International Journal of Agricultural Technology 2018 Vol. 14(7): 1787-1800
Available online http://www.ijat-aatsea.com
ISSN: 2630-0613 (Print) 2630-0192 (Online)

Isolation and characterization of keratinolytic bacteria from soil samples of poultry waste dumping sites

Reyes, A.[1,2*], Ambita, I. D.[1], Batalon, J. L.[1], Aba, B. L.[1], Cortes, A.[1], Macabecha, C. G.[1] and Montecillo, A.[1]

[1]University of the Philippine Los Baños, College Laguna Philippines; [2]College of Fisheries-Freshwater Aquaculture Center, Central Luzon State University, Science City of Muñoz, Nueva Ecija, Philippines.

Reyes, A., Ambita, I. D., Batalon, J. L., Aba, B. L., Cortes, A., Macabecha, C. G. and Montecillo, A. (2018). Isolation and characterization of keratinolytic bacteria from soil samples of poultry waste dumping sites. International Journal of Agricultural Technology 14(7): 1787-1800.

Abstract The keratinolytic bacteria isolated from soil samples which containing the degrading feathers was investigated. Thirteen bacterial isolates were selected and were subjected to preliminary screening through protease assay using Milk Agar Medium. Six isolates were detected positive for protease activity and were further characterized via biochemical and microscopic assays. Isolates were grown in basal medium containing feathers as sole nutrient source and the degree of feather degradation was monitored. Two isolates exhibited conspicuous keratinase activity. DNA from these two candidate organisms were isolated and subjected to PCR using 16S rRNA specific primers. PCR products were sequenced and analysis revealed that both of them belong to *Bacillus cereus* species. Isolation of potential keratinolytic microorganisms could have potential biotechnological used especially in processes which involved keratin hyrdolysis.

Keywords: Keratinolytic bacteria, soil, biochemical assays, *Bacillus cereaus*, 16S rRNA

Introduction

Feathers are produced in large amounts as waste by-products of poultry farms from small-scale to large-scale processing plants. This is tantamount to the numerous chickens killed for consumption worldwide. Recently, a value-added use for feathers is its conversion to feather meal, a digestible dietary protein for animal feed, using physical and chemical treatments (Riffel *et al*., 2006). However, in the Philippines, most of these feather wastes are burned, dumped or stacked in waste areas which cause problems in storage and emissions control. This is because the bulk of feather produced by farms is poorly recycled (Jeevana Lakshmi *et al*., 2013) when left alone in dumping areas unlike other animal wastes. Also, it has limited use due to the chemically unreactive nature of keratin. Consequently, with improper handling of

* **Coressponding Author:** Reyes, A.T.; **Email:** alvinreyes1845@gmail.com

discarded feathers, it could become a pool for other pathogenic microorganisms causing mycoplasmosis, chlorosis and fowl cholera (Williams *et al*., 1991).

Feathers are slowly degraded and are resistant to other soil microorganism's proteolytic enzymes, due to the complex structure of its β-keratin filaments. In addition, it has disulfide cross-links which produce a compact three dimensional network (Bradbury *et al*., 1973), resulting to intermolecular disulfide bonds between rod domains and terminal domains of the constituent molecules (Parry *et al*., 1998). These molecular configurations of its amino acids ensure the structural rigidity posing a challenge for its disposal or utilization as feather feed. Physical and chemical processes used to increase the digestibility of feather keratin require consumption of large amounts of energy and destruction of certain amino acids, thus yielding products of poor digestibility and variable nutrient quality. Dymatic hydrolysis by microorganisms that possess keratinolytic activity represents an attractive alternative to improve the nutritional value of feather wastes (Xu et *al*., 2009).

A number of studies that describe nutritional upgrading of feather meal through microbial or enzymatic treatments have been published over the years. Some of these are feather meal fermentation with *Streptomyces fradiae* supplemented with methionine (Elmayergi and Smith, 1971) and feather-lysate from *Bacillus licheniformis* with amino acid supplementation resulting to a similar growth rate in chickens fed with soybean meal (Williams *et al*., 1991). Also, the crude keratinase enzyme produced by *B. licheniformis* was found to significantly increase total amino acid digestibility of raw feathers and commercial feather meal (Lee *et al*., 1991).

Moreover, keratinolytic microorganisms and their enzymes could be used to enhance the digestibility of feathers (Odetallah *et al*., 2003) which is primarily a garbage problem for a number of poultry farms and other feathered livestock raisers in the Philippines. Furthermore, although a number of keratinolytic microorganisms have already been reported, the full commercial potential of keratinases is yet to be realized. Molecular approaches targeting the keratin gene from reported isolates yet needs to be established. The objectives were to isolate, characterize and identify keratinolytic bacteria from soil samples containing degrading feathers in the Philippines.

Materials and Methods

Sample collection

Soil samples were collected from poultry waste dumping sites at ERI Poultry Farm in Victoria, Laguna, Philippines. Composite sampling was done using sterile falcon tubes and autoclavable plastic bags (Figure 1).

Figure 1. Sampling site where the feathers were collected.

Bacterial isolation

Pre-enrichment was done by transferring 10 g of soil sample into 90 mL Feather Meal Broth (FMB) (0.5 g L^{-1} NaCl, 0.3 g L^{-1} K_2HPO_4, 0.4 g L^{-1}, KH_2PO_4 and 10 g L^{-1} cut feathers) and afterwards incubated at 37 ^{0}C for 48 hours. Sample was then serially diluted at 10^{-3}, 10^{-5} and 10^{-7} dilutions and plated into Feather Meal Agar (FMA) (FMB + 15g L^{-1} agar). Plates were incubated at 37 ^{0}C for five days. Distinct colonies were streaked into FMA and incubated for two days. Cultural characteristics of isolates were observed. Well isolated colonies were streaked into Luria Bertani (LB) Agar (10g L^{-1} tryptone, 5g L^{-1} yeast extract, 10g L^{-1} NaCl slants and 15 g L^{-1} agar) and incubated at 37 ^{0}C for 24 hours. These served as culture stocks for the succeeding steps. Isolates were Gram stained and observed under light microscope to check for purity and to determine morphological characteristics.

Preliminary screening

Initial screening of isolates was done by proteolytic activity assay using Milk Agar plates (5 g L^{-1} peptone, 3 g L^{-1} yeast extract, 100 mL L^{-1} sterile UHT non-fat milk and 12 g L^{-1} agar). Bacteria were inoculated onto plates and incubated for 24 hours. Isolates with zones of clearing are protease positive and were used for the succeeding steps.

Gelatinase assay

Pre-screened isolates were stab inoculated into 10 mL Nutrient Gelatin (NG) which contained 13 g L^{-1} dehydarated Nutrient Broth and 120 g L^{-1} gelatin. Uninoculated NG was used as negative control. Tubes were incubated

at room temperature for 48 hours. Upon incubation, tubes were placed in freezer for 15 minutes and allowed to solidify. Liquefaction of gelatin indicated positive for gelatinase enzyme.

Catalase test

Presence of catalase enzyme was tested on pre-screened isolates by teasing loopfuls of colonies into separate glass slides. Hydrogen peroxide was then poured unto the cells using Pasteur pipet. Presence of bubbles would be indicative of catalase enzyme's presence in the cells.

Spore staining

Isolates were tested for presence of spores. Schaefer-Fulton method of spore staining was utilized as staining procedure (Schaeffer and Fulton, 1933). Smears of each isolate were first prepared, air dried and heat fixed. The smears were then flooded with malachite green. Slides were steamed for 8-10 minutes and in the process, smears were kept saturated with malachite green. Smears were then washed thoroughly with water and counterstained with safranin for one minute. After which, smears were washed and dried. Microcopic observation was done under oil immersion. Spores were stained blue while vegetative cells were red.

Keratinase activity assay

Feather broth containing 0.5 g L^{-1} NaCl, 0.3 g L^{-1} K_2HPO_4, 0.4 g L^{-1} and KH_2PO_4 was prepared and transferred into big tubes. Instead of adding cut feathers, each broth was added with single whole feather and then sterilized. Added feathers were of approximately the same sizes (Figure 2). Loopful of each isolate was inoculated into separate feather broth. Cultures were then incubated at 37 ^{0}C for 14 days and were monitored for the presence of feather degradation. Isolates that exhibited signs of feather utilization were selected and subjected to DNA isolation, polymerase chain reaction and sequencing.

DNA isolation

Isolates were inoculated into five mL LB broth and incubated for 18-24 hours. Cells were collected by transfering 1.5 mL of cell cultures into 1.5 mL sterile eppendorf tubes which were centrifuged at 5,000 rpm for 5 minutes at room temperature. The process was done repeatedly in the same tubes until all

cells in the culture broths were collected. Cells were washed twice with sterile distilled water and recentrifuged at the same conditions. Cells were resuspended with 200 µL Tris-EDTA (TE) buffer and mixed briefly. Tubes were first placed into boiling water bath for five minutes and then placed in freezer for 15 minutes. This freeze-thaw cycle was repeated three times. Resulting suspension was centrifuged at 5,000 rpm for five minutes. Supernatant was transferred to new sterile eppendorf tubes and labeled accordingly.

Figure 2. Feathers used as carbon source for keratinolytic bacteria

Polymerase Chain Reaction (PCR) using 16S rRNA primers

Extracted DNA samples were subjected to PCR using 16S ribosomal RNA specific primers. Reactions of 20 µL total volume contained 1X PCR Buffer (10 mM Tris-HCL, 50 mM KCl, 1.5 mM $MgCl_2$, pH 8.3 at 25^0C), 0.2 mM dNTPs (Invitrogen, USA), 1 µL of each primer, 0.5 unit of *Taq* polymerase (New England Biolabs, USA) and 1 µL of DNA template. PCR cylcing profile included an initial denaturation at 95 ^{0}C for five minutes followed by a 25x cycling profile constituted of a denaturation step at 95 ^{0}C for one minute, an annealing temperature of 55 ^{0}C for one minute and an extension step at 72 ^{0}C for one minute. A final extension step at 72 ^{0}C for 10 minutes was included after the cycling step. All PCR products were visualized through agarose gel electrophoresis with 1.0 % agarose gel. PCR products with correct bands were sent for sequencing.

Results

Isolation of potential keratinolytic bacteria

In this study, a targeted sampling approach was utilized in order to isolate microbial populations with keratinolytic potenial. Soil with degrading feathers was used as microbial source in order to increase the chances of isolating bacteria with keratinase activity. Furthermore, samples were pre-enriched in FMB for two days in order to magnify the population of the target microorganisms from the soil samples. Initial isolation yielded 13 bacterial isolates that were capable of thriving in FMA. The cultural characteristics of the 13 isolates were provided in Table 1.

Table 1. Cultural characteristics of 13 bacterial isolates

Isolate #	Color	Colony shape	Colony elevation	Agar stroke	Gram reaction	Cell shape
1	Creamy white	Irregular	Raised	Echinulate	+	Cocci
2	Clear transparent	Pinpont	Flat	Effuse	+	Short rods
3	Clear transparent	Irregular	Flat	Filiform	+	Staphylococci
4	Yellow	Round to obvate	Convex	Echinulate	-	Short rods
5	White	Irregular	Raised	Filiform	+	Streptobacilli
6	Yellow	Irregular	Convex	Filiform	-	Cocobacilli
7	Clear transparent	Pinpoint	Flat	Effuse	+	Diplococci
8	Clear transparent	Pinpoint	Flat	Echinulate	+	Bean-shaped
9	White	Round	Flat	Echinulate	+	Cocci
10	Pale white	Round	Flat	Effuse	+	Diplococcic
11	Clear transparent	Pinpoint	Flat	Effuse	Mixed	Small rods
12	Yellow	Pinpoint	Flat	Filiform	-	Cocobacilli
13	Creamy white	Irregular	Raised	Filiform	+	Streptobacilli

Preliminary screening

Preliminary screening was done through protease assay in order to narrow down the number of isolates and to verify presence of proteolytic activity. This was done by growing the isolates in milk agar. Six isolates were able to form clearing zones indicative of proteolytic activity. Isolates 4, 6 and 12 exhibited largest clearing zones followed by 5, 13 and 7 (Figure 3). These isolates were recovered and were subjected for further testing.

Figure 3. Protease assay of the 13 isolates using milk agar medium

Biochemical and microscopic assays

Pre-screened isolates were subjected to spore staining and some biochemical assays such as gelatinase (Figure 4) and catalase (Figure 5). These tests were done in order to elucidate some characteristics of the isolates which could be useful in their identification. Results of the mentioned tests are summarized in Table 2.

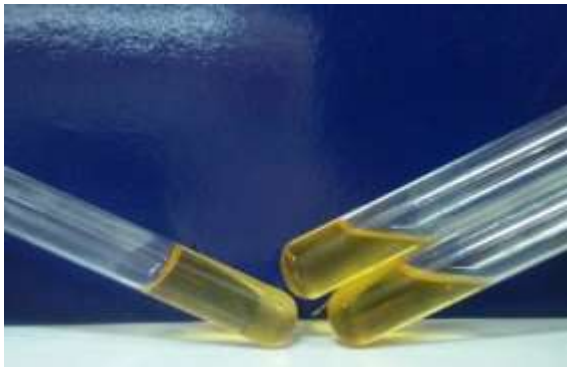

Figure 4. Representative for gelatinase activity test. All isolates are positive for gelatinase activity; left (negative control) and right (unknown isolate)

Figure 5. Catalase test of several isolates positive for keratinolytic activity

Table 2. Biochemical and microscopic assay of the pre-screened isolates

Isolate #	Gelatinase	Catalase	Spore stain
4	+	-	+
5	+	+	+
6	+	-	-
7	+	+	+
12	+	+	-
13	+	+	-

Keratinase assay

Isolates with highest keratinase activity were identified by further interrogating their capacity to degrade feathers. This was done by using feather broth, a minimal medium with feathers as sole carbon and nitrogen source. Whole feathers were supplemented in the media in order to visually inspect feather degradation. Results revealed that isolates 5 and 13 exhibited the most visible feather degradation activity (Figures 6 to 8). This was further supported by the observed highest turbidity in these isolates indicative of feather utilization for growth. These two isolates were recovered and identified.

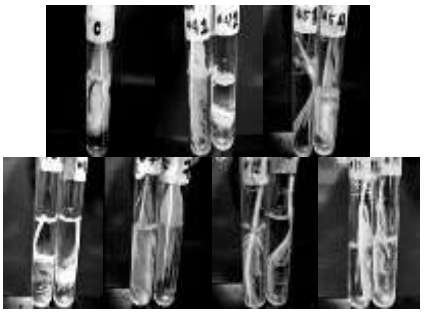

Figure 6. Keratinolytic activity assay using feather as carbon source at 0 h incubation

Figure 7. Keratinolytic degradation of several isolates after 7 days incubation using feathers as source of carbon

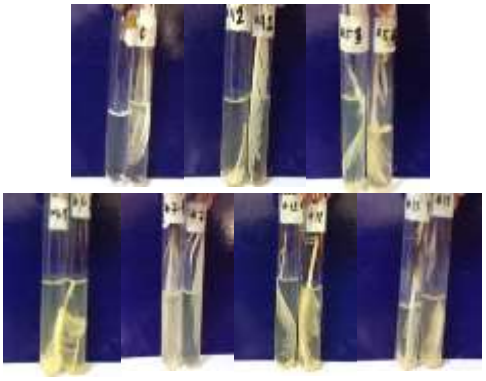

Figure 8. Keratinolytic degradation of several isolates after 15 days incubation using feathers as source of carbon

Identification by 16S ribosomal RNA sequencing

The two isolates were subjected to DNA isolation. DNA samples were used as templates for PCR amplification using 16S rRNA specific primers. Successful amplification was obtained as evidenced by the expected 1.5 kb sized amplicon in agarose gel observation (Figure 9).

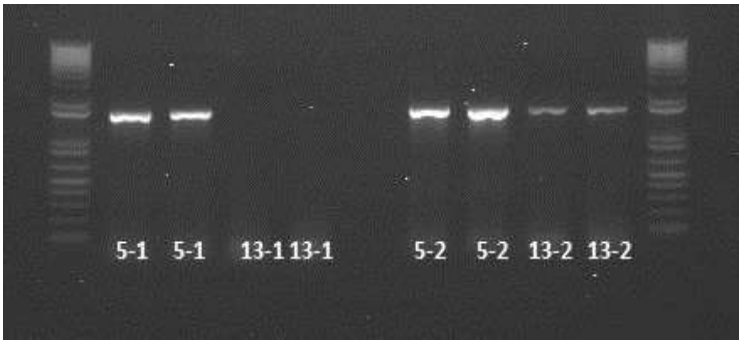

Figure 9. Result of agarose gel electrophoresis of 16S primer-PCR products using DNA from isolates as template

PCR products were subjected to sequencing using the same set of primers. BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) analysis of sequences derived from both revealed that the isolates are of *Bacillus* genus. Highest hits indicated that the isolates belong *cereus* species (Figures 10 and 11).

5-1

Bacillus cereus strain CMS17 16S ribosomal RNA gene, partial sequence

Sequence ID: gb|KU921589.1|Length: 1441Number of Matches: 1

Related Information

Range 1: 599 to 1390GenBankGraphicsNext MatchPrevious Match

Score	Expect	Identities	Gaps	Strand
1429 bits(1584)	0.0	792/792(100%)	0/792(0%)	Plus/Plus

```
Query  1     TGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGG  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  599   TGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGG  658

Query  61    CGACTTTCTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAG  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  659   CGACTTTCTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAG  718

Query  121   ATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTT  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  719   ATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTT  778

Query  181   TAGTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAAC  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  779   TAGTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAAC  838

Query  241   TCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAAC  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  839   TCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAAC  898

Query  301   GCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAACCCTAGAGATAGGGCTTCTCCTT  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  899   GCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAACCCTAGAGATAGGGCTTCTCCTT  958

Query  361   CGGGAGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGT  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  959   CGGGAGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGT  1018

Query  421   TAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCATCATTTAGTTGGGCACTCTA  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1019  TAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCATCATTTAGTTGGGCACTCTA  1078
```

Figure 10. Sequence analysis of isolate # 5

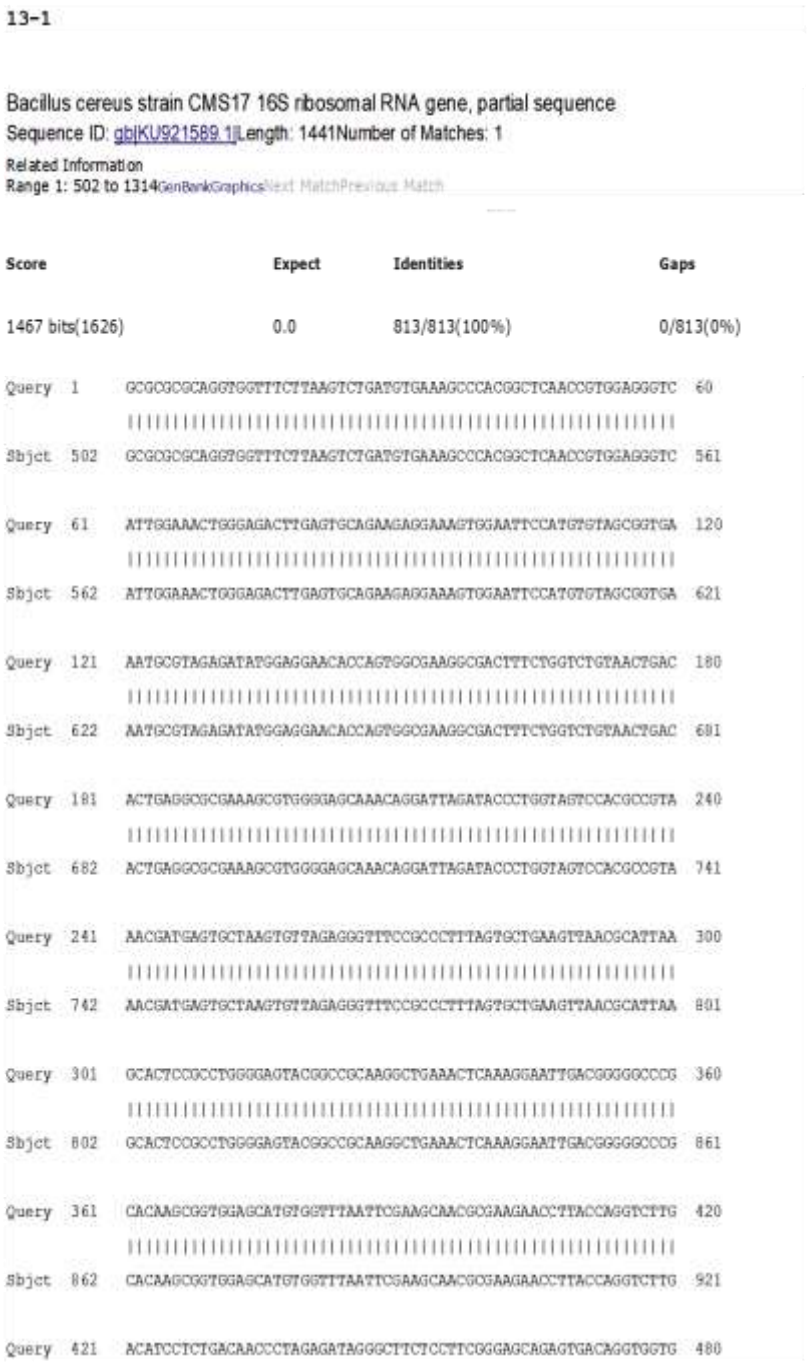

Figure 11. Sequence analysis of isolate # 13

Discussions

One of the by-products of poultry farms are feathers which are generated in large quantities. On a yearly basis, billions of chickens are slaughtered which entail tonnes of poultry feather by-products. Nowadays, these feathers are subjected to physical and chemical treatments in order to be used as dietary supplement for animal feeds. These processes, however, could destroy amino acids and will result to diminished protein quality (Kulkarni and Jadhav, 2014). Alternative solution for this is to use microorganisms with keratinase activity for keratin degradation. This idea has resulted to numerous studies which aim to isolate bacteria that could effectively utilize keratin rich substrates. In 2006, Riffel and Brandelli were able to isolate three Gram negative bacteria (belonging to *Burkholderia, Chrysobacterium* and *Pseudomonas*) and one Gram positive bacterium (*Microbacterium* sp.) in which keratinase activity was detected during growth. Similar study was done by Kulnarni and Jadlav in 2014, in which they were able to isolate keratinolytic *Breuvedimonosterrae* species.

In this experiment, keratinolytic *Bacillus cereus* were isolated. *B. cereus* is a Gram positive, rod shaped sometimes in chains, endospore forming, and facultative anaerobic bacteria. They are positive for gelatinase and catalase (Coia and Cubie, 1995). These physiological traits are consistent with the observed isolates. The ability of *Bacillus* sp. to degrade keratin has been well-documented and other studies were also able to isolate the same species from their samples. In *B. licheniformis*, the entire nucleotide sequence of the coding and flanking regions of the keratinase structural gene had been identified. Presence of keratinase, a serine protease, could be accounted for by the presence of *kerA* gene (Lin *et al*., 1995). Some serine proteases such as subtilisins share sequence and structural similarities across different *Bacillus* species. The same might be true for keratinase and this could provide an avenue for molecular approach of screening keratin degrading bacteria. In 2013, Jeevana Lakshmi *et al*. characterized two native strains which were *B. cereus* and *B. subtilis.* Furthermore, they had identified optimal conditions in which maximal production of the enzyme was reached. This further supports the current observations that *B. cereus* is indeed a keratinolytic bacterium.

Bacillus sp. had been known to exhibit unique physiological traits when exposed to environmental stresses such as nutrient limitation. When nutrients are low, one of the physiological traits that they develop would be the secretion of proteases which allow them to utilize alternative sources of carbon and nitrogen. Known keratinases, for instance are inducible and very specific towards their substrates such as hair, wool and feathers (Kaul and Sumbali, 1997). These microorganisms may have their keratinase specific genes induced

which made them adapted to utilize substrates such as feathers. Isolation of such microorganisms could be of biotechnological importance for improving nutritional qualities of feeds containing treated poultry feather wastes. Furthermore, this would provide avenues for alternative means of reducing feather wastes and at the same time preventing generation of pools of pathogenic microorganisms.

Acknowledgement

The authors would like to exend their appreciation to Prof. Andrew Montecillo for his guidance in pursuing this study.

References

Bradbury, J. H. (1973). The structure and chemistry of keratin fibers. Advances in Protein Chemistry. 27:111-211.

Coia, J. and Cubie, H. (1995). *Bacillus cereus* the immunoassay kit directory. Springer Netherlands. 1:648-649.

Elmayergi, H. H. and Smith, R. E. (1971). Influence of growth of *Streptomyces fradiae* on pepsin-HCl digestibility and methionine content of feather meal. Canadian Journal of Microbiology. 17:1067-1072.

Jeevana Lakshmi, P., Kumari Chitturi, Ch. M. and Lakshmi, V. V. (2013). Efficient degradation of feather by keratinase producing *Bacillus* sp. International Journal of Microbiology.Article ID 608321. doi:10.1155/2013/608321.

Kaul, S. and Sumbali, G. (1997). Keratinolysis by poultry farm soil fungi. Mycopathologia. 139:137-40.

Kulkarni, S. A. and Jadhav, A. R. (2014). Isolation and characterization of keratinolytic bacteria from poultry farm soils. International Research Journal of Biological Sciences. 3:29-33.

Lee, C. G., Ferket, P. R. and Shih, J. C. H. (1991). Improvement of feather digestibility by bacterial keratinase as a feed additive. FASEB Journal. 59:1312.

Lin, X., Kelemen, D. W., Miller, E. S. and Shih, J. C. H. (1995). Nucleotide sequence and expression of *kerA*, the gene encoding akeratinolytic protease of *Bacillus licheniformisi.* Applied and Environmental Microbiology. 61:1469-1474.

Odetallah, N. H., Wang, J. J., Garlich, J. D. and Shih, J. C. H. (2003). Keratinase in starter diets improve growth of broiler chicks. Poultry Science. 82:664-670.

Parry, D. A. T. and North, A. C. T. (1998). Hard a-keratin intermediate filament chains: substructure of the N- and C-terminal domains and the predicted structure and function of the C-terminal domains of type i and type II chains. Journal of Structural Biology. 122:67-75.

Riffel, A. and Brandelli, A. (2006). Keratinolytic bacteria isolated from feather waste. Brazilian Journal of Microbiology. 37:395-399.

Schaeffer, A. B. and Fulton, M. D. (1933). A simplified method of staining endospores. Science. 17:194.

Williams, C. M., Lee, C. G., Garlich, J. D. and Shih, J. C. H. (1991). Evaluation of a bacterial feather fermentation product, feather-lysate, as a feed protein. Poultry Science. 70:85-94.

Xu, B., Zhong, Q., Tang, X., Yang, Y. and Huang, Z. (2009). Isolation and characterization of a new keratinolytic baterium that exhibits significant feather-degrading capability. African Journal of Biotechnology. 8:4590-4596.

(Received: 9 September 2018, accepted: 31 October 2018)