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Keratinolytic fungi in illegal feather dump sites of condoray – Ayacucho: Isolation, characterization, and degradative potential

Saúl Alonso Chuchón-Martínez[1], Pedro Mauricio Delgadillo-Coronado[2], Rilder Nemesio Gastelu-Quispe[3*], Ruth Elsa Huaman-De La Cruz[4], Jime Jack Rivera-Villar[5], Obert Marín-Sánchez[6]
[1,2,3,4,5]Universidad Nacional San Cristóbal de Huamanga. Ayacucho, Perú; saul.chuchon@unsch.edu.pe (S.A.C.M.) pedro.delgadillo@unsch.edu.pe (P.M.D.C.) rilder.gastelu@unsch.edu.pe (R.N.G.Q.) ruth.huaman@unsch.edu.pe (R.E.H.D.L.C) jimi.rivera@unsch.edu.pe (J.J.R.V.).
[6]Universidad Nacional Tecnológica de Lima Sur (UNTELS), Lima, Perú; omarins@untels.edu.pe (O.M.S.).

Abstract: This study explores the potential of keratinolytic fungi for feather waste degradation. Soil and feather samples were collected from the clandestine feather dump in Condoray-Ayacucho and processed at the UNSCH Environmental Microbiology Laboratory. Fungi were isolated using Bushnell Haas (B-H) medium supplemented with sterilized chicken feathers, and proteolytic strains were selected using milk agar (LA). Of the 18 strains isolated, three (C.S.4, C.S.7, and C.P.2.1) exhibited significant protease activity, forming halos of 12–17 mm. Their feather-degrading capacity was assessed in B-H medium with 1% feathers, inoculated with 250 µl of spore solution (10^6 spores/100 µl), and incubated at 25°C for 8 days with manual agitation. Strain C.P.2.1 showed the highest degradation, reducing feather weight by 12%, followed by C.S.7 (10%) and C.S.4 (8%). A gradual increase in pH and protein release was observed, reaching 0.40 mg/ml for C.P.2.1, 0.32 mg/ml for C.S.7, and 0.15 mg/ml for C.S.4. These findings highlight the potential of keratinophilic fungi for enzymatic degradation of keratin. Statistical analysis revealed no significant differences among strains, but a significant difference compared to the control ($p<0.05$, Tukey test).

Keywords: *Clandestine landfills and fungi, Feathers, Keratins, Keratinolytics.*

1. Introduction

Agro-industrial waste consists of solid or liquid materials generated during agricultural and livestock production, categorized into food and non-food residues [1, 2]. The expansion of agro-industrial activities has led to an increase in waste generation, contributing to environmental contamination due to inadequate management [3]. Among these residues, poultry feathers constitute between 5% and 10% of a bird's live weight and are rich in keratin, a highly resistant fibrous protein [4, 5]. Globally, the poultry industry generates millions of tons of feather waste annually, posing a serious environmental risk when not properly handled [6-8]. These residues can act as vectors of pathogenic organisms and negatively impact water, soil, and air quality [9].

Internationally, poultry feather disposal has been a critical environmental concern due to its accumulation and slow degradation in natural ecosystems [10, 11]. Traditional disposal methods, such as incineration and landfilling, contribute to greenhouse gas emissions and soil pollution [12, 13]. Recent studies have explored sustainable alternatives, including microbial biodegradation using keratinolytic microorganisms capable of breaking down feather keratin [14, 15]. Research has demonstrated that specific bacteria (Bacillus, Streptomyces) and fungi (Trichophyton, Aspergillus, Microsporum) can degrade keratin, facilitating the conversion of feathers into valuable by-products such as biofertilizers and protein-enriched feed [16-18].

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* Correspondence: rilder.gastelu@unsch.edu.pe

In Peru, poultry production has seen a 7% annual increase, with a significant portion dedicated to live bird sales, resulting in poor by-product management, particularly in the disposal of feathers [19, 20]. The absence of strict environmental regulations and effective waste treatment processes has led to clandestine dumping sites, which exacerbate pollution and public health concerns [21, 22]. The improper disposal of feather waste not only affects local ecosystems but also creates breeding grounds for pathogens harmful to both humans and animals [23, 24]. Addressing this issue requires an urgent shift towards sustainable waste valorization strategies that promote the utilization of poultry feathers in industries such as agriculture, biotechnology, and energy [25, 26].

This study aims to analyze the presence of keratinophilic fungi in clandestine poultry feather dumping sites in Ayacucho, Peru, and their potential role in feather degradation. Given the environmental and health risks associated with improper feather disposal, understanding the microbial communities involved in natural biodegradation processes could inform strategies for waste management and sustainability [27, 28]. The research addresses the following question: How do keratinophilic fungi contribute to the degradation of poultry feathers in clandestine dumping sites in Ayacucho? The findings are expected to provide insights into microbial-driven keratin degradation and propose biotechnological applications for effective poultry waste management in Peru. This study not only contributes to scientific knowledge on keratinophilic fungi but also provides practical implications for environmental management and circular economy initiatives [29-31].

2. Theoretical Framework

2.1. Conceptual and Theoretical Definitions

Agro-industrial waste comprises solid or liquid materials generated during agricultural and livestock production, categorized into food and non-food residues [1, 2]. Among these, poultry feather waste is a major environmental concern due to its high keratin content, accounting for 5% to 10% of the bird's live weight [8]. Keratin is an insoluble fibrous protein with high mechanical stability, making feather degradation a challenging process [32, 33]. According to Kornitłowicz-Kowalska and Bohacz [11] improper disposal of these residues contributes to environmental pollution, affecting soil, water, and air quality.

Feathers contain approximately 85% to 90% keratin, a protein resistant to enzymatic degradation due to disulfide bonds between glycine and cysteine [34, 35]. However, certain keratinophilic microorganisms, including fungi, bacteria, and actinomycetes, can degrade keratin through keratinase enzymes [10, 36]. These microorganisms are found in various habitats, from Antarctic soils to landfill sites, making them key agents for sustainable waste management [37, 38].

2.2. Findings from Previous Research and Correlations

Studies indicate that poultry feather waste contributes significantly to environmental pollution due to inadequate waste management practices [9, 24]. Report that improper disposal of feathers leads to bacterial contamination in soil and water bodies, increasing health risks. In Peru, poultry production grows at an annual rate of 7%, with improper disposal of feathers exacerbating environmental hazards [19, 20].

Regarding keratin degradation, microbial activity plays a crucial role in feather decomposition. Research shows that Bacillus licheniformis can degrade 70% of feather keratin within 48 hours under optimized conditions [17, 31]. Additionally, fungal strains such as Aspergillus flavus and Trichophyton mentagrophytes exhibit keratinolytic activity, reducing feather mass by 65% to 80% in controlled studies [18, 39]. These findings confirm that keratinophilic fungi in landfills play a key role in feather degradation, supporting sustainable waste management solutions.

2.3. Relevant Theoretical Approaches

Several theoretical perspectives provide insight into the degradation of poultry feathers by keratinophilic fungi:

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a. Microbial Keratinolysis Theory Gupta and Ramnani [28]: This theory explains the biochemical pathways through which microorganisms break down keratin using proteolytic enzymes, particularly keratinases. It suggests that microbial adaptation to keratin-rich environments drives the evolution of these specialized enzymes [29].
b. Ecological Adaptation Theory Onifade, et al. [10]: This framework highlights how keratinophilic microorganisms thrive in environments rich in keratin, such as poultry landfills. According to this theory, microbial communities develop metabolic pathways to utilize keratin as a nitrogen source, promoting decomposition and nutrient cycling [37].
c. Enzyme-Substrate Interaction Model Gupta and Ramnani [28]: This theory details the mechanisms by which keratinases interact with feather keratin. It states that microbial keratinases hydrolyze keratin into smaller peptides by disrupting disulfide bonds through sulfiteolysis, a process crucial for effective keratin degradation [40].

These theories provide a comprehensive understanding of how keratinophilic fungi function in landfill environments, reinforcing the importance of studying their ecological roles in feather degradation.

The reviewed literature underscores the environmental impact of poultry feather waste and the essential role of keratinophilic fungi in its biodegradation. While microbial keratinolysis is well-documented, limited research focuses on its application in clandestine landfills, particularly in Peru. The current study addresses this gap by investigating the diversity and keratinolytic potential of fungi in illegal feather disposal sites in Ayacucho.

By examining fungal species with high keratinolytic activity, this research contributes to sustainable waste management strategies, offering insights into biological feather recycling. Additionally, it aligns with global efforts to reduce agro-industrial waste pollution, supporting environmental conservation initiatives [9]. The study's findings will inform future applications of microbial keratinases in biotechnology, paving the way for innovative bioconversion approaches to poultry waste management.

3. Materials and Methods

3.1. Sampling Site Location

The clandestine dumping site is located in the rural community of Condoray, in the Tambillo district, Huamanga province, with geographical coordinates 13° 11' 32.7" S and 74° 7' 51.3" W, at an altitude of 2953 meters above sea level [41].

A 300 m² area of the clandestine landfill was inspected, revealing a high accumulation of decomposing feathers. Five sampling points were established, from which five samples were collected (three soil samples and two feather samples). The soil samples were combined, homogenized, and treated as a single soil sample, while feather samples were kept separate. Using a sterile spatula, approximately 500 g of soil or 100 g of feathers were collected from each sampling point. Samples were placed in new polyethylene bags, sealed, labeled, and transported in a cooler under ambient temperature conditions to the Environmental Microbiology Laboratory at the Faculty of Biological Sciences, UNSCH.

3.2. Isolation of Keratin-Degrading Fungi

Soil and feather samples were dried in Petri dishes at 60°C for two hours. The soil was sieved, and the feathers were ground. Subsequently, 10 g of soil and 5 g of feathers were added to flasks containing Bushnell Haas medium supplemented with sterilized poultry feathers. The samples were incubated at 20-25°C for 15 days, with periodic manual agitation. After incubation, fungal isolates were cultured on Feather Agar (FA) and incubated at 25°C for 7 to 15 days before transferring to Sabouraud Agar to establish a fungal strain collection.

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3.3. Selection of Proteolytic Fungal Strains

To select proteolytic fungal strains, Milk Agar was used, consisting of solution "A" (5 g/L peptone, 3 g/L yeast extract, 1 g/L dextrose, and 15 g/L agar) with a pH of 7.2, sterilized at 121°C for 15 minutes. Solution "B" contained 100 mL/L of sterile UHT skimmed milk and was mixed with solution "A" at 45°C before being poured into Petri dishes. Fungal strains were inoculated via central puncture and incubated at room temperature for 7 days. Strains producing transparent halos around the colony were selected for further analysis.

3.4. Enzyme Diffusion Measurement

Fungal strains exhibiting visible casein degradation were identified as keratinolytic. The fungal growth diameter (Dcf) and the halo diameter of degradation (Dhd) were measured in three different directions. Strains without visible degradation were assigned a value of zero millimeters [42]. Enzyme diffusion was calculated using the formula:

$$\text{Enzyme Diffusion} = Dhd - Dcf$$

Strains with the highest enzymatic diffusion were selected for keratinase proteolytic activity evaluation.

3.5. Evaluation of Keratinase Proteolytic Activity

The selected fungal strains were cultured on Sabouraud Agar and incubated for 7 days. Spores were harvested in 5 mL of saline solution and adjusted to a concentration of 10^6 spores/100 µL. Each strain was inoculated into flasks containing Bushnell Haas medium with 1% poultry feathers, sterilized in an autoclave. A total of 45 inoculated flasks and 15 non-inoculated controls were prepared. The flasks were incubated at 25°C for 8 days with periodic manual agitation. The following parameters were evaluated at 0, 2, 4, 6, and 8 days:

3.5.1. pH Measurement

pH was monitored throughout the incubation period using a HANNA multiparameter meter (range 0-14, sensitivity 0.1 units).

3.5.2. Quantification of Keratinous Material Degradation

Feather samples (0.5 g) were weighed before being introduced into the Bushnell Haas medium. At days 0, 2, 4, 6, and 8, residual keratinous material was removed, washed, drained, and dried at 80°C. The dried feathers were weighed using an analytical balance (precision 0.0001 g) to assess degradation.

3.5.3. Measurement of Total Protein Production

Total protein concentration and enzymatic activity were measured at 0, 2, 4, 6, and 8 days. After centrifugation at 3000 rpm for 30 minutes, the supernatant was collected and refrigerated as an enzymatic extract [43]. Protein concentration (mg/mL) was determined using an Eppendorf Biophotometer Plus UV-170 at 280 nm and 260 nm, applying the formula:

$$\text{Total Proteins (mg/mL)} = [1.55(A280) – 0.76(A260)]$$

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3.6. Identification of Fungal Strains

The microculture technique on Sabouraud Agar was employed to observe vegetative and reproductive fungal structures. Additionally, macroscopic descriptions of colony morphology and growth patterns were recorded to support strain identification.

4. Results and Discussion

A total of 18 fungal strains were isolated from samples collected at the clandestine dumpsite in Condoray - Ayacucho, using Bushnell Haas medium supplemented with chicken feathers. Although these strains were able to grow in an oligotrophic environment, not all of them necessarily exhibited keratinolytic activity, as some may have merely survived without actively degrading keratin. Previous studies have demonstrated that avian feathers can serve as the sole source of carbon and energy for the growth of keratinophilic microorganisms [44, 45].

Timorshina, et al. [46] highlight the scarcity of research on fungal keratinases, emphasizing the need to identify new strains with high keratin degradation potential. In a study analyzing 32 fungal cultures, only four strains exhibited significant keratinolytic activity, with Aspergillus clavatus VKPM F-1593 standing out in a medium enriched with ground chicken feathers. These findings reinforce the crucial role of keratinophilic fungi in keratinase production, an essential enzyme for industrial and environmental applications, such as poultry waste bioremediation and the development of bioactive compounds for the biotechnology sector.

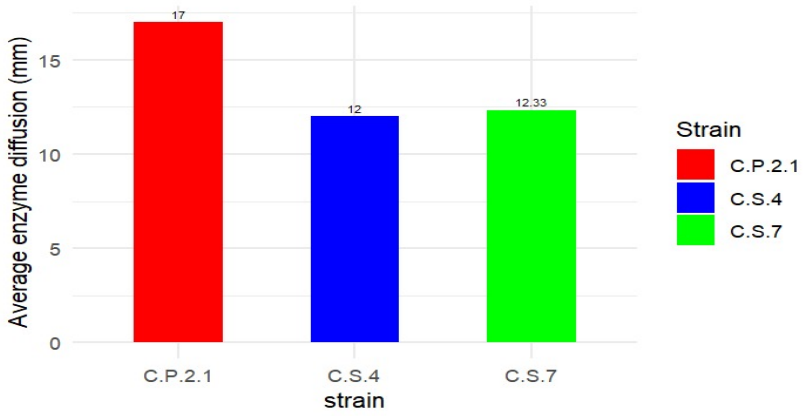

Figure 1.
Average Diameter of Proteolytic Enzyme Diffusion in Milk Agar of Three Fungal Strains Isolated from the Clandestine Dumpsite in Condoray.

Figure 1 presents the average enzymatic diffusion diameters in milk agar for three fungal strains isolated from the clandestine dumpsite. The C.P.2.1 strain exhibited the largest diffusion halo, measuring 17 mm, compared to C.S.4 and C.S.7, which displayed halos of 12 mm and 12.33 mm, respectively.

García, et al. [47] highlight the importance of fungal protease production through solid-state fermentation, emphasizing its industrial significance in a growing global market. Their study in Mexico evaluated six Aspergillus spp. strains on skim milk agar plates, revealing that three strains formed hydrolysis halos, indicating proteolytic enzyme activity. This finding aligns with the current study's results, which confirm the proteolytic enzyme production by the isolated fungal strains.

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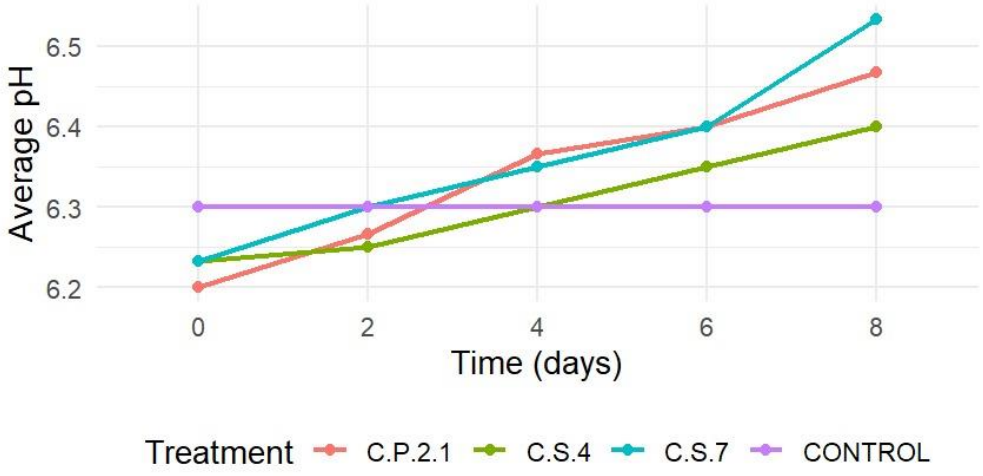

Figure 2.
Average pH Variation During Feather Degradation by Fungal Strains

Figure 2 illustrates the pH variation values recorded during the feather degradation process by fungal strains isolated from the illegal dumpsite. This figure depicts the trend of average pH variation at different incubation times. Throughout the degradation process, a tendency toward pH increase (alkalization) was observed, suggesting that this phenomenon could have been more pronounced with an extended incubation period.

According to Gupta and Ramnani [28] and Souza, et al. [48] keratinase, a serine protease, exhibits optimal activity within a pH range of 7 to 11, with pH 8.0 being identified as the optimal level for keratinolytic activity [49]. This increase in external pH during degradation could be explained by the release of alkaline byproducts such as amino acids and NH_4^+, as reported by Bohacz [50] Bohacz et al. (2017) in their studies on keratin protein mineralization by fungal strains.

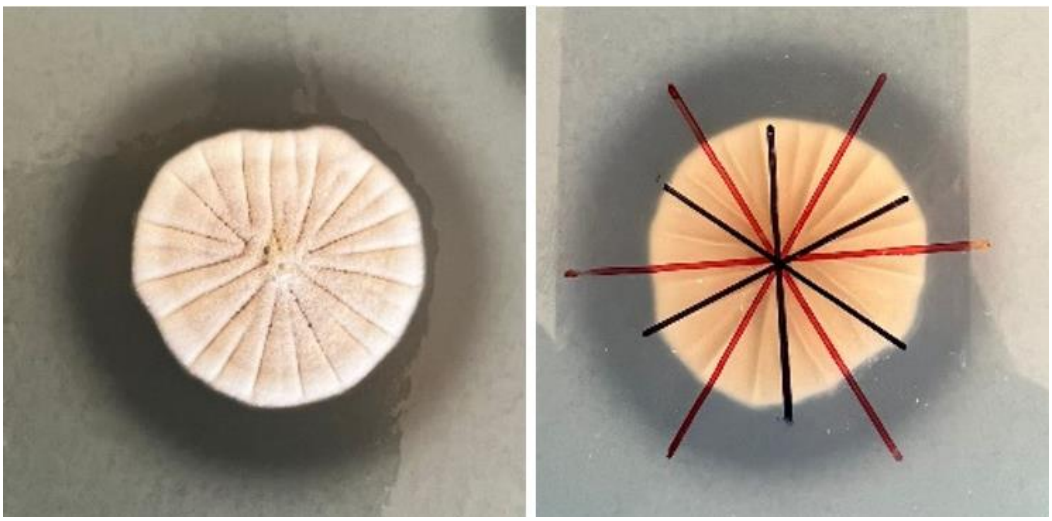

Figure 3.
Proteolytic Enzyme Diffusion and Measurement in Three Different Directions for the C.P.2.1 Strain in Milk Agar, Isolated from the Illegal Dumpsite in Condora

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Figure 4 presents the feather weight loss (g) recorded during the degradation process by fungal strains isolated from the dumpsite. This figure shows the average weight loss values at different incubation days. As incubation time progressed, feathers inoculated with proteolytic fungal strains exhibited a gradual and increasing weight loss. The C.P.2.1 strain demonstrated the highest keratin degradation capacity, with up to 12% weight loss in just eight days of fermentation, followed by strains C.S.4 and C.S.7, which showed lower degradation efficiency. Although the differences between strains were not statistically significant, a significant difference was observed compared to the control group (without fungal spore inoculation), with a p-value <0.05 according to Tukey's test.

Loja [51] reported in his study on keratin extraction from chicken feathers using enzymes produced by Bacillus spp. that hydrolytic degradation of 9.4% was achieved within 24 hours. This research highlights the efficiency of bacteria from the Bacillus genus in keratin degradation.

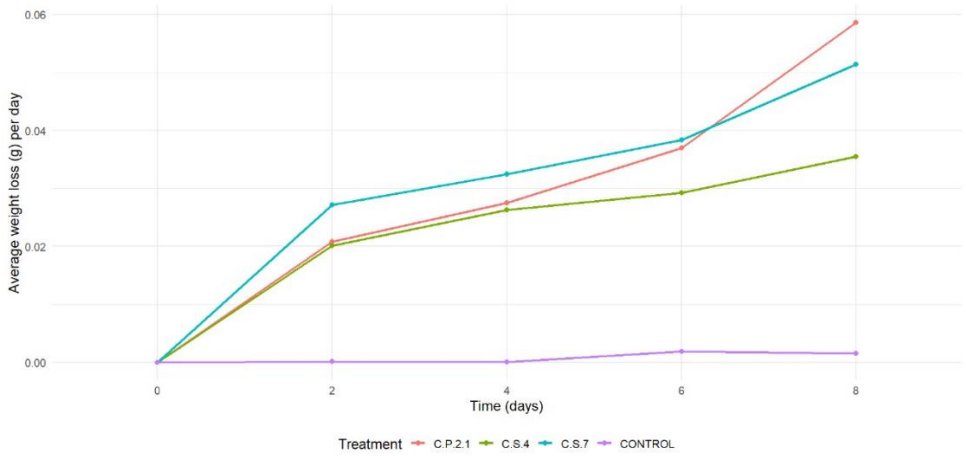

Figure 4.
Feather Weight Loss (g) During Degradation by Fungal Strains Isolated from the Illegal Dumpsite in Condoray

Bacillus species exhibit a higher metabolic rate compared to fungal strains, which have a slower metabolic process but achieve comparable results in feather degradation.

Figure 5 presents the total protein concentration (mg/mL) released during the feather degradation process by fungal strains isolated from the dumpsite. As the degradation process progresses, an increase in protein release into the liquid medium is observed. The C.P.2.1 strain exhibited the highest protein release, reaching up to 0.40 mg/mL, followed by the C.S.7 strain with 0.32 mg/mL and the C.S.4 strain with 0.15 mg/mL. These numerical differences were statistically significant compared to the control group (without fungal spore inoculation), with a p value <0.05 according to the statistical test conducted.

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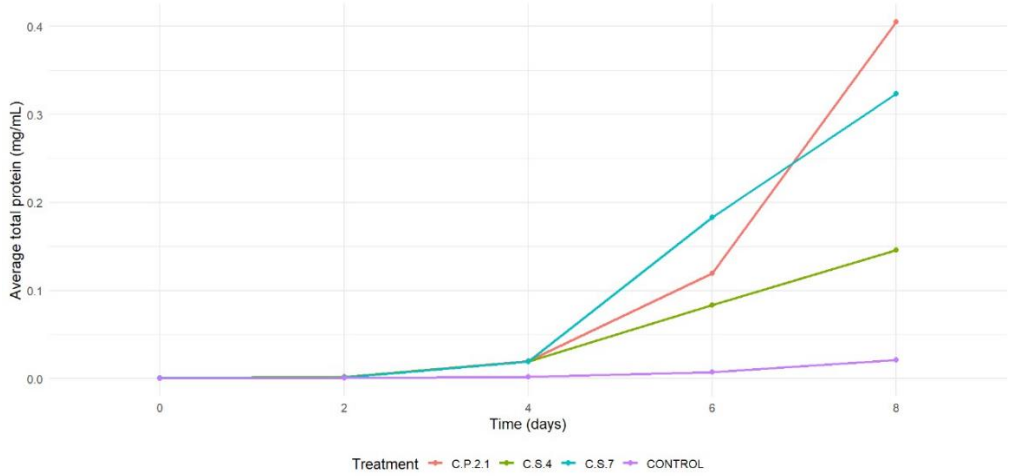

Figure 5.
Average Total Protein Concentration (mg/mL) Released During Feather Degradation by Fungal Strains Isolated from the Illegal Dumpsite

Correa [42] discusses that the increase in protein concentration in the medium is associated with the activity of keratinolytic enzymes. Keratinases, which function best under alkaline conditions, show increased activity as the medium's pH rises from 4 to 8 during the degradation process. Although keratinase activity may decline towards the end of the process, this does not necessarily mean that it has ceased entirely; other factors, such as the presence of similar substrates or high levels of end products, could be limiting its function, as suggested by Geisseler and Horwath [52]. These findings support the trend observed in our study, where protein accumulation in the medium occurred during feather degradation by the evaluated fungal strains.

The study by Bohacz [50] investigated the ability of Aphanoascus fulvescens and Chrysosporium articulatum strains to degrade keratin in chicken feathers. Using Response Surface Methodology, they optimized the culture conditions to maximize keratinolytic activity.

The stationary cultures were conducted at 28°C, with feathers as the sole carbon and nitrogen source. A correlation was observed between keratinase activity and the release of proteins and peptides, as well as between caseinolytic protease activity and sulfate ions. The Aphanoascus fulvescens B21/4-5 strain exhibited the highest substrate mass loss (65.9%) compared to the reference strain CBS104.62 (35.4%), achieving a maximum loss of 71.08% at pH 7.58 and 28.7°C. Meanwhile, Lopes, et al. [53] studied Aspergillus niger, identified through sequencing of the ITS region of rDNA, which was capable of growing in feather meal as its sole carbon and nitrogen source. This strain produced slightly acidic keratinase and acidic protease, with peak activity at 48 and 96 hours, respectively. The enzymes were characterized as serine and aspartic proteases. By using a central composite design combined with response surface methodology, they optimized pH and feather meal concentration. The optimal conditions were pH 5.0 for protease, pH 7.8 for keratinase, and 20 g/L of feather meal. Both studies highlight the ability of these microorganisms to degrade keratin, a crucial factor for biotechnological applications. Optimizing culture conditions and characterizing enzymes provide valuable insights for improving the efficiency of biotechnological processes, including the production of proteolytic and keratinolytic enzymes, which are essential across industries ranging from food processing to environmental management.

Three Penicillium strains were isolated. On Sabouraud agar, after 10 days of incubation, the colonies exhibited white hyphae that turned dark green or olive upon sporulation, with a mustard-colored reverse, a powdery texture, circular edges, and slow to moderate growth. Microscopically, the septate hyaline hyphae featured long, smooth conidiophores with metulae and phialides arranged in groups of

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three to six, forming chains of globose and hyaline conidia. Other researchers, such as Correa [42] in their study on keratinolytic fungi in cattle farm soils in Lima, Peru, have also reported molds with similar keratinolytic capacities. Similarly, Betancourt, et al. [54] in their study "Isolation of Filamentous Fungi from Cat Fur Without Skin Lesions in Temuco, Chile," reported that nearly all isolated dermatophytes matched those described in the reviewed literature, including species of the Penicillium genus, which are considered opportunistic keratinophilic fungi.

5. Conclusions

From the soil and feather samples obtained from the clandestine landfill in Condoray – Ayacucho, 18 fungal strains with potential keratin-degrading abilities were isolated. However, only three strains, identified as C.P.2.1, C.S.4, and C.S.7, demonstrated significant protein degradation capacity, qualifying them as proteolytic.

The fungal strain C.P.2.1 exhibited outstanding proteolytic capacity, evidenced by the formation of enzymatic diffusion halos in skim milk agar with a diameter of 17.00 mm. This result places it above strains C.S.7 and C.S.4, which presented halos of 12.33 mm and 12.00 mm, respectively. These measurements indicate higher enzymatic activity in C.P.2.1, suggesting its superior potential for proteolytic applications.

The degradative efficacy of the strains on bird feathers was evaluated by measuring the substrate's weight loss after eight days of incubation. The C.P.2.1 strain showed the highest degradative capacity, reducing the feather weight by 12%. Strains C.S.7 and C.S.4 achieved reductions of 10% and 8%, respectively. These results are significant compared to the control, indicating that these strains have considerable keratin degradation potential in bird feathers.

The C.P.2.1 strain also excelled in terms of total protein concentration released into the fermentation medium, reaching a value of 0.4 mg/ml. This demonstrates its high degradative capacity, followed by strains C.S.7 and C.S.4, which reached concentrations of 0.32 mg/ml and 0.15 mg/ml, respectively. These values were significantly higher than the control, indicating effective protein release during feather degradation.

The three keratinolytic strains isolated from soil and feather samples from the clandestine landfill in Condoray – Ayacucho (C.P.2.1, C.S.7, and C.S.4) were classified as belonging to the Penicillium genus.

Transparency:

The authors confirm that the manuscript is an honest, accurate, and transparent account of the study; that no vital features of the study have been omitted; and that any discrepancies from the study as planned have been explained. This study followed all ethical practices during writing.

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