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Isolation and characterization of feather degrading bacteria from poultry waste

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This study was conducted to isolate, partially purify and characterize keratinolytic microbes and their enzymes for commercial exploitation. A feather degrading bacterial strain was isolated from soil where poultry feathers were dumped by the poultry farms as a waste product. The isolated bacteria grew well at 37°C and the pH of 7.5. This isolate showed maximum feather degrading activity at 37°C. The enzyme activity could be detected in the temperature range of 30 to 37°C. Keratinolytic activity was detected during growth of microbes and complete degradation of keratin was achieved. The proteolytic character of crude enzymes was assessed using azo-casein as substrate. Crude keratinase was extracted and purified by salt precipitation and dialysis. The organism was pigment producing, rod-shaped, endospore forming, catalase positive and Gram negative bacteria. Fermentation of sugars, SIM test, indole production test, citrate test, nitrate reduction test, MR test, VP test, starch and gelatin hydrolysis test were also done. The isolated bacteria produced green pigment during its growth and the media turned greenish. Phenotypic characterization carried out in laboratory showed that this Gram negative bacterium might belonged to *Bacillus* or *Alteromonas* genus. The cell free supernatant had feather hydrolyzing activity. Therefore, it can be said that it was an extracellular protease, that is, keratinase enzyme. This keratinolytic isolate could be a potential candidate for degradation and utilization of feathers. It might also be an efficient method for the production of microbial enzymes and metabolites.

Key words: *Alteromonas, Bacillus,* Keratin, poultry waste, feather.

INTRODUCTION

The expansion of biotechnology has produced an increasing demand for high-quality inexpensive microbial growth media. Utilization of feathers from the poultry processing industry as a substrate for fermentation might

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offer an inexpensive alternative for a microbial method for the production of microbial enzymes and many other metabolites (Shih, 1993). Feathers are produced in large amounts as a by-product of poultry processing plants, reaching millions of tons per year with potential environmental impact (Azeredo et al., 2006). Feathers represent 5-7% of the total weight of mature chickens. By using physical and chemical treatments, currently feathers are used in the conversion of feather meal, a digestible dietary protein for animal feed. These methods can destroy certain amino acids and decrease protein quality and digestibility (Riffel and Brandelli, 2006). Most feather wastes are land filled or burned which involve expenses and can cause contamination of soil, air and water. Thus, an innovative solution to this problems is urgently needed (Suzuki et al., 2006). Keratins are the most abundant proteins in epithelial cells of vertebrates and represent the major constituents of skin and its appendages such as nail, hair, feather and wool. The degradation of keratinous material has medical and agricultural importance (Shih, 1993; Matsumoto, 1996). A group of proteolytic enzymes that are able to hydrolyze insoluble keratins more efficiently than other proteases are called keratinases. Bacterial keratinases are of particular interest because of their action on insoluble keratin substrates and generally on a broad range of protein substrates (Lin et al., 1992). Keratinase is usually a serine protease (Bockle and Muller, 1997; Bressollier et al., 1999; Gradisar et al., 2000). Its optimum temperatures and pH values were reported to be 40 to 80°C and 6-10, respectively (Cheng et al., 1995; Friedrich and Antranikian, 1996; Bockle and Muller, 1997). Keratinase stability is influenced by temperature, pH and chemical substances (Peek et al.,1992). Many researchers isolated and characterized keratinases with respect to various parameters from different bacterial species, for instant, *Bacillus licheniformis* (Manczinger et al., 2003; Korkmaz et al., 2004; Ramnani et al., 2005), *Chryseobacterium, Pseudomonas, Microbacterium* spp. (Riffel and Brandelli, 2006), *Chryseobacterium* spp. (Riffel et al., 2003), *Sterptomyces* spp. (Bressollier et al., 1999; Montero-Barrientos et al., 2005). A high molecular weight keratinolytic protease from feather degrading DCUW strain was purified and characterized (Ghosh et al., 2008). The aim of this study was to isolate, purify and characterize keratinolytic microbes and their enzymes for potential commercial exploitation.

MATERIALS AND METHODS

Sample collection and isolation of bacteria from soil sample

A number of samples were collected from the outskirts of poultry farms where poultry feathers together with other waste were dumped. Both waste water and soil samples were collected from these areas in Bangladesh. The samples were screened for bacteria efficient in degrading feather of chickens. Then samples were taken in a Petri dish and diluted in distilled water by a glass rod. After a few minutes when the soil settled down, the surface water (which contained the soil bacteria) was taken very carefully to liquid broth media by micropipette for overnight growth. Then, the bacterial cultures was diluted serially up to 10^{-6} times with distilled water and spread in an agar plate. Among them, 20 colonies were identified on the basis of different colony morphology. Each colony was incubated into 20 screw capped test tubes containing autoclaved feather and nutrient broth media and incubated overnight at 37°C with shaking at 160 rpm. One medium containing only autoclaved feather was used as negative control. Single isolated colonies were obtained by streaking small volume of a liquid culture on to a Petri plate containing nutrient media.

Isolation of feather degrading bacteria

The soil samples were processed according to the method described in earlier section. About 30 discreet colonies of different size and shape appeared in each of 10 nutrient agar plates. About 20 colonies from each plate were inoculated in the screw capped test tube containing 5 mL sterile nutrient broth and intact sterile feather. One tube was kept as control containing no bacterial colony. The tubes were incubated at 25°C temperature for 7 days. The organisms grew profusely in all the tubes with different feather degradation capability.

These tubes were then incubated at 37°C for 7 days. Degradation of the feather was detected in some tubes. But all of these had not satisfactory feather degrading activity. In one of the tubes, strong feather degradation was observed and it was taken for further analysis. This tube was designated as SAR1 and these bacteria were used throughout the study.

Preservation and preparation of subculture of bacteria

A colony of the isolate collected from nutrient agar plate was stored in nutrient broth containing 15% glycerol (glycerol broth) and it was kept at -20°C. A 250 mL conical flask containing 50 mL of sterile nutrient broth was inoculated with the selected isolate. It was grown overnight in a psychrotherm shaker incubator for overnight at 37°C. This was the subculture used for the preparation of batch culture.

Determination of the effect of temperature and pH on bacterial growth

In this experiment, the effect of temperature (30, 37, 40, 45 and 50°C) on bacterial growth for 72 h was determined in different time interval. The bacteria were further incubated in nutrient broth media for 20 h at 37°C. The effect of initial medium pH (7.0, 7.5, 8.0 and 8.5) on bacterial growth was determined. The bacteria were incubated in nutrient broth media for 16 h at 37°C. The growth was measured at different time interval (0, 2, 4, 6, 8, 10 and 12 h). Then, the absorbance of bacterial culture was measured at 600 nm.

Microscopic examination of bacteria and spores

Different methods staining methods, such as, crystal violet (the primary stain), iodine solution (the mordant), decolorizer (ethanol as a good choice), safranin (the counterstain), water (preferably in a squirt bottle), were used to study the microscopic appearance of bacteria.

Screening on different agar plates

Skimmed milk agar was prepared and the above isolated bacterial sample was streaked on milk agar plates for testing the caseinolytic

activity of the organism.

Determination of the effect of culturing temperature on feather degrading activity

For the determination of effect of temperature, fresh bacterial culture was inoculated on the autoclaved feathers and incubated at different temperatures (25, 30, 37 and 45°C) and feather degradation was observed.

Determination of protease activity at different time interval at 37°C

The proteolytic activity of enzymes was determined using azocasein, a quantitative assay to measure the proteolytic activity described by Kreger and Lockwood (1981).

Azocaseein was used as substrate. 1.5 mL culture was taken in an Eppendorf tube and centrifuged at 4000 rpm for 5 min. Then, 400 µl supernatant was taken and mixed with 400 µl 1% azocasein (pH 8.0) for the test sample. For control, 400 µl supernatant was mixed with 135 µl 35% TCA and incubated for 2-3 min. Then, 400 µl 1% azocasein was mixed with the control solution. Both test and control solution was incubated at 37°C for 1 h. After incubation, 135 µl 35% TCA was mixed with only test sample. Both test and control solution was freezing at 4°C for 15 min. The solution was centrifuged at 14000 rpm for 7 min. 750 µl supernatant was taken and mixed with 750 µl freshly prepared 1 N NaOH. Then absorbance was measured at 440 nm. The protease activity was measured at different time interval (1, 8 and 18 days) at 37°C.

Feather degrading activity of cell free culture filtrate

In this experiment, 24 h grown bacterial culture with highest feather degrading activity (SARI) was centrifuged and filtered through Millipore filters. A chicken feather was sterilized in a conical flask and incubated with 10 mL of this cell free culture filtrate at 37°C.

Determination of feather degradation by bacterial strain under stringent selection pressure

The bacterial isolate SARI was incubated in media containing feather as the sole source of carbon and nitrogen and salt media containing 0.2% $MgCl_2$, 0.5%K_2PO_4, 0.2% $MnCl_2$ and 1% NaCl and 0.02% cysteine. The media was incubated at 37°C to observe the degradation of feather.

Biochemical test

To identify the biochemical properties of the isolate, different tests such as carbohydrate fermentation, catalase, hydrolysis of starch, indole production, methyl red, VP (Vogues Proskauer), citrate utilization, nitrate reduction, gelatin utilization, H_2S production and motility tests were performed.

Ammonium sulfate fractionation of the enzyme

All proteins are precipitated by ammonium sulfate. This is a technique used in the partial purification of enzyme. About 100 mL of culture filtrate was treated with ammonium sulfate at of 75% saturation and most of the proteins were precipitated. The resulting precipitates were collected by centrifugation at 10,000 × g for 7 min and dissolved in minimal volume of 0.1 M Tris-HCl, pH 7.6. The enzyme activity was determined in both the precipitated and supernatant. Most of the enzyme activity lies with the precipitated fraction.

Table 1. Growth profile of the organism at different temperatures (in solid media).

Temperature (°C)	Time of incubation (in hour)	Results
30	12	-
	24	+
	48	++
37	12	++
	24	+++
	48	+++
45	12	-
	24	-
	48	-

- (No growth), + (moderate growth), ++ (good growth), +++ (very good growth).

Identification of the bacteria

Probabilistic identification of the bacterial strain was conducted by using a bioinformatic tool, named Probabilistic identification package for bacteria, “PIB”. In this tool, results of different tests for bacteria were given as input and the program utilized a preexisting database and compared the input result with it. It helped to identify the unknown bacterial strain.

RESULTS

Effect of temperature on bacterial growth

To monitor the effect of temperature on the bacterial growth, the organisms were grown both in solid and liquid media. In solid media, the growth pattern was measured by observing the media under microscope. The visible growth pattern was good at the temperature of 37°C. Growth reduced at 45°C and no growth was visualized at 50°C. The result was presented in Table 1. The growth rate was also determined by taking absorbance (Figure 1) of the bacterial culture grown in liquid media.

Effect of pH on the bacterial growth

The bacterial growth over a pH range between 7.0 and 8.5 was studied. Maximum bacterial growth was observed at the pH of 7.5. At the pH of 8.0 and 8.5, the growth was diminished markedly. The result was presented in Figure 2.

Growth profile of the organism at 37°C

The organisms were grown in nutrient broth at 37°C.

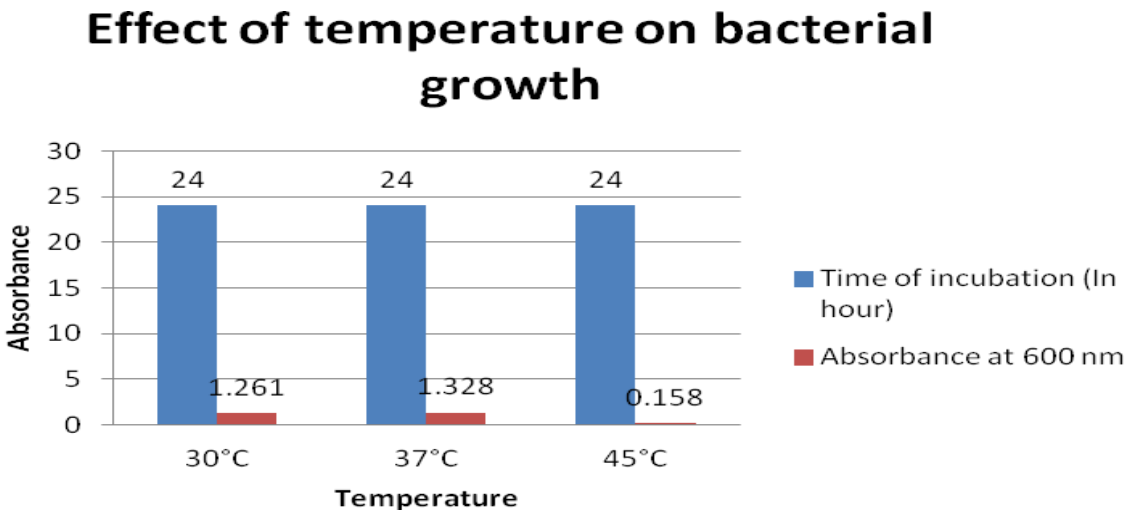

Figure 1. Growth profile of the organisms at different temperature (in nutrient broth media).

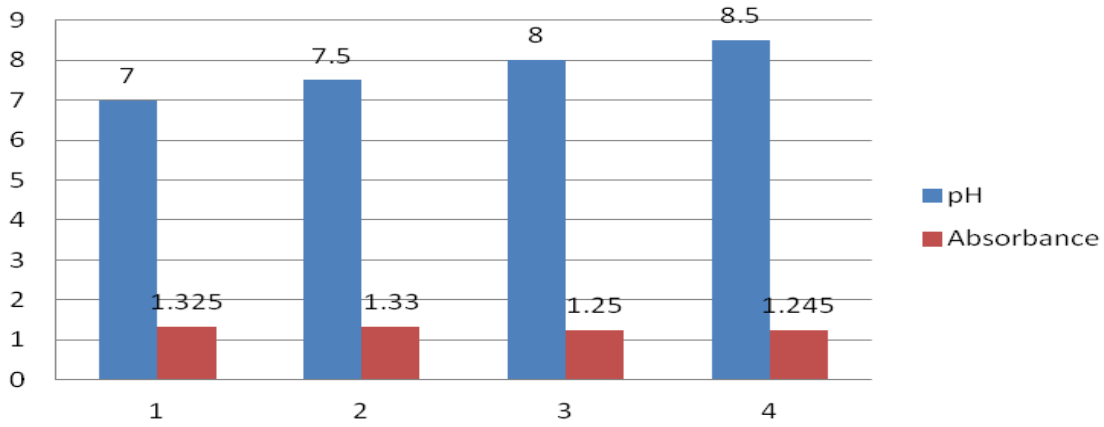

Figure 2. Growth profile of the organism at different pH.

Different samples were taken at 2 h interval and absorbance was taken at 600 nm to measure the growth. The growth profile of the organism showed that the growth reached its maximum level after 8 h of incubation. The result is given in Figure 3.

Microscopic observation after Gram staining and spore staining of isolated SAR1 bacteria

The newly isolated bacteria SAR1 was examined under microscope and found as pink colored bacteria. The bacteria looked small rod shaped and it was Gram negative bacteria. The test result was cited in Table 2. It was also observed that bacteria were endo spore forming bacteria. Spores are formed within 19 h by these bacteria. It was found that the vegetative cells were pink and the spores were green when observed under microscope.

Biochemical tests

Different biochemical tests were done for characterizing the bacteria and the results are given in Table 2.

Effect of temperature on feather-degrading activity of the organism

The bacteria were grown in the liquid broth media (pH 7.2) for 12 days at different temperatures ranging from 25 to 45°C. Maximum feather degradation was observed at 37°C within 4 to 6 days. No significant degradation was

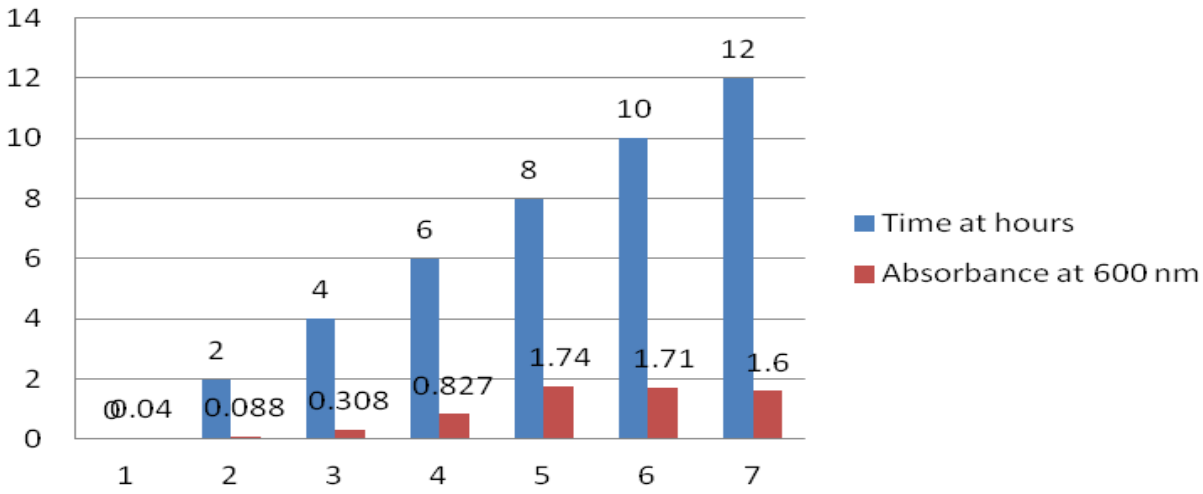

Figure 3. Growth profile of the organism at 37°C.

Table 2. Overall microscopic and biochemical characteristic of the newly isolated bacteria SAR1.

Test performed	Observation	Result
Microscopic observation		
Gram stain	Small reddish pink colonies Singly	Gram negative rods
Spore stain	Green color appeared	Spore form
Biochemical tests		
Catalase test	Bubbles formed	Positive for catalase production
Carbohydrate Fermentation tests		
Glucose	Red	Negative for acid and gas
Lactose	Red	Negative for acid and gas
Ribose	Red	Negative for acid and gas
Adonitol	Red	Negative for acid and gas
Sorbitol	Red	Negative for acid and gas
IMVIC test		
Indole (SIM) test	No red colored layer form	Negative for indole
Methyl red test	yellow colored layer formed	Negative for mixed acid production
Voges-Proskauer test	The mixture became copper colored	Negative for acetoin production
Citrate test	Change in color	Positive for citrate utilization
H2S test	Upper surface of media and along the line of stab became black colored	Positive for H_2S test
Nitrate test	No color change after zinc dust addition	Positive for nitrate reduction
Motility test	Grew upper surface on media and along the line of stab	Positive/negative
Gelatin test	Remain liqefied at 4˚C	Positive for gelatinase production
Starch test	No clear zone of hydrolysis	Negative for starch hydrolysis

Table 3. Feather degrading activity of the organism at different temperatures.

Temperature (°C)	Weight of feather (before incubation) (g)	Weight of feather (after incubation) (g)	Degradation of feather
25	0.10	0.03	70% degraded within 9 to 11 days
30	0.10	0.014	86% degraded within 7 days
37	0.10	0.01	90% degraded within 4 to 6 days
45	0.10	0.10	No significant degradation within 12 days

Table 4. Result for the test of proteolytic activity.

Time (days)	Absorbance at 440 nm	Activity (U)
1	1.931	191.3
8	1.713	171.3
18	1.599	159.9

Table 5. Result of azocasein test under stringent pressure.

Time	Absorbance at 440 nm	Activity (U)
15 days	0.2	20

Table 6. Different enzymatic properties of protease before purification procedure.

Crude culture supernatant			
Protein concentration (mg/mL)	**Total protein (mg)**	**Activity (U)**	**Specific activity (activity/concentration of protein)**
2.14	139.1	119.3	55.74

observed at 45°C. The results are given in Table 3.

Result of proteolytic activity

The proteolytic activity of enzyme was determined using the procedure described earlier (Kreger and Lockwood method, 1981). Here azocasein was used as a substrate. The azocasein hydrolysis by the culture filtrate of SARI was carried. As the organism produced greenish pigment and turned the media slightly black, it was difficult to assay the enzyme activity in cell free extract. The culture filtrate was dialyzed against buffer solution to remove the pigment. After that the enzyme activity was assayed. The result was presented in Table 4. One unit of proteolytic activity was defined as the amount of enzyme that produces an increase in the absorbance of 0.01 at 440.

Feather degradation by the isolated bacteria (SAR1) under stringent selection pressure

When bacteria was incubated in media containing feather as the sole source of carbon and nitrogen and mineral components specifically 0.2% $MgCl_2$, 0.5% K_2HPO_4, 0.2% $MnCl_2$. 1% NaCl and 0.2% cysteine under that stringent selection pressure, the organism grew and degraded the feather very slowly. Within 15 days, only 20% of total feather was degraded. The result is shown in Table 5.

Feather degrading activity of cell free culture filtrate

The degradation of feather was observed after 4 days. 86% feather degraded within 12 days. This result indicated that the culture filtrate had feather degrading activity. This result also indicated that the bacteria secreted proteolytic enzymes responsible for feather degradation.

Ammonium sulfate fraction of protein mixtures

Most of the proteins in bacterial culture filtrate

Table 7. Different proteolytic activity (by azo-casein as substrate) of enzyme after ammonium sulfate fractionation.

Sample	Activity (in unit)	Comments
Supernatant	102	Very low activity
Pellet	174	High activity

Table 8. Different enzymatic properties of protease after ammonium sulfate precipitation by dialysis.

Protein concentration (mg/mL)	Total protein (mg)	Activity (in unit)	Specific activity (activity/concentration of protein)	Purification fold
3.28	32.8	193	58.84	1.06

precipitated at 75% saturation. Enzyme activity of the pre- and pro-fractionated protein were measured. The values were given in Tables 6, 7 and 8.

DISCUSSION

The morphological and biochemical data on the growth of the organisms were analyzed by using automated software probabilistic identification package for bacteria (PIB). The analytical results indicated that the organism used in the study might belong to the genus *Bacillus* or *Alteromonas.* Some previous studies also supported these findings (Ghasemi et al., 2012; Govinden and Puchooa, 2012). Over the last few decades, bacteria, fungi and lice have been found to degrade this protein. A number of enzymes able to degrade keratin have been detected (Noval and Nickerson, 1959). These are known as keratinases. In this experiment, a number of bacteria were isolated from waste dump of feathers. These bacteria were endowed with keratinolytic activity and able to degrade keratin wastes. They used feathers as a primary source of energy, carbon, nitrogen and sulfur. The bacteria incubated at different temperatures such as 25, 30, 37 and 45°C. Growth was observed at 30 and 37□C but no significant growth was seen at 45°C, which indicated that this strain might be hemophilic. It could grow well at pH 7.5. The isolated strain was found to be Gram negative. The organism grew at 40°C but no significant proteolytic activity was detected. Many workers had shown keratinolytic activity of different organisms at different temperature (Friedrich and Antranikian,1996; De Toni et al., 2002).The optimal proteolytic activity was detected at 37°C, whereas previously described keratinolytic bacteria mostly had feather-degrading activity at elevated temperatures (Friedrich and Antranikian,1996). An optimum keratin-degrading activity at mesophilic temperatures should be a desirable characteristic because this microorganism may achieve hydrolysis with reduced energy impute. An increase of pH values was observed during feather degradation, a trend similar to other microorganisms with large keratinolytic activities (Kaul and Sumbali,1997; Sangali and Brandelli, 2000; Riffel and Brandelli, 2006). This trend might be associated with proteolytic activity, consequent de-amination reaction and the release of excess nitrogen as ammonium ions. The increase in pH during cultivation is pointed as an important indication of the keratinolytic potential of microorganisms (Kaul and Sumbali, 1997). Considering that, feather protein has been shown to be an excellent source of metabolizable protein, and microbial keratinases enhance the digestibility of feather keratin (Odetallah et al., 2003).

Conclusion

From the above discussion, it might be concluded that the phenotypic characterization carried out in our laboratory showed that this Gram negative bacteria belong to *Bacillus* or *Alteromonas,* which degrade keratins. There were circumstantial evidences to suggest that the enzymes are keratinases. This keratinolytic isolate could be a potential candidate for degradation and utilization of feather keratin to produce animal feed protein. Furthermore, this isolate possessing potential biotechnological use might be employed to remove environmental hazards like chicken feather.

Conflict of Interests

The authors have not declared any conflict of interests.

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