water at 4 °C. After dialysis, the solution was submitted to lyophilization.

Enzyme activity assays

Keratinolytic and proteolytic activities were determined using azokeratin (laboratory synthesized) or azocasein (Sigma Co., USA), respectively, as substrates. The reaction mixture contained 100 µL of enzyme preparation and 100 µL of 1% (w/v) azokeratin (or azocasein) in 0.025 g of $CaCl_2$, 0.005 g of $ZnSO_4$, 0.015 g of $FeSO_4$ and 0.05 g of $MgSO_4$ buffer, pH 5.5. The mixture was incubated for 30 min at 37 °C; and the reaction was stopped by adding 500 µL of 10 % (w/v) trichloroacetic acid (TCA). After centrifugation (10.000 x g for 5 min) of the reaction mixture, 800 µL of the supernatant was mixed with 200 µL of (1.8 M) NaOH, and the absorbance at 420 nm was measured (CORRÊA et al., 2010). One unit of enzyme activity was considered as the amount of enzyme that caused a change in absorbance of 0.01 units under the above assay conditions.

Evaluation of the percentage of degradation of the feathers

To determine the percentage of degradation, the methodology described by SUNTORNSUK & SUNTORNSUK (2003) was used. Feather degradation was carried out in 150 ml Erlenmeyer flasks containing 50 ml of basal medium (0.025 g of $CaCl_2$, 0.005 g of $ZnSO_4$, 0.015 g of $FeSO_4$ and 0.05 g of $MgSO_4$) with 0.5 g hen feathers. During fermentation, 2 mL of the culture fluid was removed after every day (10 days) and filtered using filter paper, oven dried at 105 °C overnight and then weighed. The percentage of feather degradation was calculated via the difference in residual dry weight between the control (medium with feathers, without inoculum) and the treated sample.

Screening of growth substrates for production of proteolytic activity

Casein, gelatin and peptone (Synth, Brazil), feather meal, whole feathers (from a slaughterhouse in Boa Vista, Roraima, Brazil), human hair and chicken beaks were selected as growth substrates (0.5 %) to produce proteases in medium (0.025 g of $CaCl_2$, 0.005 g of $ZnSO_4$, 0.015 g of $FeSO_4$ and 0.05 g of $MgSO_4$). The initial pH of the medium was adjusted to 5.0. Erlenmeyer flasks (250 mL) containing 100 mL of medium were inoculated with 1 mL of a spore suspension of *Aspergillus sp*. (10^5 spores/mL) and incubated at 27 °C in a rotary shaker (120 rpm) for 48 h. Experiments were performed in triplicate.

Concentration of extracellular protease

In order to analyze the highest protein precipitation, different saturation ranges were tested using ammonium sulfate (0-20, 20-40, 40-60, 60-80 and 80-100 %) (SCOPES, 1994). For this, fermentation was carried out containing 100 mL of culture medium as previously described for 48 h. At the end of the fermentation, the broth was centrifuged at 5.000 x g for 15 min at 5 °C to obtain the supernatant. Each saturation range was tested. For this, the salt was macerated until it appeared as a fine powder, which was slowly added to the filtrate. After precipitation, the samples were re-suspended in a smaller volume of buffer (0.025 g of $CaCl_2$, 0.005 g of $ZnSO_4$, 0.015 g of $FeSO_4$, 0.05 g of $MgSO_4$) then centrifuged (10.000 x g for 5 min) and the absorbance at 420 nm was measured. From these samples, the proteolytic activity was determined, as previously described. The best saturation range was used in the following steps.

Crude protease characterization

For determination of the optimum pH, proteolytic activity was assayed at 37 °C in a pH ranging from 5 to 12 using the following buffers (20 mM): phosphate (pH 5.0-6.5), Tris-HCl (pH 7.0-9.0) and carbonate (pH 10.0-12.0), according to the test conditions previously described. The results were expressed in relative activity, with the higher value of the proteolytic activity (pH 5.0) defined as 100 %. The effect of temperature on the enzymatic activity was assessed in temperatures ranging between 37 and

80 °C. The results were expressed in relative activity, and the value of the activities carried out at 37 ºC were considered to be 100 % (CORRÊA et al., 2010).

The influence of ions ($SrCl_2$, $CuSO_4$, $MgCl_2$, $ZnSO_4$, $CaCl_2$, $MnSO_4$, KCl, NaCl and $MgSO_4$) in the final concentration of 1 and 5 mM, negative control for $CuSO_4$ was performed with chemical without enzyme, detergents [sodium dodecyl sulfate (SDS, Tween 20, cetyltrimethylammonium bromide (CTAB), polyethylene glycol (PEG) and Triton X-100] and solvents [dimethylsulfoxide (DMSO), butanol, methanol, acetone, isopropanol and acetonitrile], in concentrations of 0.5 and 1 % (v/v) in proteolytic activity was investigated under the test conditions (previously described). The results were expressed in relative activity, using the control (100 %) without the addition of chemicals. The effect of chemicals on enzymatic activity was evaluated using the compounds EDTA and β-Mercaptoethanol. The enzyme was incubated for 10 minutes at room temperature (30 °C) with the inhibitors in concentrations of 1 and 5 mM. Subsequently, the enzymatic activity was verified according to the test conditions previously described. The results were expressed in relative activity, and compared to the control (100 %) without the addition of inhibitors (CORRÊA et al., 2010).

Statistical analysis

All assays were performed in triplicate and measurement data were expressed as the mean ± standard deviation (SD). All data were analyzed using R software, version 4.0.3 (R Core Team, 2020). Since we compared the effect of different treatments on enzymatic activity of *Aspergillus* sp. relative to the control samples, we performed Dunnett's many-to-one comparisons test (DUNNETT, 1955) for each assay (group of treatments). The test performed using R software with the package 'DescTools' (ANDRI et al., 2020) and the mean differences were considered when the P - values were less than 0.05.

RESULTS AND DISCUSSION

The results of the qualitative evaluation in solid media, skimmed milk agar (SMA) and feather meal agar (FMA) demonstrated the capacity of *Aspergillus* sp. to produce proteolytic enzymes after 5 days of incubation. In both media, it was possible to observe the formation of halos around the colonies, which indicate that the fungus is capable of producing these enzymes. The preliminary data of the qualitative evaluation were decisive for the follow-up of this study, due to the capacity of *Aspergillus* sp. for the production of proteolytic enzymes.

Figure 1 shows the growth characteristics of the filamentous fungus in CYA and MEA media at 25 °C and 37 °C after 7 days of cultivation. In both media at 25 ˚C, the colony showed a green tint and, at 37 ˚C, the colony showed white tones. One of the main characteristics that differentiates *Aspergillus* species is the color of the colonies, which can present

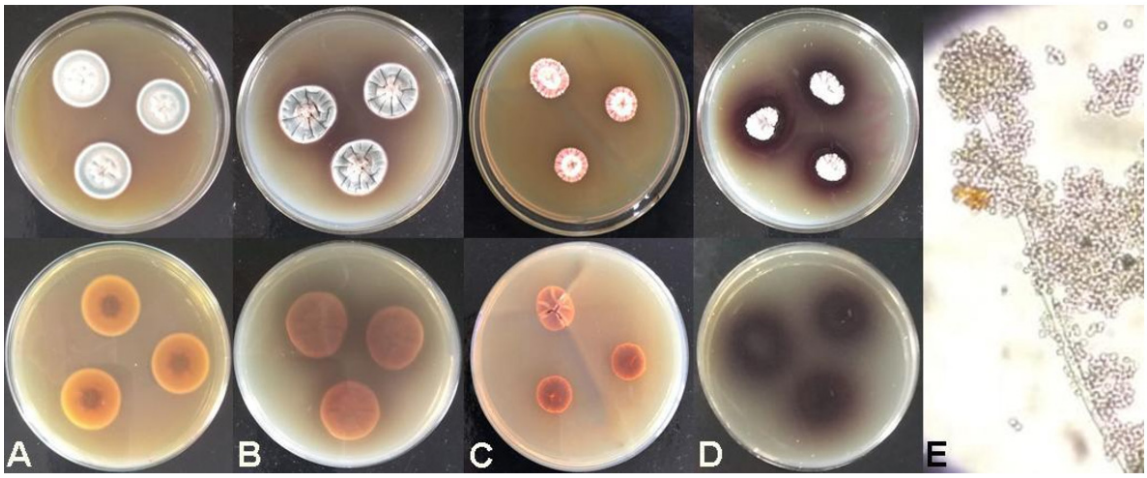

Figure 1 - Colony morphology of *Aspergillus* sp. in Czapeck yest agar (CYA) and Malt extract agar (MEA) 25 ˚C and 37 ˚C after 7 days. (A) top view and rear view in MEA 25 ˚C; (B) top view and rear view in CYA 25 ˚C; (C) top view and rear view in MEA 37 ˚C; (D) top view and rear view in CYA 37 ˚C; (E) and microscopic aspects of reproductive structures.

shades of green, black, gray, yellow, white and brown (KLICH, 2002). Characteristics, such as colony color and size after the incubation period, texture of conidiophores, size and texture of conidia, are important for taxonomic studies based on morphology, since the genus is subdivided into sections according to conidiophore arrangements and conidia. These characteristics, either together or separately, allow us to obtain a clear difference from the main genus sections (KLICH, 2002). Traditional identification, based on the morphological characteristics of the fungus, indicated that the isolate belongs to the genus *Aspergillus*, due especially due to the presence of spores (conidia) in chains from phialides that were supported by well-defined vesicles on the end of the stipe (PITT & HOCKING, 2009). This genus is considered to be cosmopolitan and widely distributed in nature. The isolation of species in soils and fallen plants is very common, and the genus has a greater abundance in regions of tropical and subtropical climates (KLICH, 2002, PITT & HOCKING, 1997).

Proteases from species of the genus *Aspergillus* have been extensively studied since they are known for their ability to secrete high levels of enzymes in the environment in which they grow and several of these enzymes that are produced in large-scale submerged fermentation have been widely used in industry over the decades (WU et al., 2006).

The determination of proteolytic activity was evaluated in submerged cultures (FM), during the 10-day incubation period. As shown in figure 2, *Aspergillus* sp. expressed its greatest activity on the second day of incubation (145.13 U/mL), with a reduction in 72 h. Results presented by IRE et al. (2011) and MUTHULAKSHMI et al. (2011) showed that the maximum production of proteolytic enzymes by *Aspergillus* species occurs between 4 to 9 days of incubation. SIVAKUMAR & RAVEENDRAN (2015) report that the process of degradation of the feathers carried out by fungi normally occurs more slowly when compared to bacteria. These are widely exploited by the industry exactly because they degrade more quickly, generally reaching the maximum peak of activity in the period of 48 h. A reduced enzyme production time is an important factor for industries since it reduces operating costs and causes less degradation of the enzymes produced (NYONZIMA & MORE, 2013). In a study carried out with 11 species of *Aspergillus* from the Amazon Fungus Collection, the proteolytic activity was evaluated using casein as substrate and presented variation between the values of 8.09 U/mL, in *A. japonicus,* to 22.49 U/mL, in *A. oryzae*, thus showing that the production proteases can vary between fungi of different species (ARAÚJO et al., 2016). In comparison with these results, the proteases produced by *Aspergillus* sp. in this study, it obtained more efficient activity in a shorter growth time, indicating its biotechnological potential as an important strategy.

Different substrates were tested in order to evaluate the production of extracellular protease by *Aspergillus* sp. in submerged growth methods. The fungus showed its ability to degrade all the substrates analyzed in this study. Cultures on the peptone and

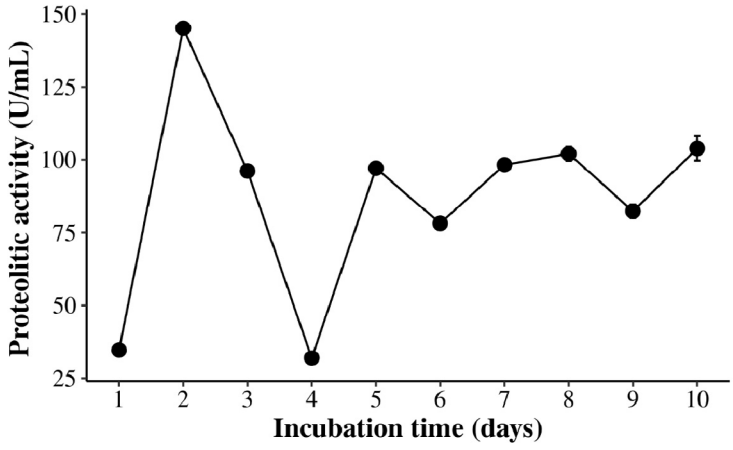

Figure 2 - Protease production by *Aspergillus* sp. during submerged cultivations (27 °C, 120 rpm) with 5 g/L feather meal as substrate. The test was performed in triplicate and the bar indicate the standard deviation.

feather meal substrates resulted in greater production of extracellular proteases, and reached maximum for enzymatic activity values within 48 h of culture (Figure 3). *Aspergillus* species are commonly known for their ability to use different substrates for their growth, as well as using different metabolic pathways for their assimilation (HAJJI et al., 2008; FLEIßNER & DERSCH, 2010). Using keratinolytic microorganisms, the production of proteases is often achieved with keratin-rich substrates, such as feathers, and mainly in the form of feather meal, as it provides greater accessibility of the enzyme to the substrate and homogeneity, which results in less resistance to hydrolysis (BRANDELLI & RIFFEL, 2005; CORRÊA et al., 2010). In contrast, the cultivation on the substrates gelatin and human hair showed lower values (47.39 and 47.69 %, respectively). Previous studies have claimed that there is a greater difficulty in hydrolysis of the hair substrate due to the conformational diversity of hair keratin in relation to feather keratin (ONIFADE et al., 1998; DAROIT & BRANDELLI, 2014). Some representatives of the Ascomycetes group have been reported to have a high capacity for degrading a wide variety of keratin substrates, including feather, hair and wool, which are considered to be structures that are very difficult to degrade (VERMA et al., 2017). This result corroborated the efficiency of *Aspergillus* sp. to produce proteases from the natural substrate (FM), which is considered the most suitable since it is a low-cost and widely available alternative, and at the same time can represent a potential ecologically appropriate management strategy, as well as adding value to these residues. *A. niger* produced a large amount of proteins to cleave feathers from the seventh day (MAZOTTO et al., 2013). According to BACH et al., 2011, a significant number of microorganisms that degrade keratin have been isolated from soil. Given the above, the feather meal substrate was considered the most suitable to be used in subsequent studies.

According to DAROIT & BRANDELLI (2014), the concentration of feathers is one of the main factors to be considered in processes of optimization of enzymatic production. In this context, the effect of different concentrations (0.5, 1, 3 and 5 %) of FM on protease production was initially evaluated. The results indicated that the best production of the enzyme occurred in cultivation with a higher amount of FM (5%), while in cultures performed with a low concentration (0.5%) less

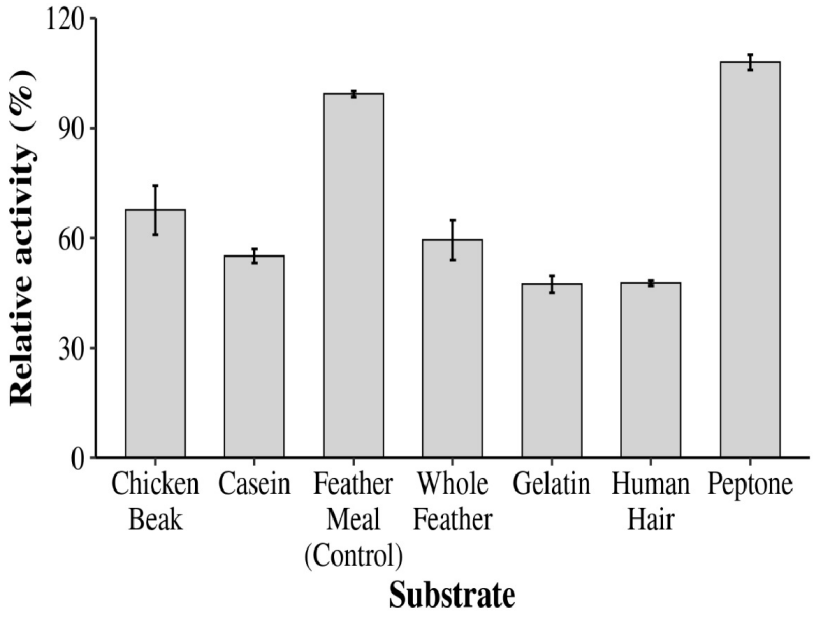

Figure 3 - Protease production by *Aspergillus* sp. in different growth substrates: chicken beak, casein, feather meal, whole feather, gelatin, human hair and peptone at a concentration of 0.5%, after 48 h of incubation in submerged culture, at 27 °C, at 120 rpm. The test was performed in triplicate and the bars indicate the standard deviation.

activity was obtained. Previous studies have observed that high concentrations of FM result in cell shear, in addition to a reduction in the transfer of oxygen to microbial growth in the medium (FAKHFAKH, 2011; DAROIT & BRANDELLI, 2014). Conversely, low concentrations of substrate can lead to under-utilization of microbial potential and less difficulty in controlling physical and chemical variables such as pH, temperature and oxygen. As this research was carried out on a laboratory scale production, we opted for the use of FM (0.5%) for the production of proteolytic enzymes. Thus, the enzyme produced in this study corroborated the previous study, which indicated the production of *Aspergillus* proteases via an agricultural residue as a substrate.

Table 1 shows keratinolytic activities in culture media (supernatants) containing 0.5% of whole feathers (WF) and feather meal (FM) after 48 h of incubation at 27 ˚C. The observed value for keratinolytic activity in the culture containing FM was 53.5 U/mL. An important factor to be observed is the type of substrate that was used (WF and FM). The results showed that FM presented the best result for the enzymatic production of *Aspergillus* sp. Since in the medium with FM the substrates are more available for the enzyme/substrate bond, there is less resistance and; therefore, hydrolysis is more efficient (CORRÊA et al., 2010). SILVA (2018) defends the idea of using microbial enzymes for the degradation of keratinous residues, mainly chicken feathers, as an alternative to reduce and/or solve the problem of accumulation of this by-product in the environment. Therefore, the search for efficient enzymes in this process has become constant.

Often, the first step used to separate proteins from crude extracts is precipitation by adding salts (ammonium sulfate) or water-miscible organic solvents. The separation in this case is based on differences in solubility presented by the proteins (MARZZOCO & TORRES, 1999). In this study ammonium sulfate was used as a precipitating agent in different saturation ranges (0-20, 20-40, 40-60, 60-80 and 80-100%) in order to determine the range of highest extracellular protease concentration. All ranges were evaluated since there was no previous knowledge on the isolate *Aspergillus* sp. studied. The results demonstrated that there was a spread of the enzymatic activity within these ranges and, therefore, the optimal saturation range for the enzyme between 0 and 60% was considered to continue the studies.

Degradation of WF and FM was evaluated after incubation at 27 °C, for 10 day. The highest percentage of degradation was obtained via cultivation with FM (16.26 %), while WP degraded 12.6%. One study carried out with *Aspergillus* sp. that was isolated from soil from the Caatinga biome reported that the greatest degradation of the cultures containing fragments of feathers was observed from the ninth to the twelfth day of incubation (FERREIRA et al., 2016). BOHACZ (2017) used 5 strains of fungi obtained from soil to evaluate the percentage of degradation of feather. In this study, all isolates were able to degrade the substrate; however, *Chrysosporium articulatum* and *Aphanoascus fulvescens* were the most active in hydrolysis, with biomass loss corresponding to 63.7 and 65.9%, respectively, after 42 days of cultivation. In this same incubation period, the strain *Chrysosporium keratinophilum* presented a lower percentage of degradation (35%). It should be noted that the primary objective (keratin hydrolysis) was achieved, since the rigid structures that constitute the feather were broken, thus reducing the time of degradation in nature. Therefore, the production of extracellular protease by the fungus *Aspergillus* sp., using chicken feathers as the only source of carbon and nitrogen, can contribute to a more suitable use of these agribusiness by-products.

The effect of pH (5.0 and 12.0) on crude keratinase produced by *Aspergillus* sp. was investigated. The maximum activity was observed at pH 5.0, with a substantial loss of activity at a higher pH (Figure 4). As in our study, proteolytic enzymes from *Lentinus crinitus* showed better activity at acidic pHs (pH 5.0 and 6.0) (MAGALHÃES et al., 2019). Enzymes of bacterial source; however,

Table 1 - Evaluation of keratinolytic activity in culture supernatants in feather meal and Whole feather at a concentration of 0.5%, after 48 h of incubation in submerged culture, at 27 °C, at 120 rpm.

Substrate	----------Keratinolitic activity (U/mL) ± SD----------
Whole feather	21.76 ± 0.12
Feather meal	53.5 ± 0.00

Assays were performed in triplicate and measurement data are expressed as the mean ± standard deviation (SD).

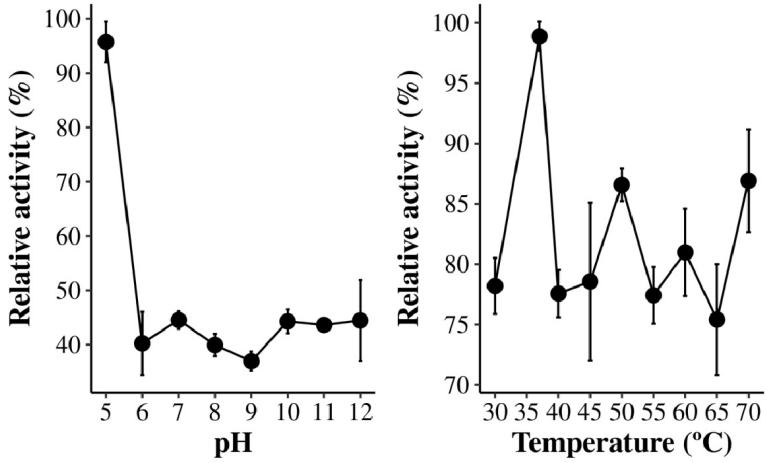

Figure 4 - Effect of temperature and pH variation on proteolytic activity. The test was performed in triplicate and the bars indicate the standard deviation.

were more active at alkaline pH, such as those studied by YAMAMURA et al. (2002), RIFFEL et al. (2003) and EL-REFAI et al. (2005). The effect of temperature on enzyme activity was evaluated in a range between 37 and 80 °C, (Figure 4). In these conditions, the enzymes of *Aspergillus* sp. demonstrated optimal activity at 37 °C, followed by a decrease at higher temperatures. In general, within the genus *Aspergillus,* the proteases produced have an optimum activity between 30 and 45 ºC (SOUZA et al., 2015). MAGALHÃES et al. (2019) report that *Lentinus crinitus* enzymes demonstrated optimal activity at 50 °C. Temperature is a relevant parameter for biocatalysis, as it is a critical variable that can cause a decrease in enzyme activity by inactivating the enzyme (ILLANES et al., 2000); hence the importance of its study in enzymatic processes.

The presence of salts in the reaction medium can influence proteolytic activity and, therefore, the effects of various salts in different concentrations were tested. According to table 2, the salts, $ZnSO_4$, NaCl, $MgCl_2$ and $SrCl_2$, caused an inhibition in the proteolytic activity of the concentrated enzyme , regardless of their concentrations, which indicated that the ions may have interacted with the active site of the enzymes and thus reduced their catalytic activity. The presence of Cu^{2+}, Fe^{2+}, and Zn^{2+} is often a negative factor for protease activity (MOALLAEI et al., 2006). In particular, excess Zn^{2+} may be inhibitory to some metalloproteases due to the formation of bridges between zinc monohydroxide ($ZnOH^{+}$) and catalytic zinc ions at the active site (RIFFEL et al., 2007). It was observed that manganese sulfate exerted a differential stimulation, and increased the enzymatic activity by approximately 55 %. Similar results were reported by MAGALHÃES et al. (2019), who reported that the proteolytic activity of *Lentinus crinitus* increased by 29.43 % with 10mM of $MnSO_4$. The stimulating effect of Mn^{2+} has also been described for *B. subtilis* keratinase S14 (MACEDO et al., 2008). According to HARER et al. (2018) metal ions such as Ca^{2+}, Mg^{2+} and Mn^{2+} increase and stabilize the enzymatic activity. Metal ions, such as Ca^{2+}, Co^{2+}, K^{+}, Na^{2+}, Cu^{+}, Fe^{2+}, Mn^{2+} and Zn^{2+}, have been shown to increase or not affect the protease activity of an *Aspergillus* sp. strain (FERREIRA et al., 2017). NAZMI et al. (2006) asserted that ions can be involved in catalytic processes, and participated in redox reactions or electron transfer. The effect of different metal ions on microbial keratinases is generally highly variable, and depends on both their nature and their concentration (WERLANG & BRANDELLI, 2005). In this perspective, the addition of specific salts to the reaction medium, mainly cations, can help in the stabilization of microbial protease through connections to specific sites in the enzyme structure (SILVEIRA et al., 2010), and thus contributed to enzymatic catalysis in bioprocesses.

Non-ionic detergents, such as Triton X-100 and Tween-20, are mild surfactants and generally do not affect protein activity (LINKE, 2009). In this research, Tween 20 (0.5 % and 1% v/v) and Triton

Table 2 - Effect of various salts on protease activity.

Salts	----------Concentration (mM)----------	----------Relative activity (%) ± SD----------
Control	-	100 ± 0.4
$CuSO_4$	1	150.80 ± 3.4*
	5	132.05 ± 3.3*
$MgSO_4$	1	151.23 ± 3.3*
	5	148.07 ± 3.5*
KCl	1	139.15 ± 2.6*
	5	150.64 ± 6.2*
$MnSO_4$	1	153.31 ± 3.8*
	5	155.55 ± 6.3*
$CaCl_2$	1	144.87 ± 7.8*
	5	141.93 ± 1.7*
$ZnSO_4$	1	49.78 ± 3.6*
	5	56.09 ± 1.9*
NaCl	1	54.01 ± 0.6*
	5	57.10 ± 2.6*
$MgCl_2$	1	57.37 ± 1.6*
	5	58.54 ± 1.7*
$SrCl_2$	1	56.51 ± 0.7*
	5	54.00 ± 5.2*

Assays were performed in triplicate and measurement data are expressed as the mean ± standard deviation (SD). * significant difference at $P < 0.05$.

X-100 (0.5 and 1% v/v) tended to stimulate enzymatic activity. FERREIRA et al. (2017) demonstrated that certain detergents at a final concentration of 2% had a positive effect on the activity of *Aspergillus* sp. CPU 1276. Similar results to our study were found with keratinolytic protease from *Aspergilllus parasiticus* which in the presence of 0.5% SDS and CTAB had an inhibitory effect on proteolytic activity (ANITHA & PALANIVELU, 2013). SDS is a strong anionic surfactant that can have inhibitory effects on several proteases (FAKHFAKH-ZOUARI et al., 2010). In our study, SDS (0.5% and 1% v/v) had a negative effect on catalysis (Table 3). At a concentration of 0.1% (m/v), the SDS did not affect the *Bacillus licheniformis* KBDL4 protease (PATHAK & DESHMUKH, 2012). BACH et al. (2011) reported that this detergent increased the activity of *Aeromonas hydrophila* K12 crude protease. The increase in solubility with hydrophobic substrates and the elimination of microbial contamination are some of the advantages of using enzymes in an organic solvent system. However, the enzyme's catalytic activity can be impaired or even inactivated. Therefore, we evaluated the enzyme activity in several organic solvents (Table 3). The proteases of *Aspergillus* sp. of this study maintained their activities in the presence of solvents, varying little in relation to the control. The stability of organic solvents is generally attributed to the disulfide bonds located on the surface of the molecule (DOUKYU & OGINO, 2010). ZANPHORLIN et al. (2011) reported that the protease of the fungus *Myceliophthora* sp. lost enzymatic activity with the addition of acetone and butanol.

The effect of chemicals on proteolytic activity was examined and is listed in table 3. The results demonstrated that the proteases were resistant to the action of β-mercaptoethanol, suggesting that it is not a cysteine protease. β-mercaptoethanol, which is a strong irreversible reducing agent that reduces the disulfide bonds of the enzyme (SABOTIČ & KOS, 2012). EDTA inhibited the enzyme, indicating that the protease is a serine protease depending on metal ions for optimal activity and/or stability. MAGALHÃES et al. (2019) reported that the relative proteolytic activity of *Lentinus crinitus* enzymes was significantly reduced in the presence of EDTA, indicating that the crude extract of the fungus contains metalloproteases. Inhibition of the enzyme keratinase by chelating agents may provide a method for temporarily inactivating them during their storage proteins, reducing the autolysis associated with proteolytic enzymes. In this case, the metalloenzymatic nature of some keratinases represents a method to immobilization, which has

Table 3 - Effect of various chemical reagents on proteolytic activity.

Reagents	----------Concentration (%)----------	----------Relative activity (%) ± SD----------
Control	-	100 ± 0.7
CTAB	0.5 (w/v)	90.70 ± 1.7*
	1 (w/v)	105.93 ± 1.8
Tween 20	0.5 (v/v)	125.53 ± 5.5*
	1 (v/v)	120.97 ± 3.5*
Triton X-100	0.5 (v/v)	122.90 ± 3.9*
	1 (v/v)	131.11 ± 0.9*
SDS	0.5 (w/v)	62.88 ± 2.5*
	1 (w/v)	92.62 ± 2.3
PEG	0.5 (v/v)	64.38 ± 3.5*
	1 (v/v)	42.56 ± 12.7*
Acetone	0.5 (v/v)	119.73 ± 28.07
	1 (v/v)	101.77 ± 2.6
Butanol	0.5 (v/v)	109.98 ± 1.2
	1 (v/v)	97.24 ± 4.9
Methanol	0.5 (v/v)	112.73 ± 1.9
	1 (v/v)	105.90 ± 2.6*
DMSO	0.5 (v/v)	97.41 ± 2.9
	1 (v/v)	101.54 ± 2.2
Acetonitrile	0.5 (v/v)	107.63 ± 1.6
	1 (v/v)	104.93 ± 0.4
Isopropanol	0.5 (v/v)	98.27 ± 0.9
	1 (v/v)	96.15 ± 1.5
β - mercaptoethanol	1mM	156.59 ± 11.6*
	5mM	163.00 ± 10.1*
EDTA	1mM	88.84 ± 2.1
	5mM	78.05 1.3*

Assays were performed in triplicate and measurement data are expressed as the mean ± standard deviation (SD). *significant difference at $P < 0.05$. CTAB – cetyltrimethylammonium bromide, SDS – Sodium dodecyl sulfate, PEG – Polyethylene glycol, DMSO – Dimethyl sulfoxide.

been identified as being able to increase the stability due to reduced enzymatic autolysis (ALLPRESS et al. 2002). MARTIM et al. (2017), analyzing the effect of inhibitors on the proteolytic activity of *Pleurotus albidus*, observed the presence of serine and cysteine proteases in the crude extract of the fungus. The reduction of protease activity by reducing agents indicated that disulfide bonds are important to maintain the active conformation of these enzymes.

CONCLUSION

This study was important for the characterization of the fermentation process of the fungus *Aspergillus* sp. for an potential use in bioprocesses of modification and hydrolysis of protein substrates. Results of feather degradation by *Aspergillus* sp. suggested future application in agro-industrial residues with a low production cost. The results obtained showed that *Aspergillus* sp. is efficient in the cleavage of keratinous residues, grows in simple culture with feathers as its only source of energy and reduced cultivation time, and obtains interesting enzymatic activity under these conditions. These results suggested that proteases obtained represent high value products aggregate obtained through feather bioprocessing. The characteristics of the crude protease and its ability to hydrolyze feathers suggested its potential use in modification bioprocesses and also in the hydrolysis of protein substrates, indicating promising perspectives for future research. As such, this study presents a strategy for processing agro-industrial waste, which adds value to these low-cost raw materials, and thus, contributes to the maintenance of environmental quality. Future studies should be performed so that

the production of this enzyme is optimized and may be used industrially.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

AUTHORS' CONTRIBUTIONS

All authors contributed equally for the conception and writing of the manuscript. All authors critically revised the manuscript and approved of the final version.

SUPPLEMENTARY DATA

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

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