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Bacillus subtilis BML5 Isolated from Soil Contaminated with Poultry Waste has Keratinolytic Activity

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ABSTRACT

The present study aim was to isolate keratinolytic bacteria from the soil contaminated with poultry waste. From amongst nine bacterial keratinase producing isolates were found. The BML5 isolate was identified as *Bacillus subtilis* based on biochemical characterization and 16S rRNA Ribotyping. *B. subtilis* BML5 showed maximal keratinase activity at 37°C and at pH 8. An increase in keratinase activity of *B. subtilis* BML5 was determined in the presence of Ca^{2+}, Mg^{2+}, and Zn^{2+} while keratinase activity was inhibited by added copper ions. A 130-kDa protein was detected from feather-treated bacterial cells, suggesting its role in degrading feather. *B. subtilis* BML5 can be used to overcome the poultry wastes based environmental pollution and keratinase could be used to improve feather meal digestibility in animal feed as an additive.

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Authors' Contribution
AR designed the study, wrote the article and supervised the work. AI performed experiments, analyzed the results and wrote the article.

Key words
Keratin, Keratinolytic protease, *Bacillus subtilis* BML5, Poultry waste.

INTRODUCTION

Keratins are classified as fibrous proteins known as scleroproteins that are present abundantly in epithelial cells. Keratin is difficult to degrade as the polypeptide (Pandian *et al*., 2012). Insoluble fibrous keratin protein, primary substance found in chicken feathers, which cannot be degraded by common proteases *i.e.* trypsin, pepsin, papain (Bernal *et al*., 2003). The firm linkage of polypeptide chains is responsible for the mechanical stability of the keratin and its resistance to biochemical degradation. Both α-Helix (α-keratin) and β-Sheet (β-keratin) structure are linked by disulfide bonds which fold into an initial 3-dimensional form (Vidhya and Palaniswamy, 2013).

Currently, physical and chemical treatments are used to increase the digestibility of feather keratin and the processed poultry feathers are used as animal feed stuff, fertilizers, glues, films and as the source of rare amino acids, such as serine, cysteine and proline (Raju and Divakar, 2013). However, these processes require a large amount of energy and may also destroy certain amino acids, thus yielding products of poor digestibility and variable nutrient quality. Hydrolysis of feather keratin by microorganisms, possessing keratinolytic activity represents an attractive alternative to improve the nutritional value of feather wastes (Bo *et al*., 2009).

Keratinase is an extracellular, robust enzyme, having a wide temperature and pH range and is largely serine or metalloprotease, produced in the presence of keratin. Keratinase attacks the disulfide bonds of keratin to degrade it. Some microbes have been reported to produce keratinase enzyme in the presence of keratin substrate (Brandelli *et al*., 2010). A huge variety of microorganisms have been reported to produce keratinase including fungi, actinomycetes and bacteria.

Among bacteria, *Bacillus* spp. appear to be promising for enzyme production on commercial scale (Thys *et al*., 2004; Banerjee *et al*., 2016). It is difficult to compare keratinase titers of different microbes due to the variety of substrates and definitions of keratinase units employed. As far as physical parameters for production are concerned, they are species-specific and thus vary according to the organism (Han *et al*., 2012). But it is reported that the feather-lysate obtained by *B. licheniformis* PWD-1 has nutritional features for feed use similar to soybean protein (Kim *et al*., 2001). Microbial keratinases have potential to be used for biodegradation of feathers, enzyme activities enhancement and the increase in yields.

The present study was aimed at characterization of bacterium isolated from poultry waste and investigation of bacterial growth in feather containing medium. Keratinase characteristics including temperature, pH and metal ions were also determined.

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MATERIALS AND METHODS

Sample collection

The soil samples from vicinity of the poultry form, situated in market of University of the Punjab, Lahore, were collected to isolate bacteria having capability to degrade the feathers. The samples were collected in small polythene bags and brought to laboratory as soon as possible, dilutions up to 10^{-3} were made in normal saline and were used to plating for bacterial isolation. The samples were kept in refrigerator.

Medium used for bacterial isolation

For bacterial isolation, Luria Bertani (LB) agar medium was prepared by dissolving yeast extract (0.5g/100 ml), trypton (1.0g/100 ml), NaCl (0.5g/100 ml) and agar (1.5g/100 ml). Finally, the volume was made up to 100 ml by adding distilled water. All the ingredients were properly mixed and autoclaved. The glasswares used were washed, autoclaved and oven dried at 70-100°C (properly sterilized) before use. For the isolation of distinct colonies spread plate method was used.

Screening for keratinase producing bacteria

To screen keratinase producing bacteria, Feathers meal medium (FM medium) was prepared by dissolving NH_4Cl (0.5g/l), NaCl (0.5g/l), KH_2PO_4 (0.4g/l), K_2HPO_4 (0.3g/l), $MgCl_2.6H_2O$ (0.1g/l), yeast extract (0.1g/l) and powdered feathers (10g/l). Final volume was made up to 1000 ml by adding distilled water. All the 9 isolated colonies were inoculated in FM medium and incubated at 37°C for 72 h at shaker. The maximum growth was shown by bacterial isolate BML5 isolated from soil sample 3 (S-3) and was selected for further research work.

Effect of feathers on the growth of bacterial isolate

Growth curves of *B. subtilis* BML5 were determined in minimal salt medium and the medium was divided into two sets. In one set powdered feathers were added and in second set glucose was added as a substrate. Then medium was inoculated (both sets) with 1 ml of log phase bacterial culture prepared in L-broth and incubated at 37°C. The readings (Optical densities) were taken by spectrophotometer at 600 nm after every 4 h interval up to 32 h of bacterial growth.

Physical, biochemical and molecular characterization

For *B. subtilis* BML5 identification, morphological and various biochemical tests such as Gram staining, catalase test, oxidase test, citrate utilization, lactose fermentation, mannitol test, starch hydrolysis, methyl red test, indole test and Voges-Proskauer test were performed. Bacterial DNA was extracted as described by Klindworth *et al*. (2013) and 16S rRNA gene amplification was done by universal primers. The conditions followed for amplification were as follows: Initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 57°C for 1 min, extension at 72°C for 1.5 min and final extension at 72°C for 10 min. The PCR products were analyzed in 1% agarose gel by electrophoresis in 1X TAE buffer with ethidium bromide and visualized under UV transilluminator. The PCR product (approximately 0.5kb) was purified through Fermentas Gene Jet Gel Extraction kit (# K0691) and sequenced from Macrogen, Korea.

Intra- and extra-cellular enzyme activity

The bacterium was cultured in 100 ml salt medium containing 1% feathers (powdered form) in 250 ml Erlenmeyer flasks for 72 h. The bacterial biomass was obtained by centrifugation at 6,000×g for 10 min. The bacterial pellet was washed with phosphate buffer (pH 7) twice and then sonication was done for 3 times for 15 sec with an interval of 60 sec. The pellet was centrifuged after sonication at 10,000×g for 10 min and supernatant was used to perform intracellular keratinase activity.

Bacterial culturing medium supernatant containing enzyme was precipitated by using amount of solid ammonium sulphate at different saturation level (40-80%). After overnight precipitation, the extracellular keratinase was obtained by centrifugation in new falcon tubes at 6,000×g for 10 min. Precipitated proteins containing keratinase fraction were re-suspended in Tris HCl buffer (50 mM, pH 7.0) and used as crude enzyme for extracellular keratinase assay (Herr *et al*., 1978).

Effect of temperature, pH and metal ions on enzyme activity

The effect of temperature on the keratinase activity was determined by incubating the reaction mixture at 20°C, 30°C, 37°C, 42°C and 55°C while reaction mixture was incubated in buffers of different pH [sodium acetate buffer (pH 5-6), sodium phosphate buffer (pH7-8) and tris-HCl buffer (pH 9)] to determine the pH effect on the enzyme activity. The effect of metal ions was determined by incubating the enzyme in 50 mM sodium acetate buffer (pH: 6) containing different salts ($CaCl_2$, $MgCl_2$, $ZnCl_2$ and $CuSO_4$) as a source of metal ion in 10 mM concentration.

Protein sample preparation and SDS-PAGE

The organism was grown in LB broth in the presence and absence of feathers. Bacterial pellet was obtained by centrifugation at 6,000×g (10 min). The pellet was

washed with deionized distilled water and re-suspended in 150 µl of lysis buffer (1% *sodium dodecyl sulfate*, 0.1% mercaptoethanol and 0.1% dithiothreitol). The bacterial cells were sonicated (Heilscher Ultrasonic Processors UP 400, S) 2-3 times at 4°C for 15 sec with 60 sec intervals, mixed with gel loading dye and incubated at 95°C in water bath for 10 min. Cell lysates were centrifuged at 10,000×*g* rpm (10 min) to remove cell debris. The supernatant was carefully collected and used for SDS-PAGE analysis. Then 12% acrylamide gel was used to resolve proteins and molecular weight standards were also run to measure the relative size of unknown protein bands. Coomassie Brilliant blue was used to stain the gels and gels were photographed after de-staining.

Degradation of whole feathers in the presence of bacterial isolate

For whole feathers degradation, minimal salt medium was prepared and divided into two sets. In one set whole feathers were added as substrate and inoculated with 1% log phase bacterial growth. In second set, whole feathers were added but not inoculated with bacterial cells (control). Both sets were incubated at 37°C for 7 days.

Statistical analysis

For each experiment, three independent measurements were taken and data shown are average values of means ± standard deviation (SD).

RESULTS AND DISCUSSION

Characterization of feathers degrading bacterium

The objective of the study was to isolate a bacterial strain, having ability to degrade the keratinous compounds. Out of 9 bacterial isolates obtained from the soil of poultry shops, BML5 showed the maximum production of keratinase and has shown the ability to grow in the medium containing powdered feather within 72 h at 37°C. This ability indicates the high metabolic rate of the bacterium and its adaptation to utilize keratin as a carbon and nitrogen source.

On the basis of morphological, biochemical tests and 16S rRNA sequencing BML5 was identified as *Bacillus subtilis*. They have irregular shaped yellowish white flat and dry colonies with undulate margins and no pigmentation. *B. subtilis* BML5 are Gram positive rods, with positive spore staining. These bacteria were positive for catalase, but negative for oxidase activity. They were positive for citrate, methyl red, Voges-Proskaner and starch hydrolysis tests, but were negative for Indole test and lactose fermentation. The 16S rRNA gene sequence showed 99% homology with 16S rRNA sequence of *B. subtilis* submitted to NCBI database. The 16S rRNA gene sequence was submitted to GenBank under accession number KF003018. Many *Bacillus* species including *B. licheniformis*, *B. pumilus*, *B. subtilis*, *B. cereus*, and *B. megaterium* have been reported for keratin degradation (Kim *et al*., 2004; Riffel and Brandelli, 2006).

Optimization of growth conditions

B. subtilis BML5 showed fair growth in the presence of feathers slightly less as compared to the control medium containing glucose as a substrate (Fig. 1). So it indicated that the feathers presence in growth medium act as an inducer for the enzyme. It has been reported that bacteria producing keratinse had different characteristics *i.e*., broad temperature and pH range for growth (Lakshmi *et al*., 2013). It has been reported that carbon and nitrogen amount exert a high influence on the growth of microorganisms. In this study, BML5 showed maximum growth at 37°C and, therefore, the bacterial isolate was classified as mesophilic. The bacterial isolate was able to grow on the pH ranging from 4 to 8 but has shown its best growth at pH 7 showing that the isolate is neutrophilic. Previous studies have even reported the bacterial isolates able to grow at alkaline pH *i.e*., 10 (Bernal *et al*., 2006).

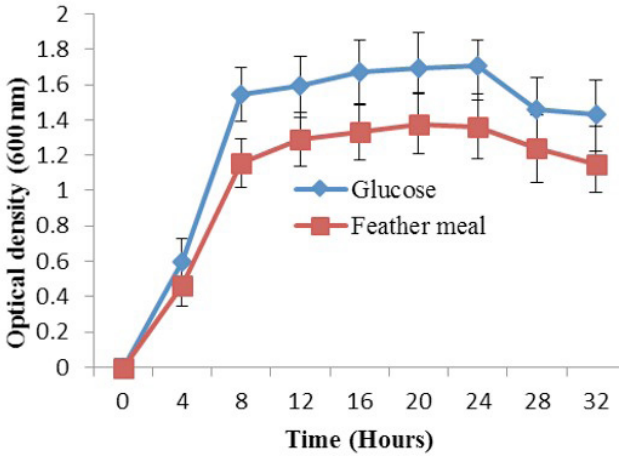

Fig. 1. Growth curves of *B. subtilis* BML5 in medium containing glucose (control) and in mineral salt medium supplemented with 1% feathers as substrate (treated) incubated at 37°C.

Intracellular versus extracellular enzyme activity

Keratinase activity, both intra- as well as extra-cellular, was performed to determine the most dominant form of enzyme. The results showed 70% extracellular activity increase as compared to the intracellular enzyme activity. The microorganisms can produce both intra- as well as extra-cellular enzymes.

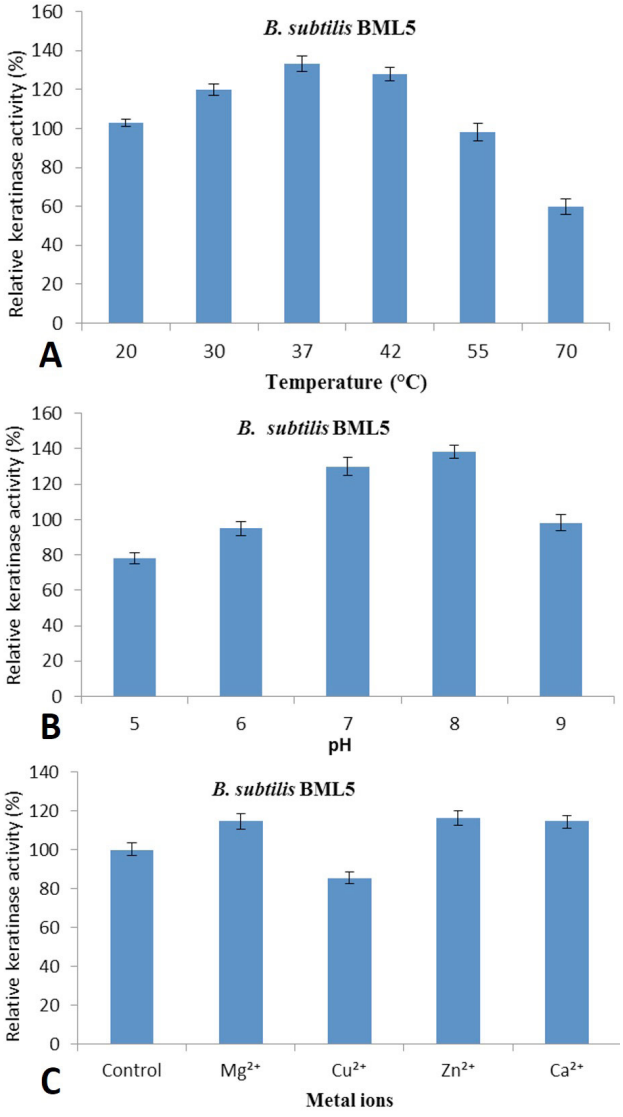

Fig. 2. Effect of temperature (A), pH (B) and metal ions (C) on keratinase activity isolated from *B. subtilis* BML5.

Temperature, pH and metal ions influence on keratinase activity

Temperature is one of the most important variables. Its effect on the activity of keratinase produced by *B. subtilis* BML5 was determined by incubating the reaction mixture at 20°C, 30°C, 37°C, 42°C, 55°C, 70°C. Keratinase was shown to be more active at 37°C (Fig. 2A), which is a bit lower as compared to the optimum temperature reported in previous studies. According to Bernal *et al*. (2006) and El-Refai *et al*. (2005) keratinase produced by the bacteria isolated from the soil mixed with chicken feathers showed maximum activity at 40°C, which is in good agreement with our results.

Enzyme activity is markedly affected by pH. This is because substrate binding and catalysis are often dependent on charge distribution on both, substrate and particular enzyme molecules (Shah and Madamwar, 2005). pH of the medium strongly affects many enzymatic processes and transport of various components across the cell membrane (Moon and Parulekar, 1991). The production of enzyme depends on the pH by particular bacterial isolates. In the present study, *B. subtilis* BML5 showed maximum activity at pH 8 (Fig. 2B). According to Rozs *et al*. (2001), *B. licheniformis* showed maximum activity at pH 7. On the other hand, Kim *et al*. (2001) reported that *B. subtilis* showed maximum keratinase activity at the pH ranging from 5-9. The alkaline pH is suitable for deamination reactions leading to the release of ammonium which increases pH and this increase in pH favors keratinolysis (Riffel *et al*., 2003).

In the present study, significant increase in keratinase activity was determined in the presence of Ca^{2+} (14.8%), Mg^{2+} (14.8%) and Zn^{2+} (16.3%) while 14.5% inhibitory effect on the enzyme activity was found in the presence of Cu ions (Fig. 2C). Similarly, metal ions like Ba^{2+}, Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, activated, while Cu^{2+}, Fe^{2+}, Hg^{2+}, inhibited keatinase activity (Lin *et al*., 1995).

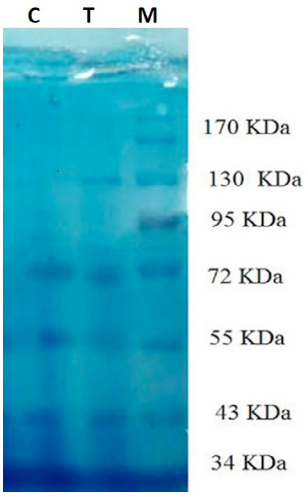

Fig. 3. Coomassie Brilliant blue-stained SDS-PAGE of partially purified keratinase from *B. subtilis* BML5. Lane C, bacterium growing without feather; Lane T, bacterium growing with feather; Lane M, molecular mass protein maker.

SDS-PAGE analysis

Keratinases have been reported (Gupta and Ramnani, 2006) with molecular masses ranging from 15 to 240 kDa. However, the majority of keratinases presented molecular masses varying from 20 to 50 kDa (Prakash *et al*., 2010).

In the present work, a protein band of 130 kDa was appeared in treated sample (feather containing medium) but was absent in control (Fig. 3). This protein band may be involved in the degradation of feathers (keratin).

Whole feather degradation

The insoluble keratinous compounds like feathers are resistant to degradation by many microbial enzymes. The major portion of protein component of feather is keratin that forms a fibrous structure, which is extensively cross-linked by disulphide, hydrogen and hydrophobic bonds. Thus the several million tons of feathers generated annually by the poultry industry leads to the troublesome environmental contamination and wastage of rich source of protein. In the present investigation, *B. subtilis* BML5 has degraded the whole feather completely after 6 days of incubation (Fig. 4). Lakshmi *et al*. (2013) reported that feather shaft degradation was achieved after 5 days of incubation and complete feather degradation was obtained after 7 days of incubation by *Bacillus* sp. It has been found that keratinase has the capacity to convert insoluble keratin into soluble material. This material (amino acids) can be used as feed for animals. Keratinase producing microbes thrive under various environmental conditions contaminated with different wastes (Bernal *et al*., 2003; Kansoh *et al*., 2009).

Fig. 4. Degradation of feathers by *B. subtilis* BML5 culture in minimal salt medium after 6 days of incubation at its optimum temperature.

CONCLUSION

From the results, we can conclude that the soil contaminated with the poultry wastes is a rich source of bacteria which are able to hydrolyze keratin. The problem of poultry wastes may be overcome by using such bacterial strains like *B. subtilis* BML5 and isolated enzyme could be used to improve digestibility of feather meal.

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Statement of conflict of interest

Authors have declared no competing interests exist.

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