SELECTION OF THE STRAIN WITH THE HIGHEST KERATINOLYTIC ACTIVITY DURING FEATHER HYDROLYSIS FOR KERATIN EXTRACTION

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ABSTRACT

The textile industry is responsible for various environmental impacts, primarily associated with the choice of raw materials, such as cotton and polyester fibers. The poultry industry is a significant source of pollution and generates a considerable amount of waste, including feathers. Approximately 90% of feathers are composed of keratin, a protein with bulky side groups, stability in water, and a molecular weight of approximately 10 kDa, making them ideal for spinning into textile fibers. However, the chemical treatments typically applied to extract keratin from feathers are costly and involve the use of toxic products harmful to the ecosystem. Therefore, this project aimed to isolate keratinolytic microorganisms, which produce keratinases, enzymes capable of degrading keratin substrates, for potential use in the production of keratin textile fibers. In this regard, microorganisms were isolated from soil with keratinous substrates, and their ability to produce proteases and keratinolytic enzymes was tested. Finally, the best strains were selected for experiments to evaluate their potential to degrade feathers and generate peptides, with strain MR8.I demonstrating the highest success.

Keywords: keratin, keratinases, feathers, peptides, fiber.

1 INTRODUCTION

The textile industry plays an important role in economies around the world[1]. Currently, the textile industry is one of the largest in the world and one of the most polluting[2]. One of the factors contributing to this situation is the choice of unsustainable raw materials in fabric production[3]. Polyester and cotton are the main fibers available in the textile market, representing 54% and 22% of global fiber production[4]. In the case of cotton, its cultivation requires around 8000 to 22000 L/kg of cotton fiber, and consumes 11% of pesticides and 26% of insecticides applied worldwide[5]. In contrast, polyester fibers are produced from fossil fuels, mainly petroleum, which involves the emission of high rates of greenhouse gases[6]; during the production of one polyester t-shirt, 5.5 kg of carbon dioxide is released[7]. Furthermore, polyester fabrics are not biodegradable and can remain in the environment for a long time[3]. In this context, many clothing brands have started to market eco-friendly fibers[8].

In parallel, Brazil is currently the second-largest producer of chicken[9], and one of the main residues generated by this industry is feathers[10], which represent 5 -7% of the chicken's weight[11]. Usually, they are buried in landfills, incinerated, or used in animal feed[12]. Keratin makes up 90% of the feather structure[13], a protein with many disulfide bonds, which are strong covalent bonds created between thiol groups (-SH) present in cysteine residues within and between keratin polypeptides[14]. The cysteine in keratin, the few bulky side groups, and a molecular weight exceeding 10 KDa make it ideal for spinning[15]. Therefore, it is evident that feathers, especially chicken feathers, are a potential protein source for the production of sustainable protein fibers. Typically, keratin extraction from keratinous substrates is carried out through chemical treatments[16]. However, chemical methods tend to be expensive[17] or unsustainable.

A possible solution to avoid the use of chemicals in keratin extraction is the degradation of keratin through keratinolytic microorganisms capable of producing keratinases[18]. These are the only proteases capable of binding to and hydrolyzing complex and insoluble protein substrates[19]. Many of the keratinolytic microorganisms used in the industry are isolated from the soil, and most of the keratinases produced come from gram-positive bacteria, mainly *Actinomycetes* sp. and *Bacillus* sp.[20].On the other hand, it has been reported that gram-negative bacteria can also be good synthesizers of keratinolytic proteases, one of which is *Pseudomonas aeruginosa*. Strains of this species have already been isolated from various soil samples and locations with keratinous waste, and in these studies, this microorganism was successful in the hydrolysis of these substrates, making this genus promising[20, 21]. In this context, the objective of our work is to isolate a keratinolytic microorganism that demonstrates high efficiency in feather degradation so that it can be applied in the future for keratin extraction from feathers and its spinning into textile fibers

2 MATERIAL & METHODS

For the first isolation, 1 gram of feathers was buried in a pot with the plant *Juniperus chinensis (*pine). After one week, 1 gram of soil near the root of this plant was removed and placed in an Erlenmeyer flask with a rich medium [g/L: yeast extract 5; sucrose 5; peptone 5; potassium chloride (KCl) 5; feathers 10], and another gram was placed in another Erlenmeyer flask with a poor medium [g/L: sodium chloride (NaCl) 0.085; feathers 10]. After 7 days, an aliquot from each flask was diluted in sterile saline in dilutions from 10^{-1} to 10^{-7} and plated on nutrient agar plates. After 24 hours, the colonies that appeared morphologically distinct to the naked eye were streaked separately on NB agar.The second isolation was carried out on the banks of the Pavuna Stream, and two soil samples were collected near the roots of some trees in the area. Then, 1 gram of each collected sample

was placed in a different Erlenmeyer flask with the rich medium. After 48 hours and 168 hours, an aliquot from these media was diluted in sterile saline in dilutions from 10^{-1} to 10^{-7} and plated on nutrient agar plates and on cetrimide agar plates (a selective medium used for isolating species of *Pseudomonas* sp.). After 24 hours, the morphologically distinct colonies were streaked separately on nutrient agar plates, as in the previous isolation.

The proteolytic activity of the isolated strains was tested on casein agar. Each microorganism was suspended in sterile saline. This suspension was inserted, in triplicate, into wells on casein agar plates. One of the wells on each plate served as the control and contained only sterile saline. After 24 hours, microorganisms capable of degrading protein formed a halo around the well. The strains that produced halos were inoculated, in triplicate, into tubes containing a medium for the production of hydrolysates [Na_2HPO_4 0.06M; KH_2PO_4 0.04M, pH 8.0; $MgSO_4$ 0.5 mM; MnCl2 0.5 mM; $CaCl_2$ 0.5 mM; chopped feathers 1.5%] The control used were tubes that were not inoculated with any strain. After 120 hours, the tubes were centrifuged, and the supernatant was used in the Lowry et al. (1951) method for protein quantification. The greater the amount of protein in the supernatant, the more feathers were degraded. The protein concentration produced by each microorganism was analyzed, and the three microorganisms with the highest protein concentrations were selected for further experiments.

The selected strains were inoculated into Erlenmeyer flasks with nutrient medium for 48 hours. After this time, the volume corresponding to an OD of 0.4 was centrifuged. The resulting cell pellets were inoculated into Erlenmeyer flasks with a medium for the production of hydrolysates and keratinases and were incubated under agitation for 48 hours, 120 hours, and 168 hours. After these periods, the contents of the Erlenmeyer flasks were filtrated with filter paper (Whatman no. 1), and the filtrate was centrifuged to extract the supernatants, which were then subjected to the Lowry et al. (1951) protein assay method. The microorganism with the highest protein production was selected for future use in manufacturing keratin textile fibers through feather biotreatment.

3 RESULTS & DISCUSSION

In the first isolation, the microorganisms isolated were named MP1, MP2.1, MP2.2, MP3, MP4, MP5, MP6, MR1, MR2, MR3, MR4.1, MR4.2, MR5.1, MR5.2, MR6.1, MR6.2, MR7, MR8, MR9, MR10, MR11, and MR12 from the pot with the plant *Juniperus chinensis* (pine) with buried feathers. However, after the 2020 pandemic, since the samples MR4.1, MR4.2, MR8, MR10, and MR12 exhibited different colonial characteristics from those recorded at the time of their isolation, they were renamed to MR4.1I, MR4.2I, MR8I, and MR10I. In the second isolation, soil samples were collected near the rhizosphere of two trees, and these samples were designated as soil A and soil B. The microorganisms isolated from soil A and soil were named BA1, A2, A3, A4, A5, A6, A7, A8, A9, A10, A11, A12, A13, A14, B1, B2, B3, B4, B5.1, B5.2, B6, B7, B8, B8.1, B9, B10, B11, B15, B16, B17, B18, B19, B20, and B21.

Keratin is a substrate that does not accumulate in nature, which indicates the existence of natural decomposers capable of degrading it[22]. In this sense, microorganisms that decompose this substrate are usually collected from the environment, such as soil samples, air, foliage, sand, and environmental residues[23]. The rhizosphere is a favorable region for the growth of microorganisms since it is an area of the soil affected by compounds secreted by the plant root, such as organic exudates. These exudates stimulate the establishment of a diversity of microbial communities that participate in biological processes and protection against pests and pathogens, which provides benefits for plant development. Research also indicates differences between the soil microbial community and the rhizosphere microbial community, with the former generally having greater diversity and the latter tending to have a higher quantity of microorganisms[24]. This may explain the small number of distinct colonies isolated from the rhizosphere of three different plants.

According to Li et al., 2021, keratinolytic microorganisms should be obtained according to the following steps. First, ideal samples for the isolation of these microorganisms need to be collected from the environment, and they can come from soils or water containing keratinolytic substrates. Next, an assay should be performed to measure the activity of proteases produced by the collected microorganisms, and the progress of keratin decomposition and the composition of the proteins resulting from its hydrolysis should also be assessed[25]. Therefore, our project aimed to follow these steps to obtain keratinolytic microorganisms so that their performance in feather hydrolysis could be evaluated. In the screening of microorganisms with proteolytic activity, the strains capable of forming halos were MP1, MP3, MP4, MP5, MP6, MR1, MR2, MR4.1I, MR4.2I, MR5.1, MR7, MR8.I, MR10I, MR11, A3, A13, B3, B4, B5.1, B5.2, B9, B10, B11, B16, B17, and B20. The strains that showed the highest average of peptides present in their supernatant after feather hydrolysis were MP5, MR4.2, and MR8.I. MR8.I and MR4.2I presented, on average, the same amount of proteins in their supernatant, 0.739 mg/mL.

After standardizing the inoculum, MR8.I showed the highest amount of proteins in its supernatant after feather degradation at all incubation periods, 48 hours, 120 hours, and 168 hours, with concentrations of 3.0405 mg/mL, 3.2945 mg/mL, and 3.6615 mg/mL, respectively. According to the Gram staining, this is a bacillus-shaped, Gram-positive bacterium. Many studies isolating keratinolytic microorganisms from soil with keratin residues have reported that among the collected strains, those showing the best keratinolytic activity were of the *Bacillus* sp. Genus[26]. Therefore, there is a high likelihood that this strain belongs to this genus. In the article of De Oliveira et al. (2017) about *Bacillus* sp. strain CL33A, its keratinolytic activity was tested using feather meal as a substrate, and the highest protein concentration in the supernatant was 4.39 ± 0.22 mg/mL[28], higher than the concentrations obtained by MR8.I in non optimized conditions.

4 CONCLUSION

This study demonstrated the isolation of keratinolytic microorganisms obtained from soil samples and provided preliminary results for the selection of the best microorganism capable of producing soluble peptides from feathers. According to the results, the strain exhibiting the highest keratinolytic activity was MR8.I. However, further experiments are still required to analyze the

peptides generated by this bacterium after feather hydrolysis so that this strain can potentially be used in the production of keratin textile fibers in the future.

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