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Title: **Isolation of Keratinolytic Bacteria from Common Ostrich (*Struthio Camelus)* and their Efficacy towards Feather Degradation of Poultry Waste**

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Isolation of Keratinolytic Bacteria from Common Ostrich (*Struthio Camelus)* and their Efficacy towards Feather Degradation of Poultry Waste

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Abstract

Keratinolytic microorganisms have the capacity to biosynthesize particular keratinases and determine their prospective application in poultry waste management. Hence, the objective of this study was to utilize keratinolytic microbes for the degradation of poultry waste. For this purpose, the feathers of common ostrich (*Struthio camelus*) were collected, scattered along the surrounding dry soil, from a private ostrich farm in Kasur, Pakistan. Bacteria were isolated by using culture enrichment techniques and screened for their proteolytic activity on skim milk agar. The isolates were characterized clonially, morphologically, and biochemically and labeled as SNO1, SNO2, and SNO3, respectively. The effects of varied pH and temperatures were recorded on bacterial growth and feather degradation. It was noticed that the bacterial cell densities and feather degradation were high at 50˚C and pH 8.0, as compared to the rest of the culture conditions. Visual observations were made using a stereomicroscope. The maximum feather degradation in the form of white powdery mass was noticed at fourth (4^{th}) week. The protein content of *Struthio camelus* feathers was determined as 6.5 mg/100 ml after degradation. Taken together, the authors concluded that the isolated strains were capable of feather degradation and can be used in poultry waste management.

1. Introduction

The poultry industry is growing worldwide progressively, with large amounts of excreta produced. These wastes could cause serious soil and water pollution if not processed and discharged properly [1-3]. Now a day's ostrich industry is growing due to certain aspects of ostrich products like ostrich meat, leather and feathers, eggs, and multipurpose oil [4-7]. Ostrich breed is much faster and regular as compared to other animals breed. Pakistan's environment is ideal for ostrich farming and it has become quite popular in the country. According to recent reports 2000 birds have been registered for farming in Pakistan [8].

All over the world, large quantities of feathers (40×10^9 kg per year) are being produced as a by-product [9]. The

composition of feathers include mainly keratin which is unyielding to normal proteolytic chemicals, for example, pepsin, trypsin, and papain [10]. Keratin is an insoluble protein macromolecule with high solidness and a low debasement rate [10]. Keratins consist of hard and soft keratins as per the sulfur content. Hard keratins are found in limbs like quills, hair, hoofs, and nails which have high disulfide bond content and are inextensible [11].

The emerging problem in recent eras is the accumulation of biowastes, chiefly produced from slaughterhouses. It triggers scientific interest towards the methods for bio-utilization which includes searching for keratinolytic microorganisms [12]. The strong nature of keratin protein causes hazardous environmental issues and transmission of diseases [3]. Developing interest in the customary recycling process is a pathway toward the utilization of keratinolytic microorganisms in the bioconversion procedure [13]. Waste materials can be converted into valuable products by microbial biodegradation [9, 14, 15]. Presently, more than 30 different microbial species including fungi, actinomycetes, and other bacteria such as *Aeromicrobium spp., Exiguobacter spp.,* and *Marinococcus spp.,* have been used to show keratinolytic properties [10, 16, 17, 18]. Among various microbial groups, numerous keratin degraders are from the bacterial genus Bacillus [12, 16, 19]. This study was conducted to isolate and characterize keratinolytic bacteria from the soil samples collected from a poultry farm. This is useful for the conversion of the poultry shed waste into value-added products in the environment. Thus, after the degradation of feather waste, keratin produced can be used in the leather industry for hair removal and in the formation of organic fertilizer.

2. Materials and Methods

2.1 Collection of Samples and Preparation of Feather Powder

Feathers of *Struthio camelus* (ostrich) along with surrounding dry soil were collected on December 13, 2017, in plastic bags from a private farm located in Kasur (31.1179˚ N, 74.4408˚ E), Pakistan. Feathers were washed and extensively autoclaved for 15 minutes at 121ºC, and dried in a hot air oven for 4 hours at 50ºC. Then, feathers were crushed in a grinder machine (RRF-250) to obtain a fine powder.

2.2 Isolation and Screening of Bacterial Isolates from Ostrich (*Struthiocamelus*) Feather

Isolation of bacterial isolates from the soil of a private ostrich farm was done by modified culture enrichment technique method of Martinus, (1901) explained by Kumar *et al*., (2016) [11]. Soil (5 grams) was weighed and added into the 250 ml flask containing 100 ml keratin media (10 g feather powder; 0.5 g NaCl; 0.3 g K2HPO4; 0.4 g KH2 PO4; 0.1 g MgSO4 .6H2O; pH 7.5). The flask was incubated for one week at 37°C in a non-shaking incubator. The 10 ml culture broth from these enriched flasks was transferred into fresh keratin media (100 ml) and 2.56 grams of *Struthio camelus* feathers as a sole carbon source and incubated again for up to 4 weeks at 37°C. After 4 weeks of culture incubation, bacteria was isolated by serially diluting the sample from the enriched flask, and 10^{-5} dilution was spread on a nutrient agar medium. The colonies were screened for proteolytic activity [20]. Plates were incubated at 37ºC for 24 hours. Thus, bacterial colonies (22 types) were obtained and purified by quadrant streaking.

2.3 Screening on Skim Milk Agar Plates for Proteolytic Activity of Bacterial Isolates

The purified isolates were further streaked on skim milk agar plates [21]) for the testing of proteolytic activity. The skim milk agar plates were incubated at 37 °C for 24 hours. Three isolates that produced clear zones around bacterial colonies on the plates were selected for the further morphological and biochemical characterization and were named as SNO1, SNO2, and SNO3.

2.4 Preparation of Bacterial Inoculums

A loop full of purified bacterial colonies from respective plates was inoculated in L-broth and incubated at 37°C for 24 hours. The optical density of bacterial inoculum obtained from fresh culture (obtained after 24 hours) was adjusted to 10^8 cell densities (OD 0.5 at 600nm) using a spectrophotometer.

2.5 Effect of Various Media, pH, and Temperature on Bacterial Growth

The effect of different physiological parameters on bacterial growth, (showing proteolytic activity), different pHs (7 and 8), temperatures (4, 37, and 50°C), and media (L-broth and N broth) were used to study their effect on bacterial cell densities. Bacterial colonies were inoculated with each isolate in L-broth and N broth (adjusted at varying pH) and incubated at varying temperatures (4, 37, and 50˚C) for 24 hours. Finally, cell densities were recorded by using a spectrophotometer (Specord 200 plus Germany).

2.6 Colony and Morphological Characteristics of the Bacterial Isolates

Cultural characteristics, morphological, and biochemical behaviour of bacterial isolates were done by Bergey's manual of determinative biology.

2.7 Biochemical Tests

Identification of the biochemical properties of the bacterial isolate was done using, different biochemical tests (such as VP (Vogues Proskauer), methyl red, catalase, urease, indole production, citrate utilization, DNase test H_2S production, and motility assay).

2.8 Feathers (*Struthio camelus*) Degradation

To study the experimental setup for feather degradation fresh bacterial cell culture of all purified bacterial isolates SNO1, SNO2, and SNO3(cell densities adjusted at 10^8 cells per ml-OD 600 nm) were added in the equal ratio (1:1:1) to flasks containing 100 ml fresh keratin media and freshly added *Struthio camelus* feathers as a carbon source (2.56 grams). The culture was incubated for 4 weeks. The controlled flasks were incubated in the absence of bacterial inoculation.

2.9 Image Analysis of Feather Degradation

Visual analysis of feathers degradation was done before and after 4 weeks of experimental setup using a stereomicroscope. A sample of feathers (1ml) of *Struthio camelus* from inoculated and non-inoculated, flasks was placed on a Petri plate and observed by using a stereo microscope before and after the completion of the experiment.

2.10 Change in pH observed during Culture Incubation

The pH of the culture was recorded three times during four week incubation period and the value of pH was noticed after every 10 days using a pH meter (HI98163).

2.11 Protein Concentration

To determine the protein analysis of degraded feathers, protein concentration was determined by the method of Lowry et al. [22] using bovine serum albumin as a standard. OD of blue color as the endpoint was recorded at 750 nm using a spectrophotometer (Specord 200plus Germany).

3. Results

From the soil of the ostrich farm, 22 bacterial isolates were isolated. However, on the basis of proteolytic ability, only three isolates were selected and their cultural and morphological identification was carried out following Cappuccino and Sherman [20, 23]. It was noticed that the bacterial isolate SNO1 showed white-colored colonies of circular form with undulated margins and umbonate elevation. While isolate SNO2 showed off white-colored colonies with rhizoid form, filamentous margins, and flat surface. Isolate SNO3 showed white-colored colonies with rhizoid form, entire margins, and flat elevation (Table 1). For all the bacterial isolates gram, spore, and capsule staining was performed and results were recorded (Table 2). Different biochemical tests were performed for characterizing the bacteria and the results were given (Table 3).

Table 1. Colonial Characteristics of Bacterial Isolates

Isolates	Colony Color	Form	Margin	Elevation
SNO1	White	Circular	Undulate	Umbonate
SNO2	Off white	Rhizoid	Filamentous	Flat
SNO3	White	Circular	Entire	Flat

Table 2. Morphological Characteristics of Bacterial Isolates

Isolates	Morphological Characterization		
	Gram staining	**Spore staining**	**Capsule staining**
SNO1	Gram negative	Negative	Positive
SNO2	Gram negative	Negative	Positive
SNO3	Gram negative	Negative	Negative

On the basis of colony morphology, staining (gram, spore, and capsule), and biochemical test it was noticed all the 3 isolates were Bacillus species. The *Struthio camelus* feather powder was obtained after oven drying was brown in color with a 157 % yield. Bacterial isolates were selected on the basis of clear zones produced on skim milk agar plates (Figure 1). Bacterial isolates showing clear zones were identified and named as SNO1, SNO2, and SNO3.

Table 3. Biochemical Tests of Bacterial Isolates

Isolates	Voges-Proskauer test	Methyl red test	Catalase test	Urease test	Cit rate test	Indol test	Hydrogen Sulphur test	Motility test	DNase test
SNO1	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
SNO2	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
SNO3	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve

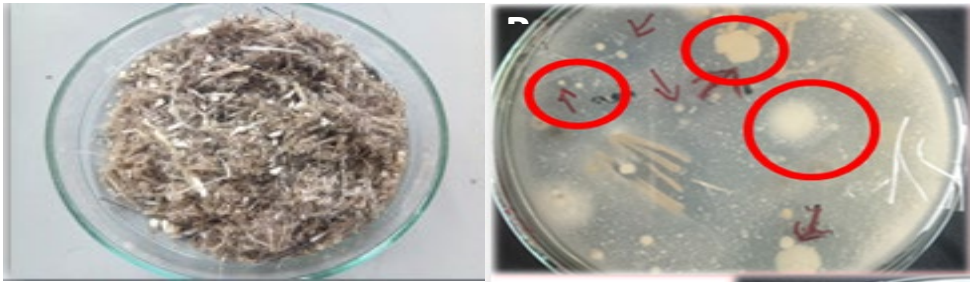

Figure 1. A. *Struthio camelus* feather's Powder,B. Zones of Proteolytic Activity of Bacterial Isolates on Skim Agar Plates

To study the effect of different physiological conditions on keratin degrading bacteria, bacterial growth in term of cell densities was recorded by growing bacterial cultures at different pHs (7.0 and 8.0) and temperatures (4ºC, 37ºC, and 50ºC). Bacterial densities were high at high- temperature 50ºC as compared to 4˚C and 37°C (Figure 2). Similarly, bacterial growth was observed high at pH 8.0 as compared to pH 7.0 (Figure 3). However, SNO2 showed maximum growth at pH 8.0 and 50 ºC among all bacterial isolates.

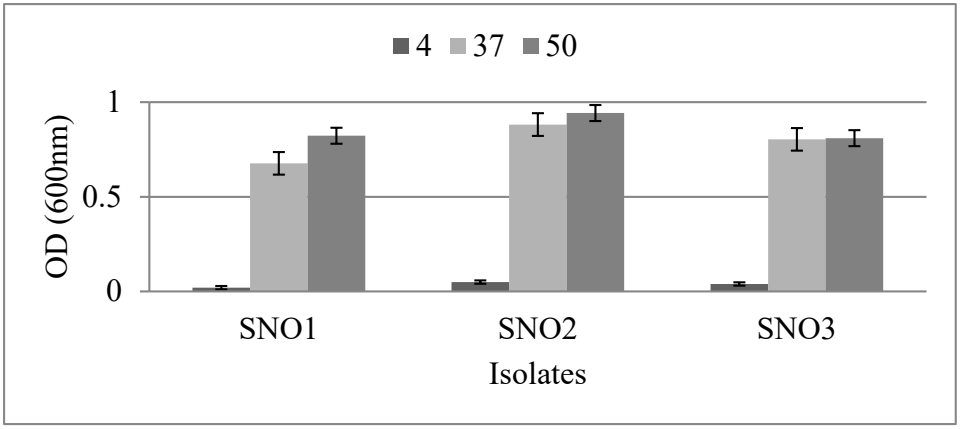

Figure 2. Effect of Varying Temperatures (4, 37, and 50 °C) on Bacterial Growth

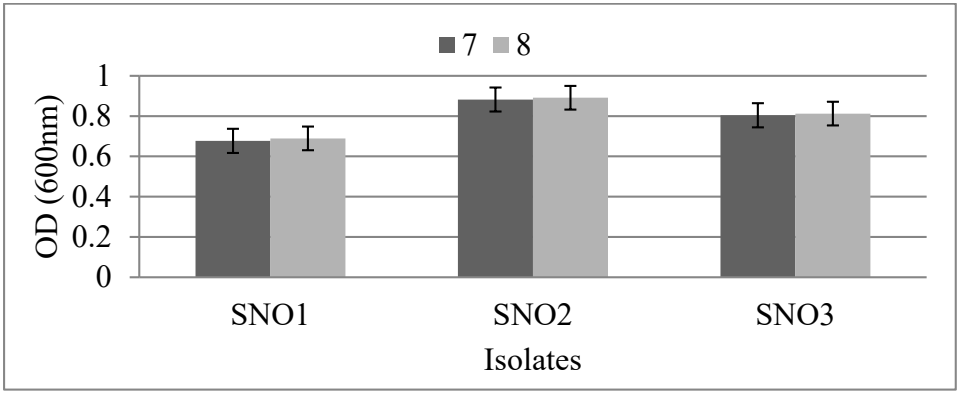

Figure 3. Effect of Different pH (7.0 and 8.0) on Bacterial Growth

The effect of different temperature on biodegradation of feathers was observed and an increase in degradation rate was noticed at high temperature. Furthermore, due to more bacterial inoculums reduction in the time period for degradation was seen by 10 days earlier as compared to non-inoculated samples. It was also observed that the amount of keratin per gram feather weight was highest at pH 8.0 and temperature 50°C as compared to the rest of the conditions. In general, high temperature and high pH favored keratin production in the presence of bacterial consortia. The graduate increase in pH during the biodegradation of feathers was observed irrespective of the incubation temperature. The timely recorded pH at 10 days increased of experimental setup showed that pH also increased with time and media became alkaline. Degradation of feathers in the presence of bacterial inoculation increased at pH 8.0 and this was associated with a significant increase in media pH. These results are in line with bacterial growth at high pH, showing alkaline environment stimulated the rate of biodegradation (Figure: 4). At the end of the fourth week white powdery mass along with complete feather degradation was noticed (Figure: 5).

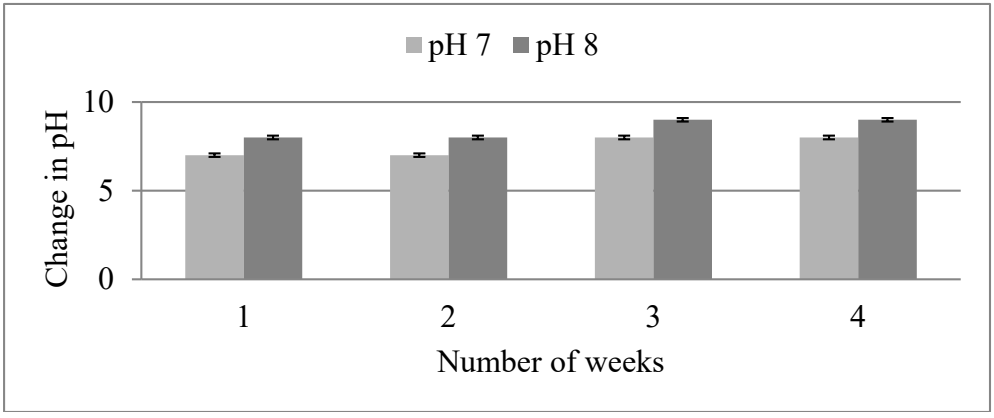

Figure 4. Change in pH Every 10 Days of Experimental Setup Incubated at Temperatures 4˚C, 37˚C, and 50˚C.

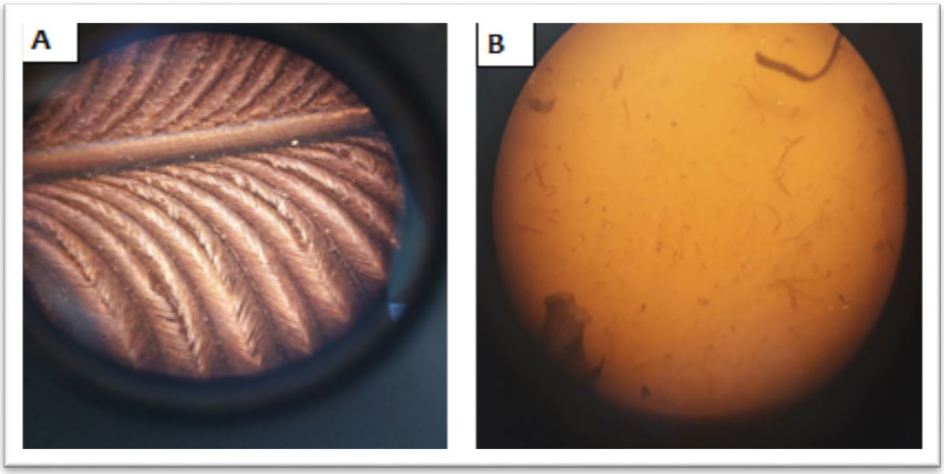

Figure 5. A. *Struthio camelus* Feather Stereo Micrograph; B. Stereo Micrograph of Degradation of *Struthio camelus* Feathers After 4 Weeks

Degradation of feather substrate was found to be associated with high temperature and a significant increase in pH of the medium to alkalinity. Thus, it served the purpose of an indicator for the efficiency of degradation.

The protein content of the inoculated flask was determined by using the method of Lowry et al. [22] and6.5 mg/100 ml was recorded in the present study (Figure 6).

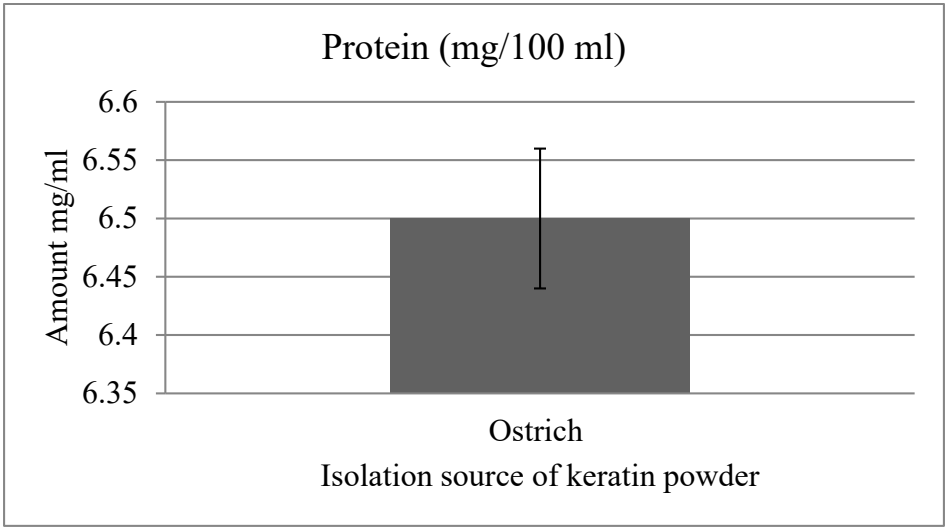

Figure 6. Amount of Protein Contents from Keratin Powder Obtained from *Struthio camelus* Feathers

4. Discussion

In the current study, bacteria were isolated from feathers by surrounding dry soil of *Struthio camelus* (ostrich) which were used to observe their efficacy for feather degradation. Samples were collected from a private farm located in Kasur. It was noticed that the feathers and soil of ostrich were rich in bacterial diversity as 22 types of bacterial colonies were isolated in the existing study. However, only three bacterial isolates showed feather degradation. This study is in line with the findings of Lang et al. [23] who reported that the soil associated with poultry farms harbours a large bacterial fauna that is also involved in biodegradation. Similarly, Veenayohini and Sangeetha [24] reported both the soil and feather samples; acted as a natural habitat for a wide variety of bacterial species.

The selected isolates were studied for their colonial, morphological, microscopic, and biochemical characteristics. SNO1 and SNO3 showed gram-negative, spore-negative, and capsule-positive staining, while SNO2 showed gram-negative, spore-negative, and capsule- positive staining, while biochemical tests showed results of the negative Voges-Proskauer test and positive methyl red, catalase, urease, citrate, indole, hydrogen sulphide, motility, and DNase test. Based on these results, isolates were determined which belong to *Bacillus spp.* In the previous studies, five bacterial isolates that were characterized and identified based on colony morphology, growth characteristics, and biochemical characteristics were found to belong to genera *Aeromicrobium spp., Exiguobacter spp., Marinococcus spp., Bacillus spp1, Bacillus spp,* respectively [10]. *Bacillus* strains were able to produce keratinases in the presence of diverse keratinic waste [25-27]. Majority of reports regarding keratin-degrading actinobacteria and other bacteria include *Bacillus spp* [17, 31]

Degradation of feather substrate was found to be influenced by a significant increase in pH of the medium to alkalinity, thus, by serving the purpose of an indicator for the efficiency of degradation. At low pH values, the enzyme systems may be disrupted and at high pH, metal solubility may be affected [32]. The optimum activity of the enzyme was at alkaline pH and used in the degradation of feathers as well as in the leather industry [10]. Bacterial growth was relatively high at pH 8.0 as compared to pH 7.0. SNO2 showed maximum growth at pH 8.0. Bacterial densities were high at temperatures of 50˚C as compared to 4 and 37˚C. The effect of temperature was noted as an increase in temperature causes an increase in degradation rate. The results of the current study showed that bio-degradation ability is enhanced by the increase of pH and temperature and is comparable with the literature [12, 13, 24, 25]. The optimum temperature for proteases and keratinases was determined over a range of 30–60°C [12]. The *Bacillus subtilis* and *Bacillus licheniformis*, studied for their enzyme activity showed a wide range of temperatures (30- 80 °C) and an optimal temperature to be 50 °C [24]. pH also increased with time and the media became alkaline. The degradation abilities of fungus isolated from dumping soil also showed maximum activity at 8pH and 35°C as reported by Anbu et al. [28]. Moreover, Tiwary and Gupta [29] reported in their study that *Bacillus licheniformis* ER-15 worked well for poultry waste degradation at 11 pH and 70 °C. According to Ni et al. [33] *Bacillus licheniformis* ZJUEL31410 showed promising results for a feather to convert into soluble proteins, particularly at pH 8.0. Furthermore, a study described

Bacillus spp. 45p of Amazonian isolate performed well at pH 7 and 70 °C [30]. In general, high temperature, and high pH favored keratin production in the presence of bacterial consortia. The bacterial isolates showed higher activity at alkaline pH as most of the bacteria, actinomycetes, and, fungi produce Keratinase of pH optima in the neutral to alkaline range. However, Yamamura et al. [34] isolated the bacteria Stenotrophomonas sp. D-1 from the soil containing deer fur and reported 20 °C as the optimum temperature for proteolytic activity. Although, the bacterium showed high efficacy and degraded poultry feathers within 2.5 days at 20°C.The *Struthio camelus* feather powder that was used in keratin media was brown in color with 157 % yield. At the end of the fourth week, the white powdery mass was observed at the end of complete feather degradation. The protein content of *Struthio camelus* feathers was 6.5 mg/100 ml which made up 100 % of the degradation. In the substrate, the protein was estimated as 74% which is relatively high compared to other animal wastes [35].

4.1 Conclusion

The keratinous wastes generated from various industries are increasing and accumulating in the environment mainly in the form of feathers, hairs, horns, hooves, and nails. It was concluded in the current study that isolated bacteria can be a useful strategy to reduce feather accumulation in the environment. The isolated strains are also useful in the leather industry for hair removal and in the formation of organic fertilizer after the degradation of feather waste. Keratin obtained could be used in cosmetics, hydrogels, and agriculture industries. However, the DNA confirmation for isolated bacteria and the wide application of these isolates are required to get the beneficial results.

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