Original Research Article

Biotechnological Strategy for the Valorisation of Feather Waste Using Keratinolytic *Actinobacteria*

Abstract

In Nigeria, both local poultry farms and industries produce significant amount of feathers, which is a rich source of keratin. The traditional method of disposal for this waste is incineration or landfill which could result to an environmental effect and depletion of resource. Complex biopolymers undergo turnover by the action of various microorganisms including *Actinobacteria*. This is achieved by the ability of these organisms to secrete an array of extracellular enzymes. This research focuses on a cost-effective biotechnological approach for the environmentally friendly valorisation of feather waste by employing *Actinobacteria* isolated from the soil compost obtained from the dumping site of feather waste. *Actinobacteria* were isolated from the soil compost obtained from the dumping site of feather waste and screened for their ability to degrade raw chicken feather and keratinase production. Among the isolated strains of the *Actinobacteria,* isolate-13 was identified as the most potent for biodegradation of feather waste. The intact native feathers were about 97% degraded after 96 hours of cultivation with the isolate-13 and the initial production of extracellular keratinase under submerged fermentation was found to be 12.35 ± 0.11 U/ml. Temperature, pH, substrate, and incubation period were all found to influence the biosynthesis of extracellular keratinase by the *Actinobacteria* isolate-13. These results highlight the potential economic and environmental benefits, as well as sustainable poultry feather waste management strategy.

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Keywords: *Actinobacteria*; Biodegradation; Feather waste; Keratinase.

INTRODUCTION

Commented [AA2]: The literature related to isolated Actinobacteria research still needs to be increased so that data on various hair disposal areas in various places can be known.

In Nigeria, poultry farming is experiencing rapid transformations and the growth of this business has increased significantly in recent years. As a result, a significant amount of feather is generated as a poultry farms waste, and the potential application of these waste has been neglected for long period. Traditionally, they are either used as cushions and comforters

filling materials, burn or landfills, which could result to polluting the environment. About 90% of feather content is keratin, and keratin is an insoluble protein that is resistant to degradation by common proteolytic enzymes (Wang et al. 2016). In keratin, the high amount of cysteine residues results to more cross-linking of disulphide bridges and consequently a high mechanical stability (Li et al. 2016). Common methods of feather wastes disposal, which includes landfill, steam pressure cooking, alkaline hydrolysis and traditional burning causes great environmental problems. Biodegradation is considered the most promising alternative method for converting feather wastes into useful environmentally friendly products (Shigeri et al. 2009). The use of microbes and natural waste materials to develop new organic products has attracted more attention in biotechnological researches. The microbial and biotechnological approach is a promising alternative in keratinous wastes recycling using microbial enzymes (Mehta et al. 2014).

Microorganisms are known to utilize feather waste due to their ability to produce keratin-dissolving protease. When present in an environment with availability of keratinous substrate, these microbes can produce keratinase (Desai et al. 2010). *Actinobacteria* are known for its ability to utilise keratin as carbon and nitrogen source. Previous study reported the successful bioconversion of non-soluble keratin into soluble constituents by submerged fermentation of poultry wastes with *Actinobacteria* (Suntornsuk et al. 2005). Microbial keratinases continue to gain more attention in the enzyme market due to an increasing demand to develop a robust alternative biotechnological method for the bioconversion of keratin-rich organic wastes into valuable products (Williams et al. 1990). This alternative approach for the valorisation of much available feather wastes into a low-cost value-added product is a method that is more convenient and environmentally friendly with potential of industrialization. In Nigeria today, there are no efficient and eco-friendly methods to convert feather wastes into value-added

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products. The current study focused on the bioconversion of chicken feather by cultivation of soil *Actinobacteria* isolated from feather waste dump site in Birnin Kebbi metropolis.

MATERIALS AND METHODS

Sample collection

Soil sample was obtained from the dump site of feather waste in Birnin Kebbi metropolis, Kebbi State, Nigeria (12º 27’ 14.00’’ N, 4º 11’ 51.00’’ E). Soil compost was collected at the depth of approximately 10 cm from beneath of feather waste and transported to the laboratory in a sterile container. Collected soil sample was dried overnight in foil tray under sterile cheesecloth then placed in an oven at 60 ºC for 90 minutes. After allowing the soil to cool, 1 gram of the soil was subjected to a two-step treatment, first with 0.1 g $CaCO_3$ and then 1.5% phenol for 30 minutes.

Preparation of Chicken Feather Powder

Commented [AA3]: Why not do an initial test of proteolytic bacterial activity before keratinolytic activity?

Poultry feathers were obtained from a local poultry processing site in Birnin Kebbi. Clean water was used to wash the raw and thereafter, they were dried at 45 ºC for 48 hrs in an oven. The dried feathers were boiled for 3 hrs and dried under the sun until constant weight was obtained. Subsequently, the sun-dried feathers were pulverized to flour and stored in a sterile container (Agrahari and Wadhwa, 2010).

Isolation and Identification of *Actinobacteria*

Commented [AA4]: This research begins with the identification of microbial proteases first

Soil sample (1g) was added into 9 ml normal saline from which a serial dilution of up to 10^{-5} was obtained. From the 10^{-5} dilution, 100µl was inoculated on starch casein agar that was previously prepared and added 50 µg/mL cycloheximide and 50 µg/mL nalidixic acid. This is to not allow the growth of fungi and gram-negative bacteria respectively (Cheng et al. 2010). The inoculated agar plate was incubated at 28 ºC for 21 days.

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Biodegradation of Chicken Feather

Digestion of chicken feathers was carried out by modified method of Kshetri and Ningthoujam (2016). Briefly, 17 selected isolates were inoculated individually in basal media (0.1% KH_2PO_4, 0.3% K_2HPO_4, and 0.02% $MgSO_4$, pH 7.5) containing sterile raw intact chicken feathers and incubated in a shaking incubator at 37 ºC with 150 rpm shaking. After every 24 h interval, the degradation of feather was monitored up to a period of 96 hours. The control was set up in a basal media containing the sterile raw intact chicken feather without inoculating the *Actinobacteria* isolate.

Production of Crude Extracellular Keratinase

Commented [AA5]: How do you know if you have keratinase if you haven't had a protease test?

The production of keratinase enzyme was set-up in a 250 ml Erlenmeyer flask which contained basal medium components as follows: chicken feather powder (10 g): KNO_3 (5g); KH_2PO_4 (1g); K_2HPO_4 (3g); $MgSO_4.7H_2O$ (0.5g); $FeSO_4$ (0.01g); $CaCO_3$ (0.02g), per litre and the pH of the media was adjusted to 7.0. A 5 ml of the best feather degrading bacterial inoculum (referred to islate-13) was added into 45 ml of the production media and incubated for 24 hrs at 37 °C with agitation at 150 rpm. After the 24h incubation period, the media was centrifuged at 5000 rpm for 20 min (Wang et al. 2017). The supernatant was assayed as crude extracellular keratinase.

Assay of the Crude Extracellular Keratinase Activity

The enzyme activity of extracellular keratinase was measured spectrophotometrically in accordance with a modified method reported by Akram et al. (2021). Briefly, 10 mg of chicken feather flour was added into 1 mL of Tris–Cl buffer at 50 mM with a pH of 8.0, then 1 mL of the crude extracellular keratinase was added and incubated at 45 °C for 30 min. After the incubation period, 2 mL of Trichloroacetic acid at 0.5M was added to stop the reaction. The reaction mixture was centrifuged at 10,000 × g for 10 min and the supernatant was

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analysed spectrophotometrically at OD_{595nm}. Both the experiment and control were performed in triplicates with the control reaction containing all the reaction components as described above except that the enzyme was not added, however, 1 mL of enzyme which was deactivated with 2 mL of TCA were added in the control after incubation to balance the colour intensity with that of the experiment. One unit (U) of enzyme was defined as the amount of keratinase that increases the absorbance of 0.01 as compared to control under the described assay conditions and this was obtained by applying the following Equation:

$EA = (\Delta A \times V \times f)/(\varepsilon \times l \times v_o)$ (Equation 1)

ΔA = change in absorbance

V = final volume of the reaction

f = dilution factor

ε = extinction coefficient

l = path length of the cell

v_o = volume of the enzyme used.

Factors Affecting the Production of Keratinase

The experiment was performed by one factor at a time method that is in each experiment, variation of a single factor was carried out, while keeping the others constant. A 5ml of fresh seed culture was added into 245 ml sterile basal medium containing 10 g/ml chicken feather powder and allowed to incubate for 72 hours with shaking at 150 rpm and enzyme activity was assayed afterwards. The temperature and pH effects on enzyme production were evaluated by setting up enzyme production at different temperature ranges (25, 30, 35, 40, 45, 50, 55, and 60 °C), and different pH ranges (5, 6, 7, 8, 9, 10, 11, and 12). In addition, different substrates including skimmed milk powder, casein, and keratin were used to assess

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the more suitable substrate for keratinase production by the isolated actinobacteria. The effect of incubation period on keratinase production was also evaluated by incubating the production media for a period of seven days and collecting aliquots on each day to assess the enzyme production by assaying the enzyme activity.

Statistical analyses

Each experiment was repeated 3 times and results are presented as Mean ± SD values. Analysis of Variance (ANOVA) and Duncan's Multiple Range Test (DMRT) were applied to obtain the significant differences of the data. Results of all the experiments were significant at $p \leq 0.05$.

RESULTS AND DISCUSSION

Commented [AA6]: Why not perform PCR analysis of bacterial DNA with 16sRNA primers on microbes?

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Isolation and identification of *Actinobacteria*

As a result of environmental pollution caused by the traditional methods (physical/chemical) for feather waste treatment, attention is now turning towards keratinases as an alternative green approach (De Oliveira et al. 2016). Microbial fermentation is considered to be a more economical method and many researches have been conducted with the aim to isolate natural feather degrading bacteria for applications in industrial feather hydrolysis (Cedrola et al. 2012; Gu et al. 2016; Callegaro et al. 2018). In our study, a total of 17 keratinase-producing actinobacteria were isolated and identified based on their distinct colony morphology appearances (Figure 1). The isolates exhibited an aerial hypha, an arranged chains of smooth spores and a non-fragmented substrate mycelia, on this basis, they were regarded to be *Actinobacteria*.

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Fig. 1. Representative *Actinobacteria* isolates.

Biodegradation of Chicken Feather

Feather degradation ability of the Actinobacteria isolates were assessed by the physical monitoring of the disintegration of the intact raw chicken feathers. In the submerged fermentation media, feather degradation by the isolate-13 was observed to be maximum after 96h, this is an indication that isolate-13 produced keratinolytic enzyme as the primary bioactive metabolite that enabled it to grow in a media containing feather as the sole source of carbon and nitrogen. Isolate-13 was able to degrade about 95% of chicken feathers after 96 hours fermentation (Figure 2), hence, it was considered the most potent keratinase-producing isolate among the 17 selected actinobacterial isolates.

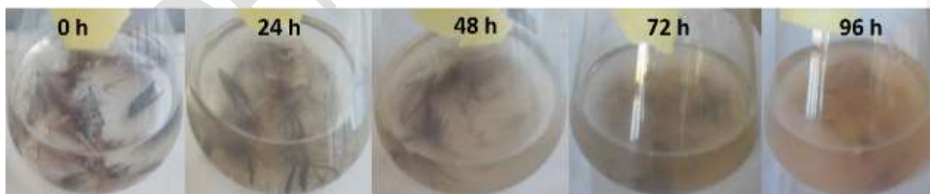

Fig. 2. Visual observation of feather degradation ability of the *Actinobacteria* isolate-13 after 96 h.

Production of Crude Extracellular Keratinase

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Microbial keratinases are extracellular in a keratinous substrate culture (Gupta and Ramnani 2006). In the present study, *Actinobacteria* isolate-13 was found to possess keratinolytic potential as it exhibited the ability to produce keratinase in submerged fermentation. The production of extracellular keratinase by isolate-13 was initially 12.35 ± 0.11 U/ml at 72 h post inoculation under culture media parameters of 37 ºC, pH 7.5 when 5 g/L of feather powder was used as substrate. Several *Actinobacteria* strains were previously reported to produce keratinolytic protease (Jain et al. 2012; Wang et al. 2017; Deniz et al. 2019; Al-Dhabi et al. 2020).

Factors Affecting the Production of Keratinase

Microbial production of keratinase is affected by factors, such as temperature, pH, and substrate type, and different species have a particular response to these factors (Kumar et al. 2010). Temperature is one of the significant factors that influence enzyme production. In this work, the temperature effect on keratinase production was assessed at different temperature points, and the highest extracellular keratinase production of 31.35 U/ml was recorded at 45 ºC (Figure 3).

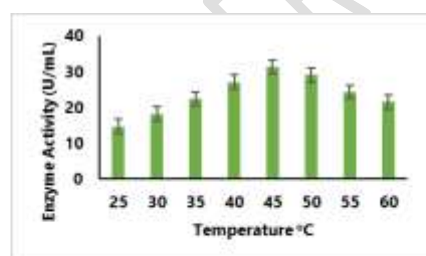

Fig. 3. Production of keratinase enzyme by *Actinobacteria* isolate-13 under different incubation temperature.

Maximum keratinase production by *Actinobacteria* was previous achieved when the incubation temperature was set at 40 ºC (Yeoman and Edwards, 1994; Al-Dhabi et al. 2020).

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This temperature variation for maximum keratinase production between our results and previously published research works may be attributed to the different strains of *Actinobacteria* under study. *Actinobacteria* isolate-13 attained maximum keratinase production of 18.57 U/ml at pH 9 (Figure 4). Studies have shown that many proteases are more effective in an alkaline pH, and this could be of benefits in industrial applications (Elbondkly, 2010). This indicates that, results of our study are in agreement with previously published literatures (Jaouadi et al. 2008; Wang et al. 2017; Al-Dhabi et al. 2020), hence, it is safe to assume that our keratinase is an alkaline protease.

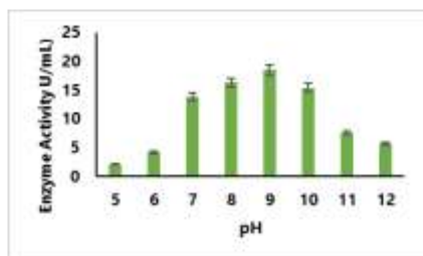

Fig. 4. Production of keratinase enzyme by *Actinobacteria* isolate-13 under different incubation pH.

To determine how substrate affect keratinase production by the isolate-13, temperature and pH of the fermentation media were adjusted to 45 °C and 9 respectively. The result shows that the maximum keratinase production of 32.72 U/ml was achieved in the presence of keratin followed by chicken feather powder with 23.37 U/ml (Figure 5).

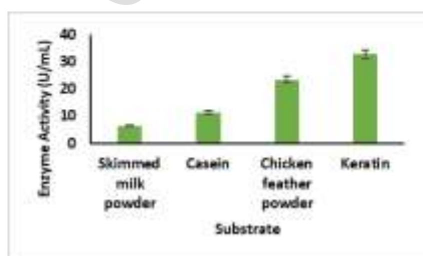

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Fig. 5. Production of keratinase enzyme by *Actinobacteria* isolate-13 using different substrates.

Finally, the effect of incubation period was investigated where the culture media temperature and pH were maintained at 45 ºC and 9 respectively and feather powder was used as substrate. Production keratinase was observed to be at maximum yield of 38.83 U/ml after 5-days of inoculation after which the production began to decline at day 6 (Figure 6) and this could be attributed to a loss of enzyme activity due to enzymatic autolysis and/or end product inhibition. Our results show consistency with other studies that reported highest yield of keratinase at 5th day post inoculation (Al-Dhabi et al. 2020).

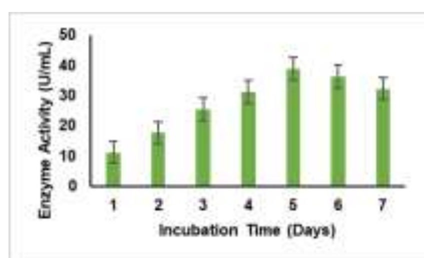

Fig. 6. Production of keratinase enzyme by *Actinobacteria* isolate-13 at different incubation periods.

Optimization of Physicochemical Factors

Optimization of the physicochemical factors including the incubation temperature (45 ºC), pH (9), substrate (10 g/L chicken feather powder) and 5 days of incubation resulted to an increased in yield of keratinase by the isolate-13 to 52.37 ± 0.21 U/ml from the initial 12.35 ± 0.11 U/ml under fermentation conditions of 37 ºC, pH 7.5, 5 g/L chicken feather powder and 72 h incubation period. These results suggest that *Actinobacteria* isolate-13 can efficiently degrade chicken feathers and also is a good source of keratinase, thereby, making it a potential alternative green technology for poultry feather waste management. We envisage

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that this strain of *Actinobacteria* and the enzymes it produces could be exploited for various applications in industries.

Bacillus spp. has become the most predominantly studied keratinolytic enzyme producing bacteria as reviewed (Andesaw, 2022). Recently, Amuthavalli and Ravi reported the isolation, characterization and optimization of keratinolytic bacteria from chicken feather waste dumping site. In their study, the isolated bacteria were identified as *Bacillus licheniformis*, and the keratinase production was maximum at 40°C and pH 8.5 (Amuthavalli and Ravi, 2022) suggesting that keratinolytic bacteria prefer high temperature and alkaline pH for optimal keratinase production.

Actinomycetes, especially the genus *Streptomyces*, are regarded as keratinolytic enzyme producers. Different strains of this bacterial group have been isolated from different ecosystems including terrestrial and aquatic and were found to be degraders of different keratin materials. For example, *Streptomyces* sp. CHA1 and *Streptomyces* sp. G11C were reported to exhibit significant feather degradation activity both strains were reported to have a maximum activity of keratinase at 50 ºC and pH 9 (González et al. 2020). In another study, a keratin-degrading *Actinobacteria* was isolated from poultry farm and their feather degrading ability was assessed. It was discovered that the isolated *Actinobacteria* was able to degrade feather and the maximum keratinase production was achieved under incubation temperature and pH of 45 ºC and 5 respectively (Alamoudi et al. 2022). Most recently, an actinobacterial strain referred to as *Streptomyces* DZ 06 (ES41) was reported to use a keratin-rich residues to produce keratinase (Hamma et al. 2024). The optimal incubation temperature and pH for microbial keratinase enzymes production may very much varies, largely due to the source and strain of microorganism. Generally, microorganisms show optimal keratinase production between 40-70 ºC incubation temperatures and incubation pH of 7.5–9.0 (Brandelli et al. 2010), which very much correlate with the results of our study. Recognition

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of keratinolytic microorganisms is based on their ability to produce specific keratin degrading proteases. Both bacteria and fungi are found to be potent producers of keratinases (Kunert, 1989; Błyskal, 2009; Habbeche et al. 2014). However, majority of research focus on bacteria for biological conversion of the keratin waste, possibly due their ability to proliferate and produce the enzyme rapidly, their potential industrial applicability, and the possibility to use the whole bacterial cell for keratin degradation, thereby removing the need to extract the enzyme.

CONCLUSION

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Bacterial isolates can utilize keratinous waste such as poultry feathers as carbon and nitrogen source in fermentation media to produce keratinases under various experimental conditions. This makes it feasible to convert poultry waste that is considered non valuable into value-added products. In this study, utilization of chicken feather as source of carbon and nitrogen facilitated the selection of an actinobacterial strain which was capable of degrading feather and also produced extracellular keratinase. Keratinase production by the *Actinobacteria* isolate-13 was influenced by temperature, pH, substrate, and incubation period. The isolated strain shows promising potential in biodegradation of poultry feather waste and this could serve as a better, safe, and eco-friendly way to convert poultry feather waste into useful raw materials and products for agricultural, industrial, and biotechnological applications.

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