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Keratin Production by Decomposing Feather Waste Using Some Local *Bacillus Spp*. Isolated from Poultry Soil

Somayeh Mousavi [a], Mojtaba Salouti [b,*], Reza Shapoury [b]

[a] *Department of Microbiology, Faculty of Sciences, Zanjan Branch, Islamic Azad University, Zanjan, Iran.*
[b] *Biology Research Center, Zanjan Branch, Islamic Azad University, Zanjan, Iran.*

Corresponding author.** ***E-mail address: saloutim@yahoo.com

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ABSTRACT

Background: Feather waste is generated in large amounts as a by-product of commercial poultry processing. The main component of feather is keratin. The main purpose of this study was to identify *Bacillus spp*. (the keratinolytic bacteria) that are able to degrade the feather for producing keratin.
Methods: *Bacillus spp*. Were isolated from the waste of poultries located in Miyaneh city. The bacteria were grown on basal medium containing 1% hen feather as the sole source of carbon ,nitrogen, sulfur and energy at 27ºC for 7 days. Then,the isolates capable of feather degrading were identified. The Bradford method was used to assay the production of keratin in the feather samples. Different pH and temperatures were studied to determine the best conditions for production of keratinase enzyme.
Results: Seven *Bacillus spp*. including: B. *pumilis*, B. *subtilis*, B. *firmus*, B. *macerance*, B. *popilliae*, B. *lentimorbus* and B. *larvae* were found to be able to degrade the feather with different abilities.
Conclusion: B. *subtilis* was found to be most productive isolate for keratinase enzyme production.

1. Introduction

Keratin is 90% of feather waste, a byproduct of the domestic poultry industries. However, due to poor digestibility, the use of feather as a dietary protein supplement for animal feed stuffs has been limited [1]. The keratin is an insoluble protein, with high stability due to a high degree of cross-linkage disulfide, hydrogen bonds and hydrophobic interactions. The keratinases belong to the group of hydrolases that are important for hydrolyzed feather, hair, wool, collagen and casein [2].

Only few bacterial species have been reported are able to produce keratin enzyme. Many of the Bacillus spp. are found to be useful in enzymatic amino acids essential metabolites [3]. As a substrate of microbial decomposers, keratin did not accumulate in substantial quantity in nature [4-6]. The previous studies reported the special advantages of Bacillus spp. for feather degradation because of its safety as well as its ability to secrete keratinases enzymes in large mass directly into the medium [7, 8].

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Feather hydrolysates produced by bacterial keratinases have been used as additives for animal feed [9-11]. Feather biodegradation by keratinases enzymes was reported as an environmental friendly process, which converts the poultry waste into the animal feed with nutrient-rich in low-cost [12]. The feather biodegradation by Bacillus spp. represent a biological method for degrading of feathers to a feed protein. The aim of this study was to isolate the *Bacillus spp* from the poultry waste capable of production of keratinase enzymes.

2. Material and Methods

2.1. Reagents

SDS (Sodium dodecyl sulfate), BSA (Bovein serum albumin), Nutrient Broth, Nutrient Agar, BHI, TSI, VP, was purchased from Merck. Size marker 1kb DNA Ladder Fermentase and size marker protein kDa 14/6-116 Fermentase, reverse and forward primer DNA was purchased from Cinagen, Iran.

2.2. Isolation of feather-decomposing Bacillus species from poultry waste

Different sites of poultry and chick farm wastes in Miyaneh city (in west north of Iran) were chosen for collecting the soil samples. The medium used for isolation of the feather-decomposing microorganism contained: 0.5 g NH_4Cl, 0.5 g NaCl, 0.3g K_2HPO_4, 0.4g KH_2PO_4, 0.1 g $MgCl•6H_2O$ and 10 g hammer-milled chicken feathers per litre in pH 7.5. Feathers were washed, dried, and hammer-milled prior to add to the medium. The mixture was incubated at 27°C for 7 d. Strains of Bacillus bacteria was detected by gram staining and spores, catalase, VP, and starch hydrolysis tests [13].

2.3. Identification of feather- decomposin bacillus

Bacillus species isolated from poultry waste soil were cultured in nutrient agar medium. Then, 2 ml of a McFarland turbidity of each sample bacillus was added to mineral medium containing autoclaved feather, and incubated at 27° C. The protein measurement was performed by Bio-Rad protein assay method for determinationof degraded keratin at 595 nm.The protein concentration was monitored on the first, third and sixth day after the incubation. Then, Bacillus spp. demonstrating the highest activity were isolated and identified based on morphological, physiological and biochemical tests. The standard curve of protein assay with different concentrations of Bovine serum albumin (BSA) ata wavelength of595 nm was plotted. The concentration of Keratin in the same wavelength with Coomassie Blue G-250 staining was measured by spectrometry. Then, the Keratin concentration was obtained using the standard curve [14].

2.4. Enzyme expression

SDS-PAGE was performed to confirm the production of keratinase by Bacillus spp. The mineral medium including feather was centrifuged at 2500 g for 20 min and the supernatant was used for SDS-PAGE [15]. The samples stained with Coomassie blue R 250 zymogram were placed on vertical slab gel according to laemmli method with some modifications. The samples were subjected to electrophoresis on 12% separating gel at 100 V with electrode buffer (pH 8.3), containing 0.025 M Tris-HCl and 0.192 M glycine, and the stacking gel containing 4% polyacrylamide in 1.5 M Tris HCl (pH 6.8) [16]. The molecules with standard weights were ß-Galactosidase (120 KD), Bovine Serum Albumin (85 KD), Ovalbumin (50 KD), Cardonic anhydrase (35 KD), ßlactoglobuline (25KD) Lysozyme (20 KD).

2.5. PCR analysis of16S rRNA gene sequence

The Bacillus bacteria were cultured in the liquid BHI medium at 37°C for 24 h. Then, 1 ml of cells was centrifuged at10,000 g for 2 min, transferred to alcohol solution and 50 µl sterile water was added to it. The final solution was shaken and boiled for 5 min and centrifuged for 3 min at 12000 g. The supernatant containing DNA was transferred to a new microtube. Gene fragments specifics for 16S rRNA-coding regions were amplified by PCR using primers:

F: 5'-GAGTTTGATCCTGGCTCAG-3

R: 5'-AGAAAGGAGGTGATCC-3

The PCR solution, containing 5 µl of DNA template (500 µg), 2 µl of Forward Primer (10pMol), 2 µl of Reverse Primer (10 pMol), 2 µl of PCR buffer, 0.75 µl of $MgCl_2$ (1.5 mMol), 0.2 µl of Taq, 0.25µl of dNTP (200 mMol) and 7.8 of deionized H_2O,was prepared (final volume of 20µl) and PCR reactions were performed under the following program: initial denaturation for 3 min at 94°C, denaturation for 1min at 94° C , primer annealing for 1min at 58°C , primer extension for 2 min at 72° C. The last three steps were repeated for 30 cycles and ultimately final elongation was done for 10 min at 72°C [14, 17].

Then, the PCR amplification product was analyzed by SDSPAGE. Then, the PCR amplification product was analyzed by SDS-PAGE.

2.6. PH optimization

The pH of mineral medium was adjusted to 7, 8,9,10 and 11 to monitor the effect of pH on the enzyme production. 2 ml of a McFarland turbidity of Bacillus spp. was added and the samples were incubated at 27°C.Then, the Bio-Rad protein assay method was used to study the enzyme activity in different pHs [18].

2.7. Temperature optimization

At optimum pH, the optimum temperature was found by incubating the enzyme solution with feather at different temperatures ranging from 20-40°C. Then, the enzyme activity was determined by the Bio-Rad protein assay method at each temperature [19].

2.8. Statistical analysis

Data were analyzed statistically using the Student t test by graph pad prism version 5. The 2-sided P values were calculated, and statistical significance was acceptable within 95% confidence intervals. All the experiments were repeated three times.

3. Results and Discussion

3.1. Isolation and identification of feather decomposing Bacillus spp.

Seven out of seventeen isolated *Bacillus spp*. from the poultry waste were found to secrete keratinase enzyme and degrade the feather (Fig. 1).

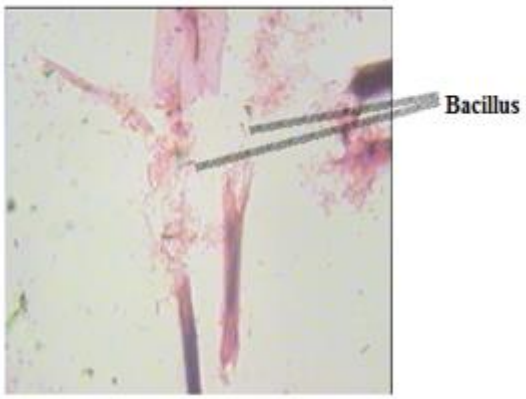

Fig. 1: A microscopic view of feather degrading by *Bacillus spp*. The slide was prepared by gram staining.se b: after used.

Based on morphology and several biochemical tests, they were identified as B. lentimorbus, B. pumilis, B. subtilis, B. firmus, B. macerance, B. popilliae, and B. larvae (Table 1).

3.2. Confirmation of keratinase production by SDS-PAGE

SDS-PAG Eanalysis revealed that the molecular weight of secreted enzyme was about 35 KD which is the same askeratinase enzyme. This test confirmed the secretion of keratinase by *bacillus spp.* (Fig. 2).

3.3. The Bacillus spp. identification by 16S Rrna gene sequence

A band in the area about 1500 bp was observed on agarose gel, which is related to the genus Bacillus. The results showed that the PCR products were specifically amplified. B. subtilis subsp. subtilis ATTC6051 was used as a positive control (Fig. 3).

Table1: Results of morphological and biochemical tests for *Bacillus spp.* isolated from poultry waste.

Bacillus	Catalase	V-P reaction	Growth in anaerobic	Growth at 50°C	Growth in 7% NaCl	Acid and gas in glucose	NO_3 reduced to NO_2	Starch hydrolyzed	Growth at 65°C	Rods 1.0µm wide or wider	pH in V-P medium <6.0	Acid from glucose	Hydrolysis of casein	Parasporal bodies
Subtilis	+	+	-	+	+	-	+	+	-	-	V	+	+	-
Pumilis	-	-	+	-	-	-	-	-	-	-	-	+	-	-
Firmus	-	+	+	-	-	-	+	+	-	+	-	-	-	+
Macerans	-	-	+	-	-	-	+	+	+	-	+	+	-	+
Larvae	-	+	+	+	V	-	+	-	-	-	-	+	+	+
Popilliae	-	-	+	-	-	-	-	-	-	+	-	+	-	-
Lentimorbus	-	-	+	-	-	-	-	-	-	-	-	+	-	-

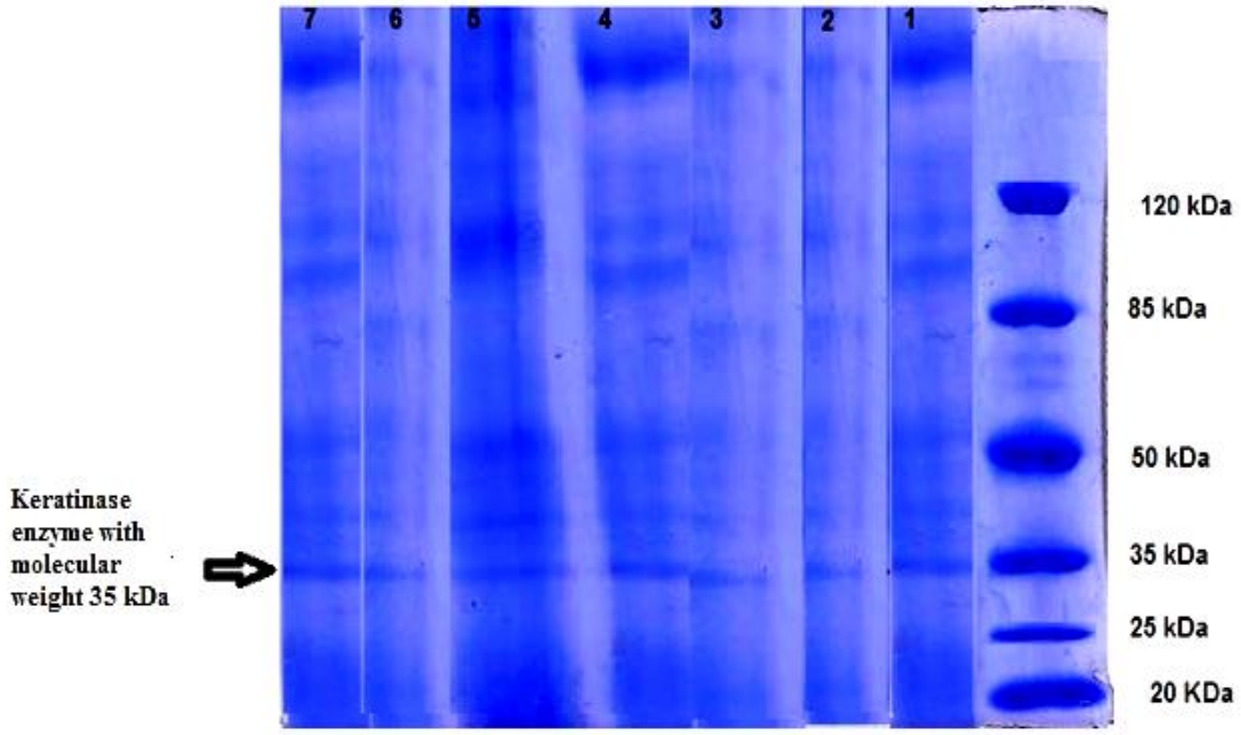

Fig. 2: SDS-PAGE of enzymes secreted by *bacillus spp*. Column 1: B. *Pumilis*, Column 2: B. *Subtilis*, Column 3: B. *Macerans*, Column 4: B. *Popilliae*, Column 5: B. *Larvae*, Column 6: B. *Firmus*, Column 7: B.*Lentimorbus*. Lane M: Molecular weight marker proteins: ß-Galactosidase (120. KD), Bovine Serum Albumin (85 KD), Ovalbumin (50 KD), Cardonic anhydrase (35 KD), ß –lactoglobuline (25 KD), Lysozyme (20 KD).

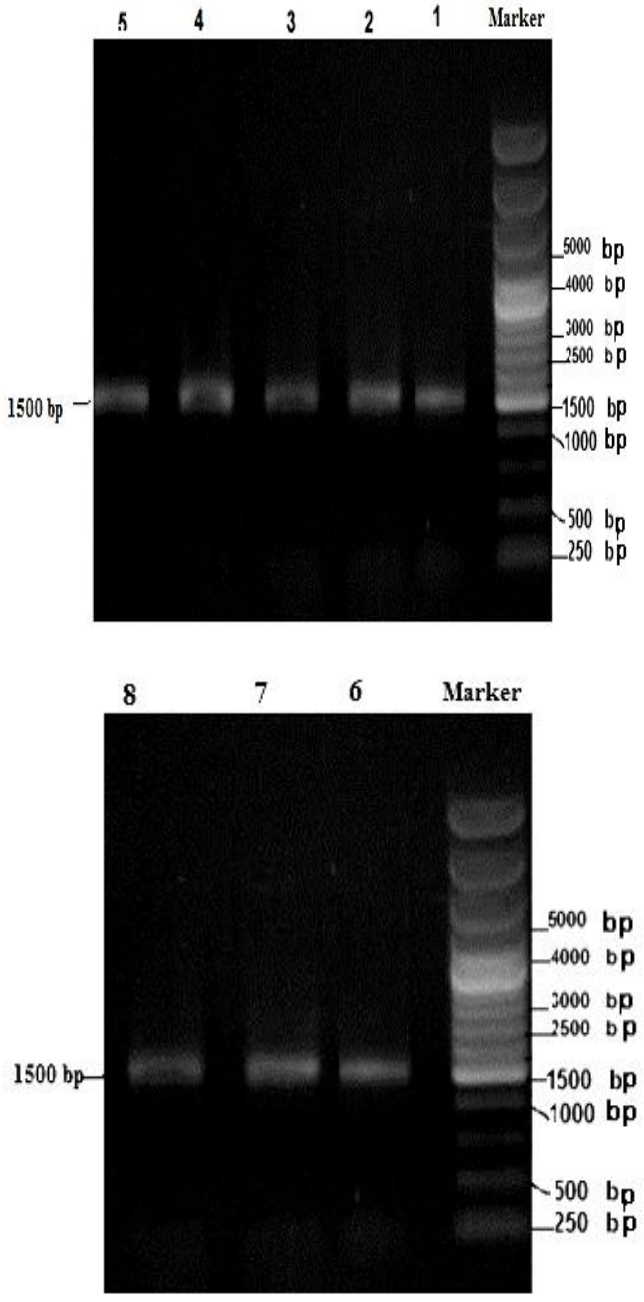

Fig. 3: Results of 16S rRNA PCR of the isolated Bacillus spp. First column: size markers, second column: B. *subtilis subsp*. *subtilis* ATTC6051, Column 3: B. *Pumilis*, Column4: B. *Subtilis*, Column 5: B. *Macerans*, Column 6: B. *Popilliae*, Column 7: B. *Larvae*, Column 8: B. *Firmus*, Column 9: B. *Lentimorbus*.

3.4. PH optimization

The results showed that the pH = 11 on the sixth day of culturing was the best for maximum production of keratinize enzyme by B. *subtilis*, B. Larvae and B. *Lentimorbus*. In this regard pH = 10 was found the best for B. *Firmus* and pH = 9 was the best for B. *Pumilis*, B. *Popilliae* and B. *Macerans* under the same conditions (Fig. 4).

The highest enzyme activity was related to *Bacillus subtilis* ($P<0.05$).

3.5. Temperature optimization

The optimum temperature for the highest production rate of keratinase activity for each isolated bacillus sp. on the sixth day of culturing and in the optimum pH was shown in figure 5. The results showed that 40ºC was the best temperature for maximum production of keratinase enzyme for B. *subtilis*, B. Larvae and B. *Lentimorbus*. In this regard 25ºC was found the best temperature for B. *Popilliae* and 35ºC was the best temperature for B. *Pumilis*, B. *Firmus* and B. Macerans under the same conditions. The highest enzyme activity was found related to *Bacillus subtilis* ($P<0.05$).

There are many microbial resources that produce Keratinase, but only a few of these resources have industrial value. Bacillus spp. Are among them with a high capacity to secrete keratinase enzyme (25 to 20 g/l) among them [8, 20, 21]. *Bacillus species* produce keratinase in the late logarithmic and stationary phases. This study showed that the environmental conditions like pH and temperature can affect the rate of Keratinase production by *Bacillus spp*. [22]. The optimum conditions for keratinase synthesis by Bacillus spp. was essential step for the production of adequate keratin. According to this study Bacillus subtilis has the maximum keratin degradation and keratinase production on the sixth day after incubation. It was concluded that the feather waste can be a renewable source for the production of keratin.

4. Conclusion

The findings showed that the isolated keratinolytic *Bacillus spp*. can be potentially considered as the candidates for the degradation of feather to produce keratin.

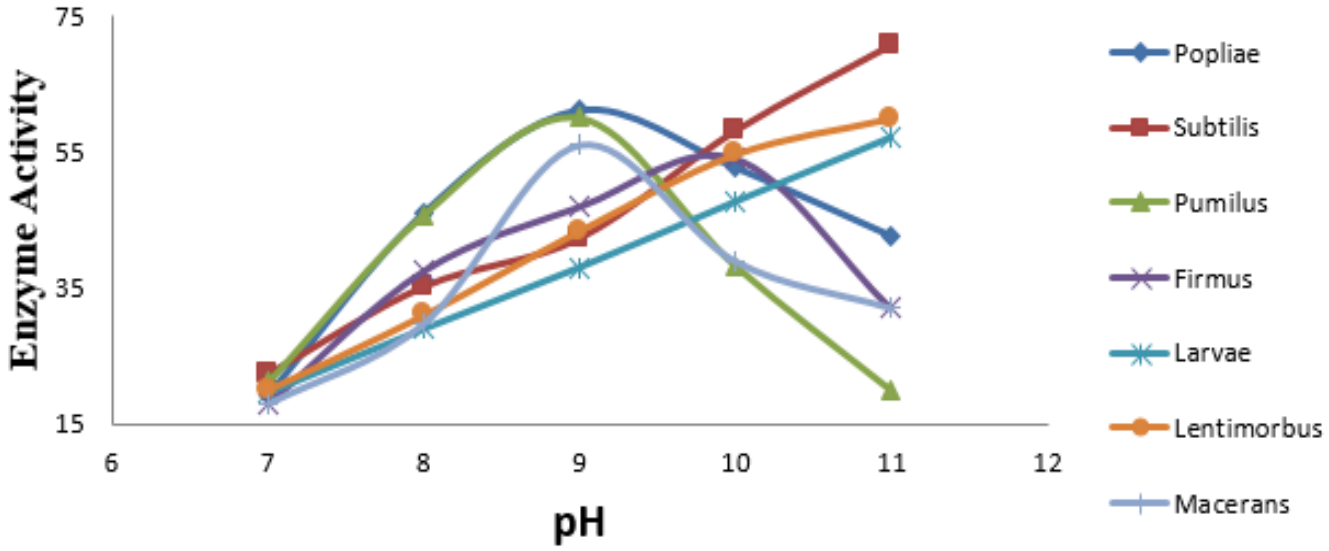

Fig. 4: Activity of keretinase enzyme at different pH for feather degrading on day 6 after feather incubation with *bacillus spp*.

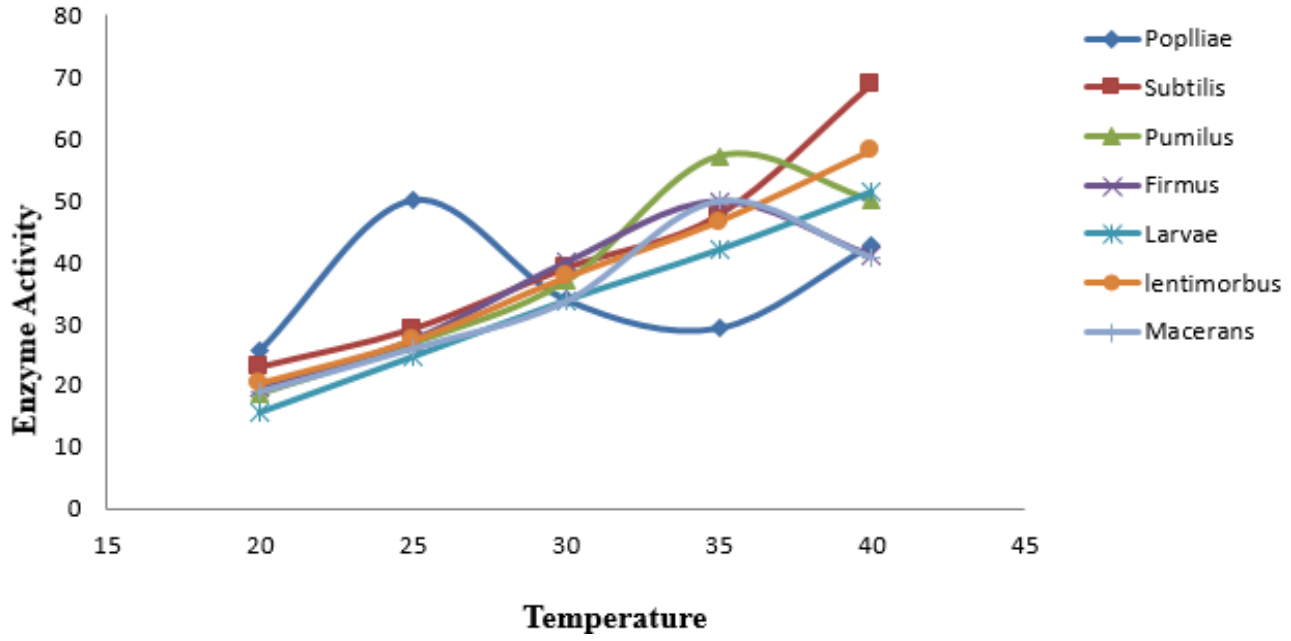

Fig. 5: Activity of keretinase enzyme at different temperatures for feather degrading on day 6 after feather incubation and in optimum pH by *bacillus spp*.

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