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Mob: +92 300 3008585, Fax: +92 41 8815544
E-mail: editorijps@gmail.com

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Isolation, Identification and Characterization of a Feather Degrading Bacterium

Savitha G. Joshi, M.M. Tejashwini, N. Revati, R. Sridevi and D. Roma
Department of Biotechnology, B.V.B. College of Engineering and Technology,
Vidyanagar, Hubli-31, Karnataka, India

Abstract: Feather constitutes over 90% protein, the main component being beta-keratin, a fibrous and insoluble structural protein extensively cross linked by disulfide bonds. This renders them resistant to digestion by animal, insects and proteases leading to serious disposal problems. In addition to this, feather waste is produced at the rate of 22 million kg per year (US alone). Use of keratinolytic microorganisms for feather degradation is an economical, environmental friendly alternative. Keratinases which are produced by these keratinolytic organisms could be used to degrade feather waste and further the digested products could be an excellent material for producing animal feed, fertilizers or natural gas. A feather-degrading bacterium was isolated from poultry waste. This bacterium was grown in basal media with feathers as its primary source of carbon, nitrogen, sulfur and energy. The organism is rod shaped, highly motile, endospore forming, catalase positive and gram negative. Phenotypic characterization carried out in our laboratory showed that this novel gram negative bacterium belongs to *Bacillus* genus. The organism is designated as *Bacillus* and named as *Bacillus* sp PW-1. The isolated strain has activity of 50 U/ml. This novel keratinolytic isolate could be a potential candidate for degradation and utilization of feather keratin.

Key words: Feather, keratin, feather degrading bacterium, poultry waste, keratinolytic activity

Introduction

Insoluble and hard-to-degrade animal proteins are ubiquitously present throughout animal bodies. Enormous numbers of these proteins are generated in the meat industry in a mixture of bones, organs and hard tissues, finally being converted to industrial wastes, the disposal of which is tremendously difficult. Most animal proteins (feathers) are currently disposed of by incineration. This method, however, has ecological disadvantages in terms of an apparent energy loss and the production of a large amount of carbon dioxide. Thus, an innovative solution to these problems is urgently needed (Suzuki *et al.*, 2006).

Feather wastes are generated in large quantities as a byproduct of commercial poultry processing. Feathers represent 5-7% of the total weight of mature chickens. Feathers are made up primarily of keratin, which is also found in the claws and armour of reptiles and the hooves, horns, hide, hair and nails of mammals. These feathers constitute a sizable waste disposal problem. Several different approaches have been used for disposing of feather waste, including land filling, burning, natural gas production and treatment for animal feed. Most feather waste is land filled or burned, which involves expense and can cause contamination of air, soil and water.

Keratin, by virtue of its insolubility and resistance to proteolytic enzymes, is not attacked by most living organisms. Nevertheless, keratin does not accumulate in nature and, therefore, biological agencies may be presumed to accomplish its removal. Several insects, including clothes moth larvae, carpet beetles and chewing lice are known to digest keratin. The common occurrence in nature of microorganisms that readily and, in some cases, preferably grow on keratinaceous substrates has supported the general belief that certain microorganisms can digest keratin (Noval and Nickerson, 1959).

Bacterial strains are known which are capable of degrading feathers. These bacterial strains produce enzymes which selectively degrade the beta-keratin found in feathers. These enzymes make it possible for the bacteria to obtain carbon, sulfur and energy for their growth and maintenance from the degradation of beta-keratin. An enzyme capable of degrading protein is known as a protease and is described as having proteolytic activity. An enzyme which degrades keratin is a keratinase, while a beta-keratinase is an enzyme capable of degrading beta-keratin. An enzyme which degrades keratin can also be described as having keratinolytic activity.

Keratinases from bacteria are isolated and characterized. For instance, keratinase from *Bacillus sp.* (Zerdani *et al.*, 2004). *Bacillus licheniformis* (Ramnani *et al.*, 2005; Korkmaz *et al.*, 2004; Manczinger *et al.*, 2003; Williams *et al.*, 1990). *Burkholderia*, *Chryseobacterium*, *Pseudomonas*, *Microbacterium sp* (Brandell and Riffel, 2006) *Chryseobacterium sp.* (Brandelli, 2005; Riffel *et al.*, 2003), *Streptomyces sp.* (Bressollier *et al.*, 1999; Montero-Barrientos *et al.*, 2005) were isolated and was studied with respect to various parameters.

Characterization based on 16S rRNA was carried out for genus *Terrabacter* (Montero-Barrientos *et al.*, 2005). On the basis of physiological, morphological and 16S rDNA studies the new isolate was found to be a member of the Thermotogales order and was identified as *Fervidobacterium pennavorans*. The strain was highly related to *Fervidobacterium islandicum* and *Fervidobacterium pullulanolyticum* (Friedrich and Antranikian, 1996). Membrane ultrafiltration and carboxymethyl cellulose ion-exchange and Sephadex G-75 gel chromatographies were used to purify the enzyme from *Bacilus licheniformis* PWD-1 (Lin *et al.*, 1992).

Bacterial keratinases are of particular interest because of their action on insoluble keratin substrates, for de-hairing processes in the leather industry, discarded feathers are currently used to produce feather meal through thermal processing, resulting in a low nutritional value product. Feather hydrolysates produced by bacterial keratinases have been used as additives for animal feed. In addition, keratin hydrolysates have potential use as organic fertilizers, production of edible films and rare amino acids (Brandelli, 2005). Because of innumerable uses of keratinases, novel bacterium having maximum activity was isolated.

Materials and Methods

Isolation of feather degrading bacteria

Enrichment: 1gm of poultry waste was serially diluted in order to reduce the initial number of micro organisms. This dilution was then inoculated into basal feather broth. Feathers was washed, dried and hammer milled prior to being added to the medium. The medium was sterilized by autoclaving. All incubations were done at 37°C with shaking at 120 rpm in a controlled-environment shaker (Shih and Michael, 1992).

Screening on Skim milk agar plates: Skim milk agar (Himedia) was prepared and the above dilutions were streaked on milk agar plates for testing the caseinolytic activity of the organism. Bacteria were inoculated onto plates and incubated at 37°C for 24 h. Strains that produced clearing zones in this medium were selected (Zerdani *et al.*, 2004).

Screening on Keratin agar plates: The colonies obtained from skim milk agar plates were transferred to keratin (Himedia) agar plates.

Subculturing: The organism screened with Keratin agar plates was subcultured by continuously growing the bacterium in basal broth medium (4 days at 37°C, 120rpm) and subsequently streaking on basal agar medium (2% agar, 2 days 37°C).

Identification of Isolated feather degrading bacteria: Gram Stain, Spore staining, Motility test, Catalase Test (Cappuccino and Sherman, 2004).

Characterization of the isolate using Biochemical assays: IMViC Test, Hydrogen Sulfide Test, Urease Test, Litmus Milk Reactions, Nitrate Reduction Test, Carbohydrate Fermentation, Starch hydrolysis and Gelatin liquefaction (Cappuccino and Sherman, 2004).

Assay for keratinase activity: The test described below was developed in order to simplify analytical work on keratinase. Azo-keratin hydrolysis provides a colorimetric assay for enzymatic activity on keratin (Shih and Michael, 1992). The inoculum was incubated in 0.9%NaCl at 37°C for 24 hours before inoculating into the basal feather broth for the crude enzyme assay. A 5% inoculum was inoculated in 100 mL of basal feather medium at 37°C and 120rpm.

Synthesis and enzymatic hydrolysis of azo-keratin

Synthesis: Azo-keratin was prepared by a similar method similar to a known procedure for azoalbumin. The use of azoalbumin as a substrate in the colorimetric determination of peptic and tryptic activity. Ball-milled feather powder was prepared as described in Williams and Shih (1989). A 1 g portion of the feather powder (the keratin source) was placed in a 100-ml round-bottomed reaction flask with 20 mL of deionized water. The suspension was mixed with a magnetic stirrer. Two ml of 10% $NaHCO_3$ (weight per volume) were mixed into the feather suspension (Lin *et al.*, 1992).

In a separate 10-ml tube, 174 mg of sulfanilic acid were dissolved in 5 mL of 0.2 N NaOH. Sixty-nine mg of $NaNO_2$ were then added to the tube and dissolved. The solution was acidified with 0.4 mL of 5 N HCl, mixed for 2 min and neutralized by adding in 0.4 mL of 5 N NaOH. This solution was added to the feather keratin suspension and mixed for 10 min. The reaction mixture was filtered and the insoluble azo-keratin was rinsed thoroughly with deionized water. The azo-keratin was suspended in water and shaken at 50°C. for 2 hr and filtered again. This wash cycle was repeated until the pH of the filtrate reached 6.0-7.0 and the spectrophotometric absorbance of the washing at 450 nm was less than 0.01 (Burtt and Ichida, 1999).

Finally, the wash cycles were repeated at least twice using 50 mM potassium phosphate buffer, pH 7.5. The azo-keratin was washed once again with water and dried in vacuo overnight at 50°C. The resulting product is a chromogenic substrate that can be incubated with enzyme solution to produce and release soluble peptide derivatives that cause an increase in light absorbance of the solution (Burtt and Ichida, 1999).

Enzymatic hydrolysis of azo-keratin: This procedure tested the keratinolytic activity of keratinase on azo-keratin. To begin the process, 5 mg of azo-keratin was added to a 1.5-ml centrifuge tube along with 0.8 mL of 50 mM potassium phosphate buffer, pH 7.5. This mixture was agitated until the azo-keratin was

Table 1: Cultural characteristics

Sl. No.	Agar plate	Characters	Results
1.	Nutrient Agar plates	Size	Moderate
		Pigmentation	Cream colored
		Form	Circular
		Margin	Lobate
		Elevation	Raised
2.	Nutrient Broth culture		Uniform with fine turbidity.
3.	Nutrient Agar slants	Abundance of growth	Moderate,
		Pigmentation	Cream colour,
		Optical characteristics	Opaque
		Form	Filiform

Table 2: Results of additional morphological, physiological and biochemical tests were conducted on the isolate

Experimental Details	Observations
Gram stain	Negative
Shape and arrangement	Rods in Single and in Chains
Endospore stain	Positive
Litmus milk reactions	Peptonization
Carbohydrate fermentations with lactose	Acid with Gas
sucrose	Acid with Gas
dextrose	Acid with Gas
Nitrate reduction	Positive
Motility test	Positive
Indole production	Positive
Methyl red test	Negative
Voges-Proskauer test	Positive
Citrate utilization	Positive
Catalase activity	Positive
Starch hydrolysis	Negative
Hydrogen sulphide test	Negative
Urease activity	Positive
Gelatin liquefaction	Positive

Collectively, these characteristics indicated that the isolate was of the genus *Bacillus*

completely suspended. A 0.2-ml aliquot of supernatant of crude enzyme was added to the azo-keratin, mixed and incubated for 15 min at 50°C with shaking. Assay conditions were the same for each enzyme sample except that pH and temperature were adjusted to their optima for the specific enzyme. The reaction was terminated by adding 0.2 mL of 10% trichloroacetic acid (TCA). The reaction mixture was filtered and analyzed for activity (Burtt and Ichida, 1999).
The absorbance of the filtrate was measured at 450 nm with a UV-160 spectrophotometer (LaboMed.Inc). A control sample was prepared by adding the TCA to a reaction mixture before the addition of enzyme solution. A unit of keratinase activity was defined as a 0.01 unit increase in the absorbance at 450 nm as compared to the control after 15 min of reaction (Burtt and Ichida, 1999).

Results

Isolation and adaptation of feather-degrading microorganism: It was found that a previously enriched, feather-degrading culture contained microorganism exhibiting keratinolytic activity. The isolate is a rod-shaped bacterium which appeared singly and in chains. It displayed clearing zone when streaked onto the skim milk agar plates. Cells of the isolate were grown on basal feather agar and transferred at frequent intervals to the basal medium, containing finely chopped feathers. Cells were also grown on keratin containing media and subcultured at regular intervals. Eventually, after several weeks of repeated selection and subculturing a pure culture of the bacterium was obtained.

Identification and characterization of feather-degrading isolate: Microscopic observation of the isolate showed a straight rod with endospores. The bacterium grew aerobically, strongly catalase positive, Gram negative and was highly motile.
Additional morphological, physiological and biochemical tests were conducted (Table 1 and 2).
The isolate was able to grow on Thrones (Atlas, 1993) medium which is specific for *Bacillus licheniformis*.
We propose “PW” as the strain designation to indicate isolation of the strain from poultry waste.

Degradation of feathers by isolate: It was observed that aerobic growth by the isolate on feathers, with the feathers as its primary source of carbon, nitrogen, energy and sulfur, resulted in nearly complete degradation of the keratin after 7 to 10 days of incubation at 37°C. Biodegradation was measured as the increase in the absorbance at 450 nm by the Azokeratin hydrolysis enzyme assay.
Isolated strain (PW) has activity of 50.0U/ml as compared to PWD-1 (30.50 U/ml). Change in absorbance at 450 with respect to days after inoculation was recorded and it was found that the maximum absorbance is on 12th day (Table 3).

Discussion

A bacterium isolated from poultry waste has been shown to degrade feather keratin by using feathers as a primary source of energy, carbon, nitrogen and sulfur. The bacterium was isolated from an anaerobic habitat; however, it showed maximum growth under aerobic conditions, as would be expected of a member of the family Bacillaceae (Williams *et al.*, 1990).
One explanation for the presence of this species in a poultry waste may be that the bacterium is indigenous to

Table 3: Change in absorbance of PW Strain with respect to days of incubation

Days	$A_{450\ nm}$
0	0
1	0.02
2	0.023
3	0.0315
4	0.0415
5	0.125
6	0.17
7	0.176
8	0.019
9	0.18
10	0.458
11	0.48
12	0.517
13	0.306
14	0.12
15	0.025

the chicken gut. However, it is more likely that, it was indigenous to the environment in which poultry excreta are collected. This environment also contains feathers and the isolate may have adapted to utilize this substrate.

When cells of *Bacillus* sp PW were grown aerobically on basal feather medium, they yielded appreciable degradation as measured by the azokeratin hydrolysis assay. Such an increase in absorbance did not occur when the cells were subjected to growth conditions in the nutrient broth in comparison with the basal feather medium. It is noteworthy that the increase in absorbance initially lags behind the growth of the bacterium. This may be due to enzymes liberated by the bacterium following a period of logarithmic growth. Such enzymes have been documented in strains of *B. licheniformis*. These enzymes appear in the extracellular medium only during the postlogarithmic phase of growth. The increase in the absorbance of the filtrate of the azokeratin medium during the degradation of azokeratin by this strain of *Bacillus* PW is evidence that this bacterium possesses a protease (s) capable of reducing the disulfide bonds of keratin. The isolated strain was found to be Gram negative (Yumoto *et al.*, 1998).

The degradation of feathers by a *Bacillus* sp. has not been reported previously; however, Molyneaux (1959) reported the isolation, from the dermoid cyst of a sheep, of a *Bacillus* sp. which digested wool keratin. He noted that although the growth of various bacilli on wool had been previously reported, his isolate was the first that could degrade nonsteam-sterilized or native wool keratin. Molyneaux (1959) conducted an extensive classification of his isolate but did not assign a species name. Many of the results of biochemical tests he conducted match those we observed for *B. licheniformis* PWD-1. However, the *Bacillus* isolated by Molyneaux (1959) grew better under mesophilic temperatures and was also unable to reduce nitrate to nitrite.

Comparison of PW with PWD-1: *B. licheniformis* strain PWD-1 with ATCC Accession No. 53757 (Shih and Michael, 1992) is found to be a gram positive (but gram variable) bacteria where as PW is gram negative. Bacterial cells are found both singly and in chains. PW is also rod shaped and found both singly and in chains. One subterminal endospore is formed per cell, the endospore being centrally located and round or oval in shape. As with PWD-1, PW is also endospore forming and motile.

PWD-1 produces acid, but not gas, from L-arabinose, D-xylose (weakly), D-glucose, lactose (weakly), sucrose and D-mannitol, where as PW is both acid and gas producer. PWD-1 can utilize both citrate and propionate as a carbon source, similar to PW. PWD-1 liquefies gelatin as that of PW. PWD-1 reduces, but does not reoxidize, methylene blue. It reduces nitrate to nitrite, but it does not reduce nitrite.

Both PW and PWD-1 is Voges Proskauer positive. It decomposes hydrogen peroxide but not tyrosine, is negative for indole and is positive for dihydroxyacetone. PW is also positive for indole. PWD-1 is negative in the Litmus milk acid test, negative in the Litmus milk coagulation test and negative in the Litmus milk alkaline test, but is positive in both the litmus milk peptonization and litmus milk reduction tests. PW is positive in litmus milk peptonization.

Conclusion: A feather-degrading bacterium was isolated from poultry waste. This bacterium was grown in basal media with feathers as its primary source of carbon, nitrogen, sulfur and energy. The organism is rod shaped, highly motile, endospore forming catalase positive and gram negative. Phenotypic characterization carried out in our laboratory showed that this novel gram negative bacteria belongs to *Bacillus* genus. The organism is designated as Bacilus and named as *Bacillus* sp PW.

We obtained an activity of 50U/ml as that compared to 30.5U/ml of PWD-1.

This novel keratinolytic isolate could be a potential candidate for degradation and utilization of feather keratin.

Future Prospective: Purification and characterization of keratinase, studying the kinetics of enzyme, Analysis of reduction in the disulfide bonds, analysis of free amino acids by free amino group assay, testing for the range of substrates, comparison of activity of keratinase with other known and commercially used proteases, testing for the dehairing activity, effect of inhibitors, enhancing the activity of keratinase, submerged state fermentation and large scale production of keratinase, immobilization of keratinase.

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