International Journal of Molecular and Clinical Microbiology 9(1) (2019) 1090-1096

International Journal of
Molecular and Clinical Microbiology

Keratinase Producing Bacteria: A Promising Approach for Poultry Waste Management

Ania Ahani Azari*[1], Neda Kouroshzadeh [2], Hamidreza Pordeli [1]

1. Assistant Professor, Department of Microbiology, Gorgan Branch, Islamic Azad University, Gorgan, Iran.
2. Department of Microbiology, Gorgan Branch, Islamic Azad University, Gorgan, Iran.

ARTICLE INFO

Article history:
Received 26 January 2019
Accepted 5 March 2019
Available online 1 June 2019

Keywords:
Isolation, Feather, Keratin-degrading bacteria, Molecular identification, Landfill.

ABSTRACT

A large amount of feather is produced each year in the poultry industries which is buried in landfill or burned in the power plant generator. Feathers are purified keratinous proteins that can be degraded by bacteria and fungi. The aim of this study was to isolate and identify keratinase producing bacteria from the feather waste dumping sites around Gorgan district, Golestan province, Iran. Soil samples were collected from six poultry farms and slaughterhouses. The keratinolytic bacteria were isolated and identified based on feather and keratin degradation in feather meal broth and keratin agar. Isolates that showed more keratinolytic activity were identified using morphological, biochemical, and molecular tests. Then, the effect of pH and temperature was examined on the growth of the isolates. Based on the results, keratinase-producing isolates belonged to the *Bacillus* genus, among the isolates, the most keratinolytic activity was related to *Bacillus megaterium* strain SR7 and it showed the best growth at pH 6.8 and 37°C in feather meal broth and keratin agar. The results of the present study were consistent with the results of many similar studies and found that *Bacillus* strains are important producers of keratinase enzyme and therefore promising organisms for the management of chicken feather wastes through efficient biodegradation.

1. Introduction

A large amount of feather wastes, over 5 million tons, is produced every year, which is buried in landfill or burned in the Power Plant generator (Chhimpa et al., 2016). The buried feathers tightly increase the nitrogen level and groundwater is contaminated with chemicals and bacteria, the burning leads to emissions and various diseases in human beings, including chlorosis and mycoplasmosis (Agrawal and Dalal, 2015).

Feathers are purified keratinous proteins that are infinitely resistant to decomposition. Today, using high temperature and grinding the feather wastes break down into a powder, which can be used as a **p**rotein dietary supplement in animal meal and many other applications. Of course, the production of meal from feather with this method is a costly process that destroys essential amino acids such as tryptophan, methionine and lysine and produces a low nutritional quality (Iruolaje et al., 2016).

Recently, due to environmental considerations, microbial keratinases has received attention of researchers. The use of microbial keratinase as an environmentally-friendly approach for feather decomposition is a

*Corresponding author: Dr. Ania Ahani Azari
Tel: 00989111777377
E-mail address: ania_783@yahoo.com

good and cheaper substitution in compare with physical and chemical methods. These enzymes can hydrolyze a number of keratinous materials. Bacterial keratinases have potentiality because of keratin substrates degradation and a variety of protein materials. They work as novel biocatalysts that have many applications in several industries and agro-industrial waste degradation (Barman et al., 2017).

Some microorganisms include mesophilic fungi and actinomycetes are the main producers of keratinase enzymes. The Keratinolytic activity of *Bacillus* spp., *Fervidobacterium* sp., *Thermoanaerobacter* sp., *Xanthomonas* sp, *Vibrio* sp., *Microbacterium* sp., *Streptomyces* sp., *Bacillus licheniformis*, *Pseudomons aeruginosa* and *Chryseobacterium* and *Candida parapsilosis* also has been revealed (Iruolaje et al., 2016, Lakshmi et al., 2013).

Therefore, it seems necessary to find natural sources of keratinases, especially new sources of microbial keratinases that meet industrial needs. The aim of this study was isolation and molecular identification of promising keratinase producing bacteria from the soil of poultry waste dumping sites around Gorgan district, Golestan province.

2. Materials and Methods

2.1. Sampling

Soil samples were collected from six poultry waste dumping sites such as poultry farms, slaughterhouses and landfills located around Gorgan, Golestan province. Samples were taken from a depth of 5 to 20 cm and the transferred to the laboratory in sterile zippered bags and kept in the refrigerator until cultivated (Godbole et al., 2017, Khodayari and Kafilzadeh, 2018). It should be noted that all the materials and media used in this study were purchased from Merck Germany.

2.2. Cultivation and purification

The 10^{-7} dilution of each soil samples were made in sterile saline. Then, 0.5 ml of each sample was inoculated into peptone broth medium and incubated at 37 °C for 24 hours. 0.1 ml of this medium was poured onto the nutrient agar and spread with a diffuser rod and incubated at 37 ° C for 24 hours. Single colonies with different morphological characteristics (size, shape and color) were streaked on nutrient agar as a pure culture (Mehta et al., 2013).

2.3. Isolation of caseinase-positive bacteria

To detect protease production by the isolates, the pure colonies were transferred to Skimmed milk agar and incubated at 37 ° C for 48 hours. Since caseinase is a type of protease, caseinase-producing bacteria were identified based on the clear halo induced by casein degradation (Mehta et al., 2013).

2.4. Isolation of keratinase-producing bacteria

For isolation of keratinase producing bacteria, caseinase positive isolates in feather meal broth (NH_4Cl, 0.5; NaCl, 0.5; K_2HPO_4, 0.3; KH_2PO_4, 0.4; $MgCl_2 \cdot 6H_2O$, 0.24; $CaCl_2$, 0.1; yeast extract, 2.0; feather meal, 10.0 g l-1) were inoculated. Chicken feathers were used as a substrate and source of keratin in this medium. The pH was measured with a pH meter (Sapna & Yimini, 2011). In addition, keratin agar (nutrient agar containing 1% keratin) was also used. The media were incubated at 37°C for one week. In the Keratin agar, the bacteria with a clear halo around the colony and degradation of the feathers in the feather meal broth were considered positive for Keratinolytic activity (Mehta et al., 2013).

2.5. Identification of keratinase-producing bacteria

The phenotypic characteristics of bacterial isolates were studied using microscopic observations and gram staining. To identify bacteria at this stage, tests such as Gram staining, Sporulation staining, catalase, oxidase, carbohydrates fermentation, motility test, starch, lecithin, gelatin and urea hydrolysis, and indole production and other biochemical tests were achieved (Femi Olla et al., 2015).

2.6. Molecular Identification of keratinase-producing bacteria

The bacterial isolate was cultured in nutrient broth overnight. Then, the genomic DNA of isolate overnight culture was extracted using Geno Plus TM Genomic DNA Extraction Miniprep System (Viogene, China). The

qualification and quantity of the extracted DNA was determined by agarose gel electrophoresis (1%). The genomic DNA was used as a template for 16S rDNA gene amplification using consensus primers. To do PCR, 2X Master Mix (Thermo scientific, USA) was applied. The reaction mixture was prepared by adding 1 µl of each primer (20 pmol), 5 µl of DNA template and 19 µl of double-distilled water (DDW). The PCR amplification was performed with 30 cycles of denaturation at 94˚C for 30 s, annealing at 56˚C for 30s, and extension at 72˚C for 30 s. The initial denaturation and final extension were 94˚C for 3min and 72˚C for 10min respectively. The primers used in this study are summarized in Table 1. The 16S rDNA genes were successfully amplified with consensus primers and anticipated fragments with 1500 bp were observed on 1% agarose gel. Then, the purified PCR products were sent to Macrogen (South Korea) for sequencing. The obtained nucleotide sequences were searched for homology in the NCBI nucleotide database using BLAST tool. The 16S rDNA gene sequences were aligned against the reference nucleotide sequences retrieved from GenBank (Ebrahimi and Ahani Azari, 2016).

Table 1. Primers used in this study (Weisburg et al., 1991).

Primer name	Primer sequence 5'-3'	PCR product
16S-F	AGAGTTTGATCCTGGCTCAG	1500bp
16S-R	ACGGCTACCTTGTTACGACTT	

2.7. Effect of pH and temperature on growth of isolates

To examine the effect of pH and temperature on the growth of the isolates, each suspension was prepared with a half-McFarlane turbidity suspension and inoculated in feather meal broth at different pHs (6, 6.8, 7.2 and 8). This medium was then incubated at various temperatures (25, 37 and 55 °C) for 72 h (Sangali and Brandelli, 2000). After this period, the growth of the isolates was checked directly by counting (CFU/ml) (Manjula, 2014).

3. RESULTS

From different soil samples, 29 different colonies were isolated and purified on the basis of morphological differences, gram and sporulation staining, and oxidase and catalase tests. Of the 29 purified colonies, 17 colonies produced clear halo on the skimmed milk, indicating presence of caseinase and possible keratinolytic activity (Fig. 1).

Figure 1. Clear halo induced by casein degradation on the Skimmed milk agar

The isolates were then transferred to the Feather meal broth and Keratin agar, all of them were capable of degrading feather and Keratin in these media (Fig. 2). Growth of the isolates was also measured by direct counting method (CFU /ml) in Feather broth medium. 5 isolates with the best growth in foresaid media were selected and then, identified using microscopic observations, biochemical and molecular tests.

Based on the results of sequencing these isolates were most similar (more than 90% identity) to *Bacillus licheniformis* strain RG1 (B1), *Bacillus cereus* strain Wu6 (B2), *Bacillus megaterium* strain SAK (B3), *Bacillus coagulans* P38 (B4), and *Bacillus pumilus* strain C4 (B5) (Figure 3). Therefore, all the isolates belonged to the *Bacillus* genus and among them *B. megaterium* strain SAK had more growth and degradation activity in the keratin-containing media. The *B. megatrium* strain SAK showed the best growth at pH 6.8 and 37°C in feather meal broth and keratin agar.

Table 2: Morphological and biochemical features of 5 selective isolates

Isolates	B1	B2	B3	B4	B5
Morphology	Rod	Rod	Rod	Rod	Rod
Gram reaction	Positive	Positive	Positive	Positive	Positive
Spore forming	Subterminal	Central	Terminal	Subterminal	Subterminal
Motility	+	+	-	+	+
Catalase	+	+	+	+	+
Oxidase	-	-	-	-	-
Gelatin hydrolysis	+	+	+	+	+
Starch hydrolysis	+	+	+	+	-
Lecithin hydrolysis	-	+	-	-	-
VP test	+	+	-	+	+
Citrate	+	+	+	-	+
Acid from glucose	+	+	+	+	+
Gas from glucose	-	-	-	-	-
Nitrate reduction	+	+	+	-	-

Figure 2. Right fig. Clear halo on Keratin agar due to Keratin degradation
Left fig. Degradation of feather by keratinase-producing isolate in Feather meal broth

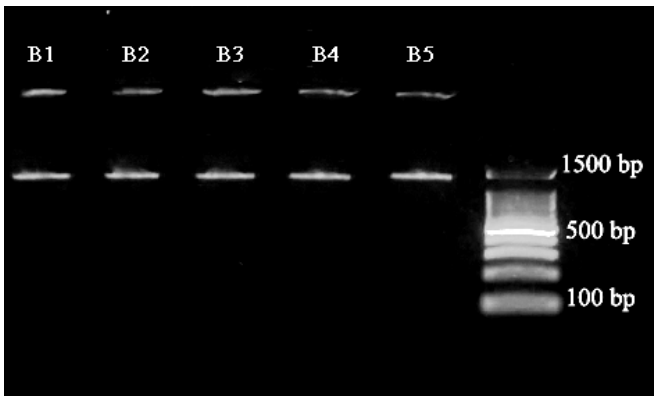

Figure 3. Lane 1: DNA ladder Mix (100-1500bp) as a size standard, Lanes 2-6: PCR result on the genomic DNAs of isolates

4. Discussion

This cross-sectional study is descriptive and the keratinase producing bacteria from the soil of poultry waste sites around Gorgan, Golestan province, were isolated and identified. Various studies have been conducted by researchers in this field (Lakshmi et al., 2013, Sapna and Ymini, 2011, Iruolaje et al., 2016, Chhimpa et al., 2016). Most of them reported that different species of *Bacillus* are able to produce keratinase as the present study.

In a study conducted by Iruolaje et al, three isolates belonged to *Bacillus* including *Bacillus licheniformis*, *Bacillus subtilis* and *Bacillus cereus*, and one of the isolates was *Serratia marcescens*. Among the isolates, *Bacillus licheniformis*, had the most keratinase activity (Iruolaje et al., 2016). In a research in India, *Bacillus licheniformis*, *Bacillus cereus* and *Staphylococcus aureus* were found Keratinase producer (Raju and Divakar, 2013). Khodayari and Kafilzadeh also reported that all keratinase producer bacteria isolated from the soil of poultry farms belonged to different strains of *Bacillus* (Khodayari and Kafilzadeh, 2018). Mehraban et al. used biochemical, morphological and molecular characterization to identify the isolates with keratinase activity. Based on the results of sequencing, two isolates were most similar to *Bacillus mojavensis* RO-H-1 and ATCC strain *Bacillus licheniformis* (Mehraban et al., 2016).

In a similar study by Masih and Singh, three isolates showed keratinolytic activity, it was found that two of them belonged to *Bacillus subtilis* strains (Masih and Singh, 2014). Manjula also isolated four keratinolytic strains from soil of a buried chicken wastes in Vellore, India (Manjula, 2014). In a similar study, keratinase producing bacteria were isolated from the dumping sites of chicken waste and Ghazi pour Recycling Plant. Upon identification, these isolates were identified as *Bacillus megaterium* SN1, *Bacillus thuringenesis* SN2, and *Bacillus pumilis* SN3, which were able to degrade the chicken waste (Agrahari, and Wadhwa, 2010).

In another study, Sapna and Ymini examined the degradation of Keratin by native bacteria isolated from soil. Several bacteria and fungi showed keratinolytic activity. Among the bacteria isolated, four species of *Bacillus* (BS-1, BS-2, BS-3 and BS-4) had the highest keratinase activity (Sapna and Ymini, 2011). Hoq et al. isolated 8 *Bacillus* species from poultry effluent by enrichment technique and identified them by 16S-rRNA, physiological tests and carbohydrate fermentation. Three isolates were *Bacillus licheniformis*, two were *Bacillus cereus* and three were *Bacillus subtilis*, *Bacillus borstlensis* and *Bacillus sphericus* (Hoq et al., 2005).

Riffel also isolated keratinolytic bacteria from various keratinous wastes such as feather powder, feather, chicken nail, hair and wool. Three of these gram-negative isolates belonged to the *Burkholderia*, *Chrysobacterium* and *Pseudomonas* genera, and one of the isolates was gram-positive (*Microbacterium*) (Riffel, 2006). One of the results highlighted in two recent studies was the isolation of Gram-negative bacteria with keratinolytic activity that were not identified and reported in other studies, including the present study.

In some researches the effect of pH and temperature on the growth and keratinase production of keratinase producing bacteria has been studied. The researchers reported that optimum pH and temperature for keratinase production is different for every producer. In our study, the *B. megatrium* strain SAK showed the best growth at pH 6.8 and 37°C in feather meal broth and keratin agar. In a study performed by Xu et al. a new strain of *Bacillus licheniformis*, K-19, produced high amounts of heat-stable keratinase that was resistant to a wide range of pH (Xu et al., 2009). In the study of Khodayari and Kafilzadeh the optimum conditions for keratinase production by *Bacillus cereus* was at pH 8 and 35°C (Khodayari and Kafilzadeh, 2018). In a similar study Mousavi et al. reported that maximum keratinase activity by *Bacillus subtilis* isolated from a poultry waste was at pH 11 and 40°C on the sixth day of culture (Mousavi et al., 2013). Prasad et al. in Vellore, India, isolated a *Bacillus* sp. with the best keratinase activity at pH 7 and 30°C (Prasad et al., 2010). Subugade et al. also described that the optimum conditions for keratinase production by a *Bacillus* sp was at pH 7.4 and 37°C (Subugade et al., 2017). Therefore, after isolation and identification of keratinase producers, optimization for the maximum kratinolytic activity is necessary.

In this study we could not measure the keratinase activity because of some limitations that we had in providing the required materials

for the test. So, based on the growth and keratin degradation in media included in this study we selected the isolates and evaluated the effect of pH and temperature on them.

Conclusion

The results of the present study were consistent with the results of similar studies. We found that *Bacillus* strains are important keratinase producers and therefore promising organisms for the management of chicken feather wastes through efficient biodegradation.

Acknowledgements

It is worth noting that this study was a thesis performed by Mrs. Neda Koroushzadeh for a Master of Science degree in microbiology. The Department of Microbiology, Islamic Azad University, Gorgan branch is acknowledged for providing necessary laboratory facilities.

Refereces

Agrahari, S. and Wadhwa, N. (2010). Degradation of Chicken Feather a Poultry Waste Product by Keratinolytic Bacteria Isolated from Dumping Site at Ghazipur Poultry Processing Plant. Int J Poult Sci., 9 (5): 482-489.

Agrawal, B., Dalal, M. (2015). Screening and characterization of keratinase enzyme obtained from keratin degrading microorganism isolated from Sanjan poultry waste dumping soil. Eur Academic Res., 2(11): 13986-13994.

Barman, N., j Zohora, F., Chandra Das, K., Mowla, M., Akhter Banu, N., Salimullah M. and Hashem, A. (2017). Production, partial optimization and characterization of keratinase enzyme by Arthrobacter sp. NFH5 isolated from soil samples, AMB Expr.7: 181.

Chhimpa, S., Shekhar Yadav, C., John, PJ. (2016). Isolation and identification of keratin degrading (keratinolytic) bacteria from poultry feather dumping sites. J Biodiver Enviro Sci., 8(6): 109-119.

Ebrahimi, M. and Ahani Azari, A. (2016). Study on Lead Biosorption by Lead Resistant Bacteria Isolated from Industrial Wastewater of Plants in AqQala Industrial Park, J Microbial World. 9(3): 258-267.

Femi-Ola, T.O, Akinbobola, O.S, Oluwaniyi, T.T. (2015). Isolation and characterization of feather degrading bacteria from poultry soil. Agric Biol J N Am. 6(5): 146-15410.

Godbole, S, Pattan, J, Gaikwad, S, Jha T. (2017). Isolation, Identification and Characterization of Keratin degrading microorganisms from Poultry soil and their Feather degradation Potential. JEAB. 2(4): 2060-2068.

Hoq, M.M., Siddiquee, KAZ. Kawasaki, H., Seki, T. (2005). Keratinolytic activity of some newly isolated Bacillus species. J Biol Sci. 5 (2): 193-200.

Iruolaje, FO. Ogbeba, J., Tula, MY. Ijebor, JA. , Dogo, BA. (2016). Isolation and Identification of Keratinolytic Bacteria that Exhibit Feather-degrading Potentials, J. adv. biol. Biotechnol. 5(2): 1-9.

Khodayari, S. and Kafilzadeh, F. (2018). Separating Keratinase Producer Bacteria from the Soil of Poultry Farms and Optimization of the Conditions for Maximum Enzyme Production. Euro J Exp Bio. 8 (6): 1-8.

Lakshmi, P.J., Kumari, Ch., Chitturi, M. and Lakshmi, V.V. (2013). Efficient Degradation of Feather by Keratinase Producing Bacillus sp. Int J Microbiol. Article ID 608321, 7: 1-7.

Masih, H. and Singh, S. (2014). Degradation of Keratinous Waste Products by Keratinolytic Bacteria Isolated from soil. Int. j. computer sci. eng. Commun 3 (8): 7588-7595.

Mehta, R., Jholapara, R., Sawant, C. (2013). Optimization of cultural condition for extracellular keratinase production by Bacillus species isolated from poultry from soil. Int J Pharm Bio Sci. 4(2): 454-463.

Mehraban, S., Sharifmoghadam, M, Bahraini M. and Asoodeh, A. Identification of kertinase Producing Bacteria Isolated from Soil around Mashhad. (2016). First National Conference on New Biological Findings, Zahedan, Sistan and Baluchestan University, Iran.

Manjula, R. (2014). Isolation and characterization of keratinolytic Bacteria from poultry waste dumping soil. Int J Enhanced Res Sci Technol & Eng. 3 (8): 238-243.

Mousavi, S., Salouti, M., Shapoury, R., Heidari, Z. (2013). Optimization of keratinase production for feather degradation by Bacillus subtilis. Jundishapur J Microbiol. 6 (8): e7160.

Prasad, VH., Kumar, G., Karthik, L. and Rao KV, B. (2010). Screening of Extracellular Keratinase Producing Bacteria from Feather Processing Areas in Vellore, Tamil Nadu, India. J. Sci. Res. 2 (3): 559-565.

Raju, E. and Divakar, D., (2013). Screening and Isolation of Keratinase Producing Bacteria from Poultry Waste. IJPRAS. 2(1): 70-74.

Riffel, A. (2006). Keratinolytic bacteria isolated from feather waste. Braz J Microbiol. 37:395-399.

Sapna, R. and Ymini, V. (2011). Study of keratin degradation by some potential bacterial isolates from soil, J. Soil Sci. 1(1): 01-03.

Sangali, S. and Brandelli, A. (2000). Feather keratin hydrolysis by a Vibrio sp. Strain. J Appl Microbiol. 89: 735-743.

Subugade, S., Gupta, S.G., Mokashe, S. (2017). Isolation and Screening of Keratinase Producing Bacteria from Chicken Feather Dumping sit. Int. j. chemtech res. 10(5): 900-905.

Weisburg, W.G, Barns, S.M, Pelletier, D.A. and Lane, D.J. (1991). 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol. 173(2): 697–703.

Xu, B., Zhong, Q., Tang, X., Yang, Y. and Huang, Z. (2009). Isolation and characterization of a new keratinolytic bacterium that exhibits significant feather-degrading capability. Afr. J. Biotechnol. 8 (18): 4590-4596.