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UTILIZAÇÃO DE RESÍDUOS DA AVICULTURA NA PRODUÇÃO DE PROTEASES POR FUNGO FILAMENTOSO

BOA VISTA, RR
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Dissertação apresentada ao Programa de Pós-graduação em Recursos Naturais-PRONAT, da Universidade Federal de Roraima, como parte dos requisitos para obtenção do título de Mestre em Ciências Ambientais (Recursos Naturais). Área de concentração: Bioprospecção

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DEDICATÓRIA

À minha mãe, que com amor e muito
carinho me instruiu a alcançar
grandes conquistas e acreditar
em minha capacidade.

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"*Entrega o teu caminho ao Senhor,*
confia Nele, e tudo Ele fará"
Salmos 37:5

RESUMO

A avicultura é um dos setores de grande impacto na economia brasileira. Nos últimos anos, tem sido observado um aumento na produção de frangos de corte, fazendo com que este setor da indústria seja responsável pela geração de toneladas de penas, que possuem potencial poluente quando descartadas de forma inadequada no meio ambiente. O descarte das penas pode se tornar um problema econômico para as empresas, que devem investir na sua correta destinação para evitar possíveis impactos ambientais. Diante disto, surge a necessidade por alternativas sustentáveis para a reciclagem desses resíduos ricos em proteína. O objetivo deste estudo foi investigar a produção de proteases secretadas por fungo filamentoso, isolado de solo de Floresta Amazônica em Roraima, utilizando penas de frango como única fonte de energia, caracterizar a cepa quanto à temperatura e pH ótimos de produção, bem como os efeitos de produtos químicos, além de avaliar o efeito de diferentes substratos de crescimento sobre a atividade proteolítica. Inicialmente, foi realizado um *screening* de 40 linhagens de fungos filamentosos obtidos de amostras de solo do Parna Viruá (preservados na Coleção de Culturas do Laboratório de Microbiologia-PRONAT), a fim de selecionar um isolado com potencial para a produção de protease extracelular. A identificação do fungo selecionado, foi realizada por meio de observações macroscópicas e microscópicas inoculando o isolado em meios de cultura específicos, o qual foi identificado como *Aspergillus* sp. O fungo foi inoculado em meio ágar leite e ágar farinha de penas para verificar a produção qualitativa de proteases e queratinases, respectivamente. A formação de halo indicou positividade para ambas as produções enzimáticas. Quanto a avaliação quantitativa das atividades proteolítica e queratinolítica, obtidas através da espectrofotometria a 420 nm, os resultados indicaram a habilidade de *Aspergillus* sp. em produzir tanto proteases quanto queratinases. A capacidade de secretar proteases foi avaliada em diferentes substratos de crescimento (pena inteira, farinha de penas, peptona, caseína, bico de frango, cabelo e gelatina). A maior produção de enzimas ocorreu em meios de cultivo com peptona e farinha de penas, indicando que *Aspergillus* sp. é um microrganismo queratinolítico, capaz de degradar materiais queratinosos com eficiência. A protease apresentou atividade ótima em pH 5,0 e temperatura moderada de 37 °C. A atividade enzimática foi potencializada com a adição de $CaCl_2$, $MnSO_4$, KCl, $MgSO_4$ e $CuSO_4$. Os detergentes Tween 20 e Triton x-100 tenderam a estimular a atividade. As enzimas foram resistentes aos solventes orgânicos (metanol, acetona, butanol, acetonitrila, isoprapanol e DMSO), mantendo a atividade enzimática próxima ao controle (100 %). Os inibidores β-mercaptoetanol e o ácido etilenodiaminotetracécio (EDTA) não inibiram a atividade proteolítica em ensaios enzimáticos, sugerindo que as enzimas presentes no hidrolisado enzimático não são metaloproteases nem cisteína proteases. Na perspectiva da microbiologia industrial, os resultados desta pesquisa sugerem, que o extrato da protease bruta pode ser potencialmente utilizado na bioconversão de resíduos queratinosos, considerados de difícil degradação no meio ambiente, e a possibilidade de aplicação do hidrolisado proteico na suplementação de ração animal e no uso como biofertilizantes.

Palavras-chave: Biotecnologia. Avicultura. Resíduos. *Aspergillus* sp. Enzimas microbianas

ABSTRACT

Poultry farming is one of the sectors of great impact on the Brazilian economy. In recent years, there has been an increase in the production of broiler chicken, making this sector of the industry responsible for the generation of tons of feathers, which have polluting potential when improperly disposed of in the environment. The disposal of feathers can become an economic problem for companies, which must invest in their correct destination to avoid possible environmental impacts. Given this, there is a need for sustainable alternatives for recycling these protein rich waste. The objective of this study was to investigate the production of proteases secreted by filamentous fungus, isolated from the soil of the Amazon Forest in Roraima, using chicken feathers as the only source of energy, to characterize the strain as to the optimum temperature and pH of production, as well as the effects chemical products, in addition to evaluating the effect of different growth substrates on proteolytic activity. Initially, a screening of 40 strains of filamentous fungi obtained from soil samples from Parna Viruá (preserved in the Culture Collection of the Microbiology Laboratory-PRONAT) was carried out, in order to select an isolate with the potential for the production of extracellular protease. The identification of the selected fungus was carried out through macroscopic and microscopic observations, inoculating the isolate in specific culture media, which was identified as *Aspergillus* sp. The fungus was inoculated on milk agar and feather meal agar to verify the qualitative production of proteases and keratinases, respectively. The formation of halo indicated positivity for both enzyme productions. As for the quantitative assessment of proteolytic and keratinolytic activities, obtained through spectrophotometry at 420 nm, the results indicated the ability of *Aspergillus* sp. in producing both proteases and keratinases. The ability to secrete proteases was evaluated on different growth substrates (whole feather, feather meal, peptone, casein, chicken beak, hair and gelatin). The highest production of enzymes occurred in culture media with peptone and feather meal, indicating that *Aspergillus* sp. is a keratinolytic microorganism, capable of efficiently degrading keratinous materials. The protease showed optimal activity at pH 5.0 and temperature of 37 ° C. The enzymatic activity was enhanced with the addition of CaCl2, MnSO4, KCl, MgSO4 and CuSO4. The detergents Tween 20 and Triton x-100 tended to stimulate activity. The enzymes were resistant to organic solvents (methanol, acetone, butanol, acetonitrile, isoprapanol and DMSO), keeping the enzymatic activity close to the control (100%). The β-mercaptoethanol inhibitors and ethylenediaminetetraacetium acid (EDTA) did not inhibit proteolytic activity in enzymatic assays, suggesting that the enzymes present in the enzyme hydrolyzate are neither metalloproteases nor cysteine proteases. From the perspective of industrial microbiology, the results of this research suggest that the crude protease extract can potentially be used in the bioconversion of keratinous residues, considered difficult to break down in the environment, and the possibility of applying protein hydrolyzate in animal feed supplementation in use as biofertilizers.

Keywords: Biotechnology. Poultry farming. Waste. *Aspergillus* sp. Microbial enzymes

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1 INTRODUÇÃO

O crescimento da demanda global de carne de frango está diretamente relacionado à mudança de hábitos e preferências alimentares dos consumidores, e, principalmente, ao crescimento populacional, que tem gerado a necessidade de aumentar a produção de vegetais e carnes. Como consequência dessa demanda, tem-se a geração de resíduos derivados da indústria de carne, por exemplo, penas de frango.

A produção mundial de carne de frango, alcançou aproximadamente 24 bilhões de toneladas em 2018, de acordo com a Food and Agriculture Organization (FAO), sendo o Brasil o segundo maior produtor de frangos e o principal país exportador (FAO, 2019; IBGE, 2020). Segundo a Associação Brasileira de Proteína Animal (ABPA), foram produzidos 13,2 milhões de toneladas de carne de frango no Brasil no ano de 2019. Desse total, a região Norte foi responsável por 1,7 % do abate de frangos no 3º trimestre de 2020 (ABPA, 2020; IBGE, 2020). Em Roraima houve um aumento na produção de frango a partir de 2019 devido a instauração de novos estabelecimentos de processamento de aves, de modo que em 2020 foram abatidos 15.070 frangos (RORAIMA, 2020). Em virtude da crescente produção de frango de corte, o descarte de penas vem aumentando progressivamente e, aliado a destinação ecologicamente inadequada, propiciam o aumento da poluição ambiental e o desenvolvimento de vários tipos de patógenos, gerando problemas ambientais e de saúde pública, além do desperdício de uma fonte potencial de proteínas. Diante deste cenário, faz-se necessário investigar alternativas para o desenvolvimento de processos sustentáveis que permitam o aproveitamento e a reciclagem desses resíduos.

As penas geradas a partir do processamento de aves, que correspondem aproximadamente de 5 a 10 % do peso total de frangos adultos, têm usualmente como destino a incineração ou a utilização na alimentação de outros animais na forma de farinha de penas (DAROIT; BRANDELLI, 2014). Contudo, estes processos requerem alto aporte energético, e no caso da farinha de penas, gera-se um produto de baixa digestibilidade. Desta forma, são necessárias a adoção de estratégias adequadas para o reaproveitamento das penas, com a possibilidade de geração de renda, e proteção ambiental.

A tecnologia enzimática tem se tornado um dos campos mais promissores da biotecnologia. Essa área visa a utilização de processos e organismos vivos no desenvolvimento e melhoramento de técnicas e produtos, não apenas para a produção de compostos de alto valor agregado por meio de processos produtivos industriais, como

também por requerer menos recursos renováveis, possibilitando o uso desses recursos de forma mais eficientes e, por conseguinte, reduzir o impacto ambiental.

Neste sentido, microrganismos queratinolíticos e queratinases vêm sendo explorados quanto à sua aplicação na bioconversão de materiais queratinosos através de abordagens biotecnológicas. No caso das penas, o uso do potencial microbiano pode resultar tanto no manejo dos resíduos quanto na agregação de valor comercial, representando uma estratégia eficiente, de baixo custo e ecologicamente segura. Dessa forma, as penas passam a ser consideradas matérias-primas e não mais como um resíduo. Além do manejo e da produção de hidrolisados, a bioconversão pode resultar em outros produtos de interesse biotecnológico, como enzimas proteolíticas e biomassa microbiana.

Nesta perspectiva, os microrganismos são considerados como uma das principais e preferencial fonte de enzimas industriais, sendo utilizados em uma ampla e vasta variedade de processos biotecnológicos na produção de enzimas com potencial para aplicações tecnológicas e industriais (FLORENCIO; BADINO; FARINAS, 2017; MONTEIRO; SILVA, 2009). A obtenção de enzimas microbianas tem despertado o interesse das indústrias devido às dificuldades operacionais e principalmente econômicas dos processos de extração de enzimas de origem vegetal e animal, bem como devido à facilidade no controle dos processos de produção de enzimas microbianas, o que indica vantagens adicionais na produção em larga escala com capacidade para atender as necessidades do mercado. Vale ressaltar que, a partir dos processos metabólicos dos microrganismos pode-se obter uma maior diversidade de enzimas com diversas aplicações industriais.

Neste contexto, o objetivo geral deste estudo foi caracterizar enzimas proteolíticas produzidas por fungo filamentoso, isolado de solo de Floresta Amazônica em Roraima, utilizando resíduos da avicultura, visando a potencial utilização em processos biotecnológicos, na tentativa de agregar a esses resíduos valor comercial, e contribuir para a redução do impacto ambiental. Para alcançar o objetivo geral, foram propostos os seguintes objetivos específicos: I) selecionar isolados de fungos potencialmente produtores de proteases; II) investigar a produção de proteases e queratinases por fungo filamentoso, utilizando resíduos da avicultura como única fonte de energia para o microrganismo; III) avaliar a produção de proteases em diferentes substratos de crescimento; IV) caracterizar a atividade proteolítica quanto à temperatura, pH e efeitos de produtos químicos.

A metodologia desse estudo baseou-se inicialmente na coleta das penas, fornecidas por uma empresa local de processamento de aves, para o preparo dos cultivos submersos, seguido do *screening* de 40 isolados de fungos filamentosos obtidos de amostras de solo do Parque Nacional do Viruá-RR, pertencentes à Coleção de Culturas do Laboratório de Microbiologia-PRONAT, com o intuito de selecionar o melhor microrganismo capaz de secretar proteases com um rendimento desejável, e em um menor tempo de cultivo. A partir dessa etapa preliminar, foram realizados ensaios qualitativo (em meio sólido) e quantitativo (em cultivos submersos) com a finalidade de investigar o potencial proteolítico e queratinolítico do fungo. Ademais, a inoculação do isolado em cultivos contendo diferentes substratos de crescimento foi avaliado, a fim de verificar a produção enzimática em substratos queratinosos e estabelecer comparações com outras fontes de carbono e nitrogênio. As proteases foram caracterizadas quanto à temperatura e pH, sendo estes parâmetros importantes de controle em processos de otimização de produção enzimática, bem como os efeitos de produtos químicos, incluindo sais, detergentes, solventes e inibidores foram determinados, pois esses fatores influenciam diretamente na atividade enzimática. Alguns ensaios complementares foram realizados, como: a avaliação da degradação dos substratos queratinosos utilizados nesse estudo (pena inteira e farinha de penas), além da determinação da melhor concentração de substrato. Os ensaios foram conduzidos no Laboratório de Microbiologia do PRONAT.

Este estudo será apresentado de forma compacta, conforme previsto na Resolução nº 008/2017-CEPE da Universidade Federal de Roraima (UFRR, 2017). Nesta seção Introdução foi apresentado a contextualização deste estudo. Na segunda seção, os resultados obtidos são apresentados no formato de um manuscrito intitulado Bioconversion of poultry residues for the production of proteases by *Aspergillus* sp. isolated from Amazon Forest soil submetido à revista "*Waste Management*", na área de ciências ambientais, com Qualis A1, ISSN 0956-053x e fator de impacto 5,4. A redação do artigo segue as normas de publicação da revista, constantes no subitem 2.1. Na terceira seção são apresentadas as conclusões finais referentes ao desenvolvimento da pesquisa, além das referências citadas na introdução.

Os dados apresentados neste trabalho, configuram um conjunto inédito de informações sobre a bioconversão de resíduos da avicultura na produção de proteases por fungo filamentoso no estado de Roraima. No Brasil, pesquisas direcionadas para a produção de enzimas utilizando resíduos da indústria avícola, estão concentradas na

região sul do país. Ademais, os trabalhos são, em sua maioria, voltados para a produção de proteases utilizando bactérias como produtoras de enzimas extracelulares.

2 ARTIGO

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

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Abstract

Feathers are by-products rich in recalcitrant proteins called keratins, generated in wide quantities by the poultry industry. There is a growing demand for economical and ecologically appropriate methods for handling this waste. Microbial bioconversion has been investigated as a promising strategy for the recycling 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 as raw materials for obtaining proteases. Within this context, the objective of this work was to investigate and characterize the production of extracellular proteases by *Aspergillus* sp., isolated from soil in the Amazon Forest. The enzymatic production was evaluated against several growth substrates (whole feather, feather meal, human hair, casein, gelatin, peptone and chicken beak). The highest enzymatic production occurred with feather meal (FM) and peptone, suggesting the efficiency of this strain in degrading keratinous substrates. The protease showed optimal activity at pH 5.0 and 37 °C. The enzymatic activity was enhanced with the addition of CaCl2, MnSO4, KCl, MgSO4 and CuSO4. The detergents Tween 20 and Triton x-100 tended to stimulate activity. The enzymes were resistant to organic solvents (methanol, acetone, butanol, acetonitrile, isoprapanol and DMSO), keeping the enzymatic activity next to the control (100%). β–mercaptoethanol and ethylenediaminetetraacetium acid (EDTA) increased activity proteolytic in enzymatic assays, suggesting that the enzymes present are neither metalloproteases nor cysteine proteases. This study suggests that the proteases produced by *Aspergillus* sp. can be used in the bioconversion of recalcitrant residues through an environmentally friendly solution and an energy saving process, producing commercially valuable with potential for future use in industry.

Keywords: Agro-industrial by-products, *Aspergillus* sp., feather residue, proteases

1. Introduction

The consumption of processed meats generates enormous amounts of organic waste and by-products. Viscera, bones, blood, skins, and meat trimmings are the main waste products that need to be managed (Lemes et al., 2016). As a source of healthy protein, and because it is more economically viable, chicken meat is increasingly consumed. According to the Food and Agriculture Organization (FAO), around 24 billion chickens were produced worldwide in 2018. Assuming that a chicken weighs about 2 kg and that the average percentage of feathers is approximately 5 %, the total amount of chicken feathers produced in 2018 can be estimated at 2.4 million tons (FAO, 2019). Most of the feathers produced by the poultry industry end up in dumps, landfills and incinerators. These used methods can cause contamination of the environment generating greenhouse gases (Acda, 2010). Feathers are considered a wide 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).

Feather residues can be treated physically, chemically and/or biologically. Each treatment has pros and cons when it comes to feather degradation, as well as protein and amino acid recovery. During physical treatment the feathers are degraded at high temperature and/or pressure, which causes the denaturation of some amino acids and requires a large amount of energy. In chemical treatment, there may also be a loss of essential amino acids, since strong acids and alkali are used (Cheong et al., 2018). Biological treatment involves keratinolytic microorganisms or keratinases and is being considered with some degree of success, as it breaks down the rigid bonds in feather (Cheong et al., 2018; Onifade et al., 1998). Keratinolytic enzymes are very active in the keratin substrate that is available, and act on peptide bonds, converting them into more

simplified forms (Gopinath et al., 2015). Bacteria and fungi that produce keratinolytic enzymes are 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, in addition to offering the advantage of separating mycelium by simple filtration (Jisha et al., 2013). With this, it is important to identify new keratinolytic microorganisms, since there is production of keratinases that can be used in industries and also in the production of keratin hydrolysates (Ghaffar et al., 2018; Fontoura et al., 2019).

Therefore, the use of the keratinolytic potential of microorganisms emerges as an economically and environmentally appropriate approach to the recycling of feathers, aiming at obtaining protein hydrolysates and adding value to these underutilized materials (Lasekan et al., 2013). In addition to ecologically correct handling and production of protein hydrolysates, bioconversion can result in other products of biotechnological interest, such as proteolytic enzymes and microbial biomass (Pleissner and Venus, 2016). In this context, the objective of this work was to evaluate the production of proteolytic enzymes, as well as to know the influence of different sources of carbon and nitrogen and also to characterize these enzymes produced by *Aspergillus* sp. aiming at its potential utility in biotechnological processes.

2. Material and methods

2.1 Microorganism and culture media

The fungus was isolated from soil samples from the Virua National Park, Roraima, extreme north of the Amazon, Brazil, from the collection of the microbiology laboratory of the Federal University of Roraima was 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. The fungal spore suspensions with a final concentration of 10^5 spores/mL (Alves and Pereira, 1998) were used, and incubation was performed at 27 °C for up to 10 days with shaking at 120 rpm.

2.2 Qualitative evaluation of protease production

Protease production was qualitatively detected by inoculating *Aspergillus* sp. on skim milk agar (SMA) plates (Riffel and 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, indicating the production of proteolytic enzymes. The ability of microorganism growing up in FMA, was prepared as described by Riffel and 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.

2.3 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 and Hocking, 2009).

2.4 Azokeratin synthesis

Azokeratin produced in the laboratory was prepared according to the methodology described by Tomarelli et al., (1949). The feathers were ground (15 g) and added in 680 mL of distilled water, 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 (0.12 M) NaOH, adding it to the feather meal mixture. Sequentially, the initial mixture was added 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, being stirred for another 5 min and then dialyzed against distilled water at 4 °C. After dialysis, the solution was submitted to lyophilization.

2.5 Preparation of chicken feather waste and inoculum

The feathers were supplied from a local chicken processing industry. To remove impurities, the feathers were washed with water at 50 °C, and then taken to the circulating air oven, at 60 °C for 48 h, for drying according to the methodology of Tesfaye et al. (2017), with modifications. After this time, grinding was carried out in a Willey knife mill to produce feather meal. The microorganism was grown in Erlenmeyer tubes, with a capacity of 250 mL, 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, in different concentrations of chicken feather meal (0.5, 1.0, 3.0 and 5 % w/v), to determine the best proteolytic activity.

2.6 Enzyme activity assays

Keratinolytic and proteolytic activities were determined using azokeratin (synthesized in laboratory) 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; 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 were 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 at the above assay conditions.

2.7 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, a fermentation was carried out containing 100 mL of culture medium as described in item 2.1 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 added slowly to the filtrate. After precipitation, the samples were resuspended 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 $MgSO_4$) centrifuged (10.000 x g for 5 min) and the absorbance at 420 nm was measured. From these samples, the proteolytic activity was determined according to item 2.6. The best saturation range was used in the following steps.

2.8 Screening of growth substrates for production of keratinolytic proteases

Casein, gelatin and peptone (Synth, Brazil), feather meal, whole feathers (slaughterhouse in Boa Vista, Roraima, Brazil), human hair and chicken beak were selected as growth substrates (0.5 g/L) to produce keratinolytic proteases in buffer (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 1 mL of a spore suspension of *Aspergillus sp*. (10^5 spores/mL) and incubated at 27 °C on a rotary shaker (120 rpm) for 48 h.

2.9 Evaluation of the percentage of degradation of the feathers

To determine the percentage of degradation, the methodology was followed described by Suntornsuk and Suntornsuk (2003). At the end of fermentation, the supernatant was filtered on filter paper, oven dried at 105 ^oC overnight and weighed. The percentage of feather degradation was calculated through the difference in weight residual dry between a control (medium with feathers without inoculum) and the treated sample.

2.10 Determination of soluble protein

Culture broth filtrates were centrifuged (10.000 x g for 10 min) and supernatants were utilized for determining the soluble protein concentration by the method of Bradford (1976). This method is based on the colorimetric reaction between the aromatic groups of the protein and the Coomassie blue dye in an acid medium. Bovine serum albumin (BSA) was used as a standard.

2.11 Effects of pH and temperature on enzyme activity

For pH optimum determination, proteolytic activity was assayed at 37 °C in a pH range 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 described in item 2.6. The results were expressed in relative activity, with the value of the activities proteolytics (pH 5.0) defined with 100 %. The effect of temperature on enzymatic activity was assessed in temperatures between 37 and 80 °C. The results were expressed in relative activity, being the value of the activities carried out at 37 ºC considered 100 % (Corrêa et al., 2010).

2.12 Effect of chemicals on enzyme activity

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, detergents (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 (described in item 2.6). The results were expressed in relative activity, with the control (100 %) without adding chemicals. The effect of inhibitors on enzymatic activity was evaluated using the following compounds: EDTA and β-Mercaptoethanol. The enzyme was incubated for 10 minutes at room temperature (30 °C) with the inhibitors in a concentration of 1 and 5 mM. Subsequently, the enzymatic activity was verified according to the test conditions described in item 2.6. The results were expressed in relative activity, with the control (100 %) without the addition of inhibitors (Corrêa et al., 2010).

2.13 Statistical analysis

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

3. Results and discussion

3.1 Qualitative evaluation of protease production

The results of the qualitative evaluation in solid medium, skimmed milk agar (SMA) and feather meal agar (FMA), showed 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 (complementary figure), indicating that the fungus is efficient in the production of these enzymes. In the SMA medium, a translucent halo was formed around the colonies, while in the FMA medium, the halo formed allowed the observation of degraded feather meal around the colonies. The preliminary data of the qualitative evaluation were decisive for the follow-up of this study, due to the capacity of *Aspergillus* sp. in the production of proteolytic enzymes.

3.2 Preliminary fungal identification

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 color showed white tones. One of the main characteristics that differentiates *Aspergillus* species is the color of the colonies, which

can present 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 together or separately allow a clear difference from the main genus sections (Klich, 2002). Traditional identification, based on the morphological caracteristics of the fungus, showed that the isolate belongs to the genus *Aspergillus*, especially due to the presence of spores (conidia) in chains from phialides which were supported by well-defined vesicles on the stipe end (Pitt and Hocking, 2009). This genus is considered cosmopolitan and widely distributed in nature, the isolation of species in soils and fallen plants is very common, the genus has a greater abundance in regions of tropical and subtropical climates. (Klich, 2002, Pitt and Hocking, 1997). These data corroborate our results, where the fungus under study was isolated from the soil in the Virua National Park, located in northern Amazonia. 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 growing environment. Several of these enzymes produced in large-scale submerged fermentation have been widely used in industry over the decades (Wu et al., 2006). Thus, the enzyme produced in this study meets the previous study, showing the production of *Aspergillus* proteases from the use of an agricultural residue as a substrate. Future studies must be done so that the production of this enzyme is optimized and used industrially.

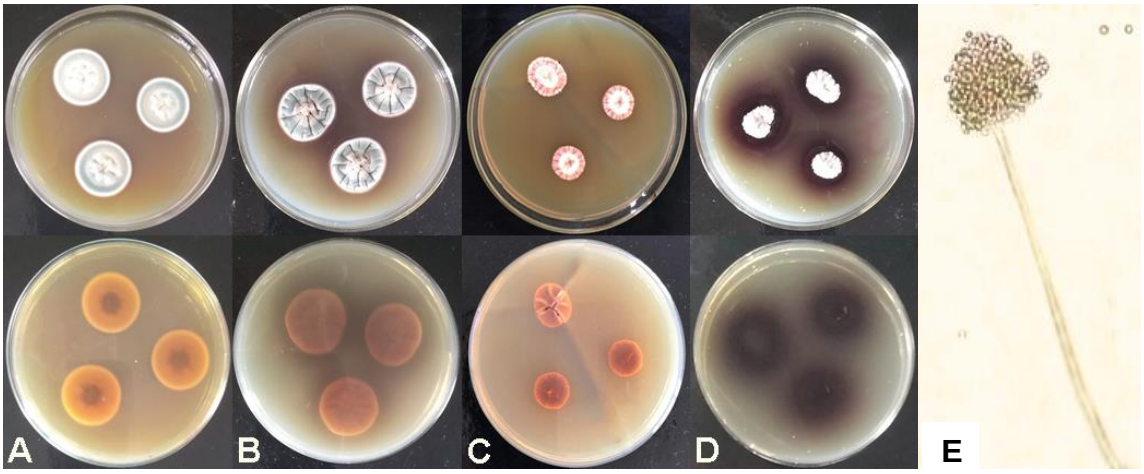

Figure 1. Colony morphology of *Aspergillus* sp. in CYA and MEA 25 ˚C and 37 ˚C after 7 days. (A) up and reverse site in MEA 25 ˚C; (B) up and reverse site in CYA 25 ˚C; (C) up and reverse site in MEA 37 ˚C; (D) up and reverse site in CYA 37 ˚C; (E) and microscopic aspects of reproductive structures.

3.3 Preparation of chicken feather waste and inoculum

According to Daroit and 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 greater amount of inoculum (5 %) (supplementary material), while in the culture of lower concentration (0.5 %) less activity was obtained. Previous studies claim that high concentrations of FM result in cell shear, in addition to reducing the transfer of oxygen to microbial growth in the medium (Fakhfakh, 2011; Daroit and Brandelli, 2014). On the other hand, low concentrations of substrate can lead to underutilization of microbial potential and less difficulty in controlling physical and chemical variables such as pH, temperature and oxygen. As this work was carried out on a laboratory scale, we opted for the use of FM (0.5 %) for the production of proteolytic enzymes.

3.4 Assay of enzymatic activity and concentration of soluble proteins

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. The protein concentration was verified in 48 hours of cultivation, which showed greater activity, with values of 0.07 mg/mL. Results presented by Ire et al., (2011) and Muthukshmi et al., (2011) showed that the maximum production of proteolytic enzymes by Aspergillus species occurs between 4 to 9 days of incubation. Sivakumar and Raveendran (2015), report that normally the process of degradation of the feathers carried out by fungi, occurs more slowly when compared to bacteria. These are widely exploited by the industry exactly because they degrade mare quickly, generally reaching the maximum peak of activity in the period of 48 h. Reduced enzyme production time is an important factor for industries as it reduces operating costs and less degradation of the enzymes produced (Nyonzima and More, 2013). In a study carried out with 11 species of *Aspergillus* from the Amazon Fungus Collection, the proteolytic activity showed a variation between the values of 8.09 U/mL, in *A. japonicas* to 22.49 U/mL, in *A. oryzae*, 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 efficiently better activity in less time of growth, proving its potential in a biotechnological perspective.

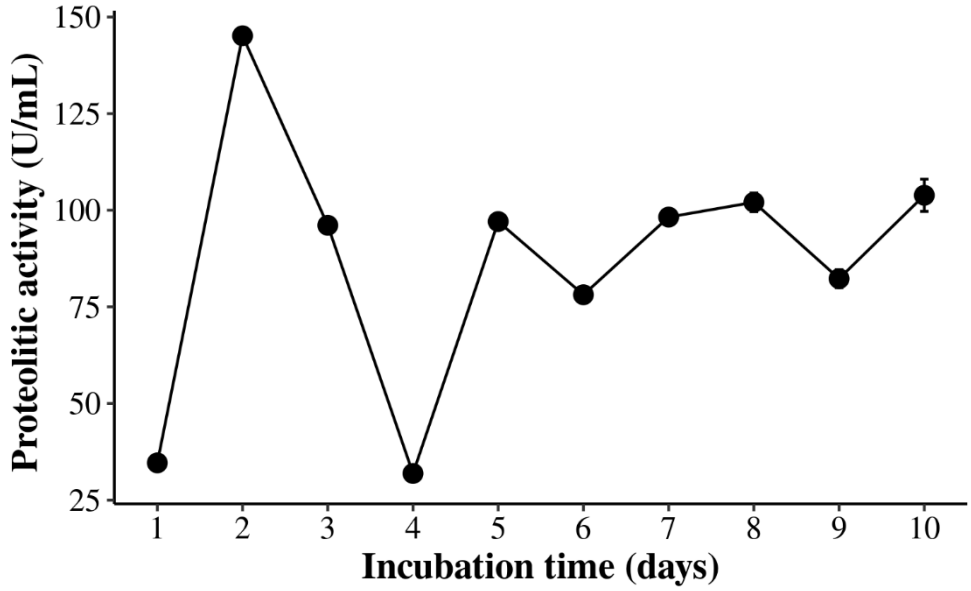

Figure 2. Proteolytic activity of *Aspergillus* sp. Obtained through submerged culture for 10 days of incubation, at 27 °C, at 120 rpm**.** The test was performed in triplicate and the bars indicate the standard deviation.

3.5 Keratinolytic activity

Table 1 shows the keratinolytic activity of the substrates whole feather (WF) and feather meal (FM) in submerged culture medium containing 0.5 % of substrate 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 is the best substrate 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.

Substrate	Proteolitic activity (U/mL) ± SD	Keratinolitic activity (U/mL) ± SD
Whole feather	45.5 ± 0.04	21.76 ± 0.12
Feather meal	145.13 ± 0.7	53.5 ± 0.00

Table 1 Evaluation of enzymatic activity. Assay were performed in triplicate and measurement data were expressed as the Mean ± Standard Deviation (SD).

320

321 *3.6 Concentration of extracellular protease*

322 Often, the first step used to separate proteins from crude extracts is precipitation by adding
323 salts (ammonium sulfate) or water-miscible organic solvents. The separation in this case
324 is based on differences in solubility presented by the proteins (Marzzoco and Torres,
325 1999). In this study ammonium sulfate was used as a precipitating agent in different
326 saturation ranges (0-20, 20-40, 40-60, 60-80 and 80-100 %), in order to determine the
327 range of highest extracellular protease concentration. All ranges were evaluated, since
328 there was no previous knowledge about the isolate *Aspergillus* sp. studied in that work.
329 The result demonstrated that there was a spread of the enzymatic activity within these
330 ranges, and, therefore, the optimal saturation range for the enzyme between 0 and 60 %
331 was considered to follow the studies.

332

333 *3.7 Screening of growth substrates for production of keratinolytic proteases*

334 Different substrates were tested in order to evaluate the production of extracellular
335 protease by *Aspergillus* sp. in submerged growth. The fungus showed to degrade all the
336 substrates analyzed in this study. Cultures on the substrates peptone and feather meal
337 resulted in greater production of extracellular proteases, reaching maximum values for
338 enzymatic activity in 48 h of culture (Fig. 3). *Aspergillus* species are commonly known
339 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 and Dersch, 2010). The synthesis of microbial proteases is often induced by keratin substrates such as feather and mainly in the form of feather meal, as it contains greater accessibility of the enzyme to the substrate and homogeneity, which results in less resistance to hydrolysis (Brandelli and Riffel, 2005; Corrêa et al., 2010). In contrast, the cultivations on the substrates gelatin and human hair showed lower values 47.39 and 47.69 %, respectively. Previous studies claim that there is a greater difficulty in hydrolysis of the hair substrate due to the conformational diversity of the hair keratin in relation to feather keratin (Onifade et al., 1998; Daroit and Brandelli, 2014). Some representatives of the Ascomyctes group have been reported to have a high capacity to degrade a wide variety of keratin substrates including feather, hair and wool, which were considered very difficult to degrade structures (Verma et al., 2017). This result corroborates the efficiency of *Aspergillus* sp. to produce proteases from the natural substrate FM, considered the most suitable because 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. Given the above, the feather meal substrate was considered the most suitable to be used in subsequent studies.

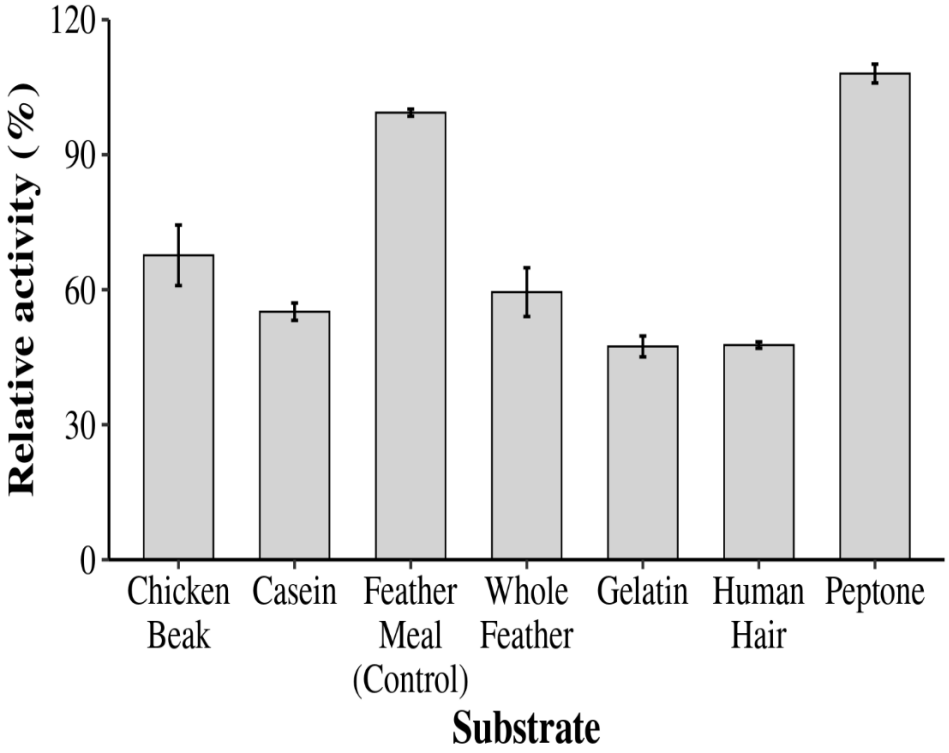

Figure 2. Protease production by *Aspergillus* sp. in different growth substrates: chicken beak, casein, feather meal, whole feather, gelatin, human hair and peptone at a concentration of 0.5 %, after 48 h of incubation in submerged culture, at 27 °C, at 120 rpm. The test was performed in triplicate and the bars indicate the standard deviation.

364

365 *3.8 Evaluation of the degradation of the feathers*

366 Degradation was evaluated in submerged cultures (WF and FM), incubated at 27 °C, for
367 48 h. The highest percentage of degradation was obtained in cultivation with FM (15.82
368 %), while WP degraded 9.24 % (supplementary figure). A study carried out with
369 *Aspergillus* sp. isolated from Caatinga soil, reports that the greatest degradation of growth
370 containing fragments of feathers was observed from the ninth to the twelfth day of
371 incubation (Ferreira et al., 2016). Bohacz (2017), used 5 strains of fungi obtained from
372 soil, to evaluate the percentage of degradation of feather meal. In this study, all isolates
373 were able to degrade the substrate, however, *Chrysosporium articulatum* and
374 *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 %). Although the percentage of degradation has not reached maximum values during the tests, we observed that with the addition of salts, detergents, as well as solvents, the activity was satisfactorily elevated. It should be noted that the primary objective, keratin hydrolysis, was achieved, so the rigid structures that constitute the feather were broken, 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 the better use of these agribusiness by-products.

3.9 Effects of pH and temperature on enzyme activity

Temperature and pH are important control parameters to be investigated in submerged growth, to ensure maximum microbial growth and consequent enzyme production (Sharma et al., 2017). The effect of temperature on enzyme activity was evaluated between 37 and 80 °C, (Fig. 4). In these conditions, the enzymes of *Aspergillus* sp. demonstrated optimal activity at 37 °C, followed by a decrease in higher temperatures. In general, within the genus *Aspergillus*, the proteases produced have an optimum activity temperature of 30 to 45 ºC (Souza et al., 2015). Unlike our work, the fungi *Aspergillus parasiticus* and *Aspergillus niger*, showed maximum proteolytic activity at 50 and 45 ºC, respectively (Devi et al., 2008; Anitha and Palanivelu, 2013). *Aspergillus* sp. it had an optimum temperature at 40 °C (Ferreira, et al., 2017). Magalhães et al. (2019), report that *Lentinus crinitus* enzymes demonstrated optimal activity at 50 °C. Temperature is an important tool in the industry, 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

400 of its study in enzymatic processes. The initial pH of the reaction medium is capable of
401 affecting the solubility of salts, function of the cell membrane, absorption of nutrients.
402 cell morphology, product biosynthesis, and consequently protease production. Even
403 within the same species or even between fungal isolates it is possible to find growth
404 differences at different pHs (Hung and Trappe, 1983; Fang and Zhong, 2002). Therefore,
405 pH control is of great importance in the production of protein hydrolysates from microbial
406 protease (Surówka et al., 2004). The effect of pH (5.0 and 12.0) on crude keratinase
407 produced by *Aspergillus* sp. was investigated. The maximum activity was observed at pH
408 5.0, with a substantial loss of activity at a higher pH (Figure 4). As in our study,
409 proteolytic enzymes from *Lentinus crinitus* showed good stability at acid pH (pH 5.0 and
410 6.0) (Magalhães et al., 2019). Other enzymes, however, were more active in alkaline pH,
411 such as those studied by Yamamura et al. (2002), Riffel et al. (2003) and El-Refai et al.
412 (2005).

413

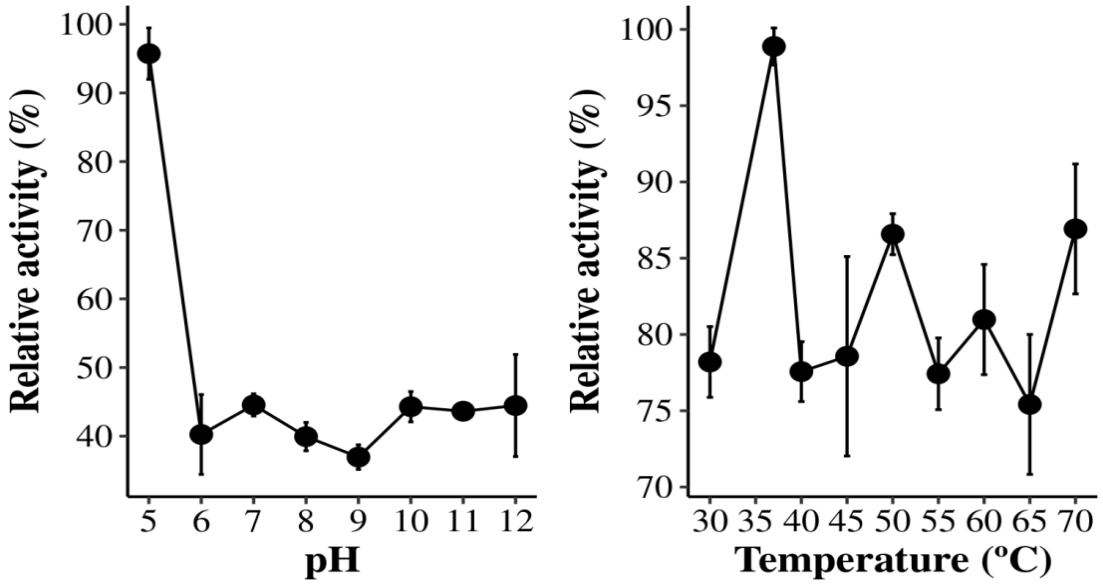

Figure 4. Effect of temperature and pH variation on proteolytic activity. The test was performed in triplicate and the bars indicate the standard deviation.

3.10 Effect of chemicals on enzyme activity

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 fungal extract regardless of their concentrations, indicating that these ions interacted with the active site of the enzymes and thus reduced their catalytic activity. The presence of Cu^{2+}, Fe^{2+}, 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, increasing the enzymatic activity by approximately 55%. Similar results were found by Magalhães et al., (2019), who reported that the proteolytic activity of *Lentinus crinitus* was increased (129.43 %). 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) assert that ions can be involved in catalytic processes, participating in redox reactions or electron transfer. The effect of different metal ions on microbial keratinases is generally highly variable, depending on both their nature and their concentration (Werlang and 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 contribute to enzymatic catalysis in bioprocesses. Non-ionic detergents like Triton X-100 and Tween-20 are mild surfactants and generally

do not affect protein activity (Linke, 2009). In this work, Tween 20 (0.5 % and 1% v/v) and Triton 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 and Palanivelu, 2013). SDS is a strong anionic surfactant that can have inhibitory effects for 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 (Patak and Deshmukh, 2012). Bach et al. (2011), report 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 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 enzymatic stability 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 to organic solvents is generally attributed to the disulfide bonds located on the surface of the molecule (Doukyu and Ogino, 2010). Zanphorlin et al. (2011) reported that the protease of the fungus *Myceliophthora* sp. lost enzymatic activity with addition of acetone and butanol.

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*

Table 2 Effect of various salts on the production of proteases. Assay were performed in triplicate and measurement data were expressed as the Mean ± Standard Deviation (SD). * significant difference at $p< 0.05$

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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

Table 3 Effect of various chemical reagents on proteolytic activity. Assay were performed in triplicate and measurement data were expressed as the Mean ± Standard Deviation (SD). * significant difference at $p < 0.05$.

468

469 The effect of inhibitors on proteolytic activity was examined and is listed in Table 4. The

470 results demonstrate that the proteases were resistant to the action of EDTA and β-

mercaptoethanol, suggesting that it is not a metalloprotease or cysteine protease. EDTA is a chelating agent that inhibits the action of metalloprotease and β-mercaptoethanol, which is a strong irreversible reducing agent that reduces the disulfide bonds of the enzyme (Sabotič and Kos, 2012). Unlike our results, 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. Martim et al. (2017), analyzing the effect of inhibitors on the proteolytic activity of *Pleurotus albidus*, verified the presence of serine and cysteine proteases in the crude extract of the fungus. Tests with specific inhibitors need to be done to classify the enzyme under study.

Inhibitor	**Concentration (mM)**	**Relative activity (%) ± SD**
Control	-	100 ± 1.0
β - mercaptoethanol	1	156.59 ± 11.6*
	5	163.00 ± 10.1*
EDTA	1	88.84 ± 2.1
	5	78.05 1.3*

Table 4 Effect of inhibitors on proteolytic activity. Assay were performed in triplicate and measurement data were expressed as the Mean ± Standard Deviation (SD). * significant difference at $p < 0.05$.

Conclusion

The work was important for the characterization of the fermentation process of the fungus *Aspergillus* sp. for an efficient and economical production of extracellular proteases. The reduction in production cost was attributed to the fungus ability to produce extracellular protease in liquid medium using feathers, an agro-industrial residue, as carbon source. The results obtained showed that *Aspergillus* sp. it is efficient in the cleavage of

keratinous residues, growing in simple cultive with feathers as its only source of energy, requiring a low concentration of substrate and reduced cultivation time, obtaining excellent enzymatic activity in these conditions. These results suggest a future application of protein hydrolysates as a supplement in animal feed and biofertilizers. Therefore, this study presents a strategy for recycling agro-industrial waste, enabling the addition of value to these low-cost raw materials, and thus, contributing to the maintenance of environmental quality.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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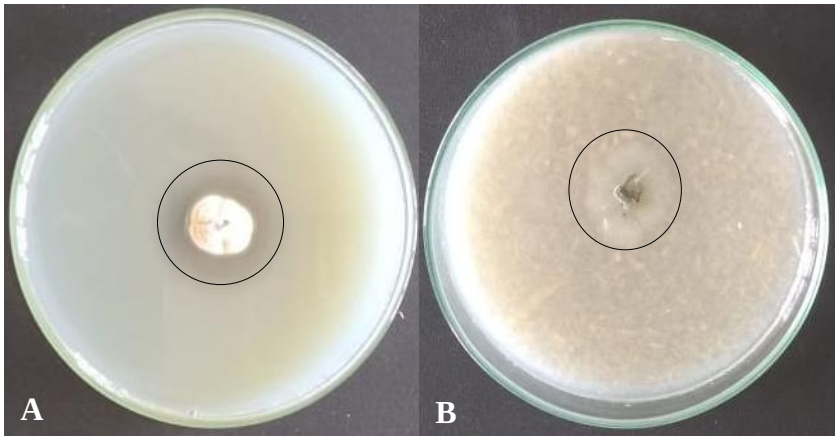
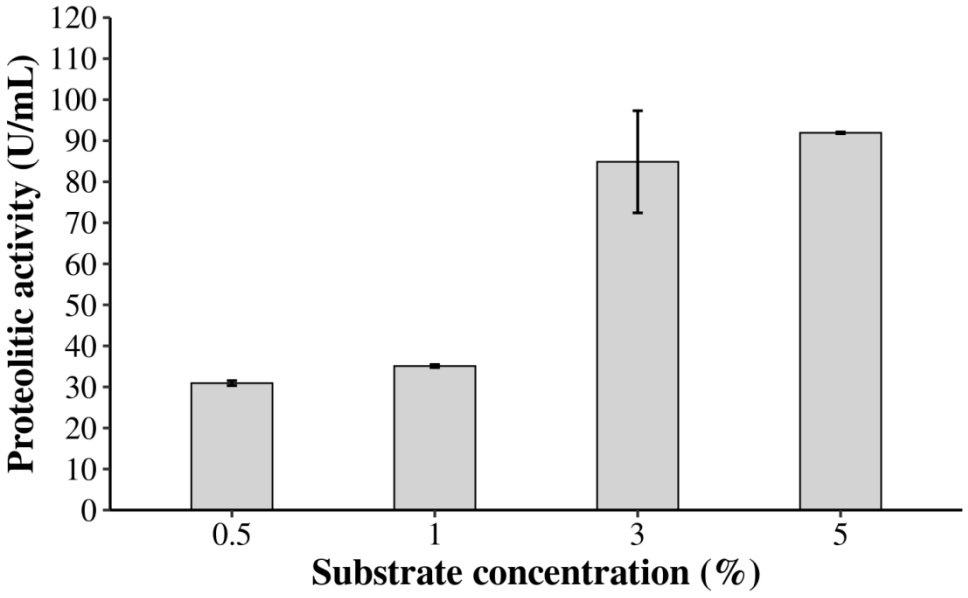
751 **Appendix A. Supplementary data**

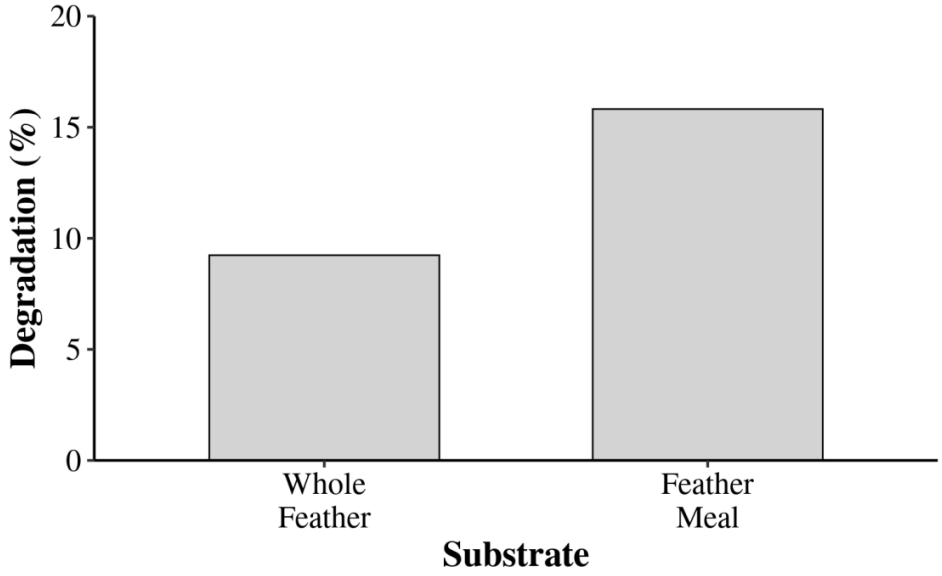


Qualitative evaluation in solid media, incubated at 27 ° C, for 5 days. (A) Proteolytic activity determined on milk agar medium, (B) Keratinolytic activity evaluated on feather meal agar medium. The presence of clear zones around the colonies of *Aspergillus* sp., indicates the production of proteases. The black circle shows the degradation zone.



Evaluation of concentrations of feather meal as a substrate in the production of proteases by Aspergillus sp., after 48 h in submerse cultivation. The test was performed in triplicate and the bars indicate the standard deviation.

752



Determination of the percentage of degradation in submerged cultures of whole feather and feather meal (0.5%).

2.1. INSTRUÇÕES DE PUBLICAÇÃO DA REVISTA WAST MANAGEMENT

Este manuscrito foi submetido a revista Wast Management, Qualis A1 na área de Ciências Ambientais. Segue as instruções da revista:

ELSEVIER

WASTE MANAGEMENT

International Journal of Integrated Waste Management, Science and Technology

AUTHOR INFORMATION PACK

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ISSN: 0956-053X

DESCRIPTION

Waste Management is devoted to the presentation and discussion of information on **solid waste generation**, **characterization**, **minimization**, **collection**, **separation**, **treatment** and **disposal**, as well as manuscripts that **address waste management policy**, **education**, and **economic and environmental assessments**. The journal addresses various types of solid wastes including municipal (e.g., residential, institutional, commercial), **agricultural** and **special** (e.g. construction and demolition, household hazardous, sewage sludge, and non-hazardous industrial) wastes.

We welcome both fundamental and applied research that can be related to problems of interest to solid waste researchers, practitioners and/or policy makers. Well documented case studies will be considered but they must describe results that can be applied beyond the specific location of the case study. Manuscripts that focus on the use of a waste material in a new product are often more suitable for a journal that focuses on the material properties of the product. In considering whether a manuscript is suitable for publication in Waste Management, consider whether the

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AUDIENCE

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GUIDE FOR AUTHORS

INTRODUCTION

Journal scope

Waste Management is devoted to the presentation and discussion of information on solid waste generation, characterization, minimization, collection, separation, recycling, treatment and disposal, as well as manuscripts that address solid waste management policy, education, and economic and environmental assessments. The journal addresses various types of solid wastes including municipal (e.g., residential, institutional, commercial), agricultural, construction and demolition, household hazardous, coal combustion residues and other non-hazardous industrial wastes.

Manuscripts that describe processes related to materials production with no application to the solid waste system will not be considered. Manuscripts on the treatment and disposal of biosolids from wastewater treatment will only be considered if they describe a process that is also applicable to other solid wastes (e.g., anaerobic digestion, char production, thermal treatment, but not dewatering). Manuscripts that focus on human behavior must discuss practical policy implications. While manuscripts on facility siting are in scope, it is essential for the authors to explain the new contribution in the cover letter as we get many submissions that do not represent significant innovation.

The following topics are not in the journal?s scope: wastewater, mining waste, hazardous industrial waste, radioactive waste, material science, land application of waste-derived products. Manuscripts on waste valorization are welcome in cases where the waste is a major part of the valorization process.

We welcome both fundamental and applied research that can be related to problems of interest to solid waste researchers, practitioners and/or policy makers. Well documented case studies will be considered but they must describe results that can be applied beyond the specific location of the case study. Manuscripts that focus on the use of a waste material in a new product are often more suitable for a journal that focuses on the material properties of the product. For example, studies on the use of a waste in transportation materials (concrete, asphalt) should be sent to journals that focus on those materials. In considering whether a manuscript is suitable for publication in Waste Management, authors should consider whether the information is of potential use to solid waste researchers, practitioners and/or policymakers. The following are some of the major areas in which papers are solicited: Generation and characterization Minimization Recycling and reuse Storage, collection, transport, and transfer Treatment (mechanical, biological, chemical, thermal, other) Landfill disposal Environmental assessments Economic analysis Policy and regulations Education and training Planning

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3 CONCLUSÃO

Dentre as linhagens de fungos filamentosos avaliadas quanto a produção de proteases, *Aspergillus* sp. foi o isolado que melhor produziu enzimas em meio líquido, utilizando pena inteira e farinha de penas como única fonte de carbono e nitrogênio.

A atividade proteolítica da linhagem estudada em temperatura moderada de 37 °C e pH de 5,0, nas quais as proteases apresentaram melhores atividades, indica que essas enzimas podem ser potencialmente utilizadas no bioprocessamento de resíduos queratinosos, concomitante com produção de hidrolisados proteicos, biomassa microbiana e enzimas proteolíticas e queratinolíticas.

Na presença de íons $CaCl_2$, $MnSO_4$, KC_l, $MgSO_4$ e $CuSO_4$ a atividade proteolítica foi potencializada. As enzimas foram resistentes aos solventes orgânicos metanol, acetona, butanol, acetonitrila, isopropanol e DMSO, bem como aos detergentes Tween 20, Triton X-100, PEG, SDS e CTAB. Os inibidores proteolíticos β - mercaptoetanol e EDTA, tiveram efeito positivo sobre a atividade enzimática, sugerindo que as enzimas presentes no extrato bruto não são da classe das metaloproteinases nem das cisteína proteases. Testes com inibidores específicos devem ser realizados para classificar a proteína.

Aspergillus sp., foi capaz de hidrolisar todos os substratos de crescimento avaliados, principalmente peptona e farinha de penas, sendo os resíduos queratinosos mais indicados e adequados para a síntese de proteases por representar um meio de cultivo simples, de baixo custo, rico em proteína e abundante. Vale ressaltar que a substituição de substratos sintéticos por naturais, pode ser uma alternativa que contribua para a redução nos custos do processo de fermentação, fator importante para as indústrias.

As características da protease bruta e sua capacidade de hidrolisar queratina apresentadas nesse trabalho, sugerem a reciclagem de resíduos agroindustriais, com concomitante obtenção de enzimas com potencial biotecnológico, indicando perspectivas promissoras para pesquisas futuras.

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