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Feather Keratin Degradation by *Stenotrophomonas* sp. AB20 Screened from Poultry Waste

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Microbial keratinase is biotechnologically important enzyme which has ability to hydrolyze highly rigid, strongly cross-linked structural polypeptides as well as recalcitrant and insoluble fibrous keratin proteins. In the present investigation, 25 feather waste dumped soil samples were screened for keratinolytic bacteria. Samples were enriched in mineral salt medium containing 1% feather followed by screening for protease activity using casein agar, milk agar and keratinase acitivity with feather meal agar. Amongst 82 bacterial isolates, 39 showed maximum proteolytic and 17 showed keratinolytic activity. Moreover isolate *Stenotrophomonas* sp. AB 20 showed highest potential in both the proteolytic and keratinolytic activities. In addition to this, isolate AB 20 showed more than 87.3% feather degradation within 6 days using submerged degradation process and crude enzyme activity was 52.82 U/ml. Identification of potential keratinolytic isolate trough 16S rRNA analysis revealed that it belongs to Stenotrophomonas sp. (Gene bank accession number KF201645). The isolate due to its potential keratinolytic activity may serve as a candidate for feather waste management and number of different biotechnological and industrial applications.

Key words: Keratin waste, Feather hydrolysis, Bacterial Keratinase.

Keratin is a most abundant fibrous and insoluble structural protein, present on epidermis of vertebrate's epithelial cells of skin appendages such as nails, hairs, feathers, horns, scales and wool[1]. It is extensively cross linked with hydrogen, disulphide bonds and hydrophobic interactions which make it resistant for degradation[2]. Chicken bird contributes about 10 % feathers to its body weight, while dried feather contains 85-99 % proteins. Large amount of this feather waste is generated through poultry as higher as millions of tons per year which is deposited in the environment and leads to pollution as well as creates threat of occurrence of epidemic disease from such waste[3]. However, so far less emphasis has been given for feather waste management and its control. Since feather keratin exhibits an elevated content of several amino acids such as Leucine, Isoleucine, Serine, Valine, Methionine, Glycine, Cystein, Tyrosine, Tryptophan, and Lysin[4], it has been explored for number of applications. However, compared to other organic waste such as cellulose and hemicelluloses, keratin waste is utilized on a limited basis as a dietary protein supplement for animal feed and fertilizer for plants[5]. Physical and chemical methods for feather meal production are expensive which require significant amount of energy. Moreover, these processes destroy certain amino acids, yielding a product with poor digestibility and variable nutrient quality. As compared to physicochemical methods, enzymatic hydrolysis effectively converts this waste into

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valuable and superior quality feather meal[6-7]. Keratin dumped soil contain large number of microorganisms, having potential to hydrolyze feather, it includes fungi, bacteria, actinomycetes[8]. Keratinase production is reported from *Bacillus licheniformis* PWD-1[9], B. licheniformis, *Bacillus pumilus* FH[9] and *Bacillus subtilis* KD-N2[1], *B. cereus*, *B. amyloloquefaciens* and *B. megaterium*[10], *stenotrophomonas* sp. D1[11] *Leuconostoc* sp., *Pseudomonas microphilus*[12] and fungi such as *Trichophyton*, *Aspergillus flavipes, Cephalosporium, Penicilium, Microsporum*[13] and *Actinimycetes* such as *Streptomyces* sp. *Streptomyces albidoflavus*[5]. Recently, keratinase is being explored in number of industrial applications including leather industry for quality leather, in pharmaceutical, detergent industries and supplement for livestock feeds etc[14-15].

The present study deals with screening and isolation of extracellular keratinase producing bacteria from poultry and feather waste dumped soil samples from North Maharashtra region, India. Screening was performed on the basis of protease and Keratinase production abilities, percentage of feather degradation and crude enzyme activity. The potential of a new isolate *Stenotrophomonas* sp. AB20 is also explored for keratin degradation.

MATERIALS AND METHODS

Source of keratin

Chicken feathers collected from a local poultry farm were thoroughly washed with tap water, followed by mild detergent wash, boiled and oven dried overnight at 50°C. Fine powder prepared with the help of hand blender was used as sole source of carbon and nitrogen during preparation of screening media[16]. The purity of the feather keratin powder was analyzed using FTIR (Shimadzu) and compared with standard keratin powder (Hi-media).

Isolation and enrichment of keratinase producing Bacteria

Twenty five different soil samples were collected from a poultry farm, chicken market, and feather waste dumping areas in North Maharashtra region. Multiple samples were collected from surface and deep soil and labeled with habitat details. One gram soil was dispersed in a 250-ml Erlenmeyer flask containing 100 ml sterile mineral salt enrichment medium with feathers (containing g/L) NaCl 0.5, K_2HPO_4 1, KH_2PO_4 0.6, $MgCl_2.6H_2O$ 0.1, Feather 10, pH 7.5. It was incubated at 30°C for 5 days at 120 rpm[3, 4]

Isolation and screening of keratinase producing Bacteria

Casein agar and Skimmed milk agar (Hi-media) was used to determine proteolytic ability as primary screening approach described by Nagal and Jain[17]. Distinct colonies observed using morphological features were selected, isolated, and plated on nutrient agar to obtain pure cultures. The isolates exhibiting proteolytic activity were further screened for keratinolytic activity using feather-meal agar (FMA) containing 1% feather powder in basal medium[18]. Plates were incubated at 37°C for 5 days and the isolates producing clear zone around the growth were selected for further analysis[19].

DNA extraction PCR amplification and sequencing of the keratinolytic strains

Total genomic DNA was isolated using standard phenol/chloroform extraction protocol described previously[20]. The 16S rRNA gene was amplified with universal primers[21] 16F27 (5'-CCA GAG TTT GAT CMT GGC TCA G-3') and 16R1525XP (5'-TTC TGC AGT CTA GAA GGA GGT GWT CCA GCC-3'). PCR reaction mixture (25 µl) included 200 µM of dNTP and 10 picomole each primer, 50ng DNA template and 1U Taq DNA polymerase with reaction buffer containing 1.5 mM $MgCl_2$ (Bangalore Genei, Bangalore, India). Polymerase chain reaction was performed in a thermal cycler (Applied Biosystem® 2720) with the following conditions: 1 cycle of 5 min at 94°C; 35 cycles of 1 min at 94°C, 1min at 55°C, and 1min at 72°C followed by a final extension of 10 min at 72°C. PCR products were run on 1% agarose gels, stained with SYBR® Safe (Invitrogen). These PCR products were purified using PEG-NaCl method20. The successfully amplified products were sequenced using BigDye Terminator Cycle Sequencing kit version 3.1 (Applied Biosystems) by following manufacturer protocol. Sequences were obtained using an automatic DNA sequencer (3730xl DNA analyzer, ABI).

Phylogenetic analysis

Sequences of all the type species and strains of genus Stenotrophomonas (N=9) along with some representative of Pseudomonas genus

(N=4) was retrieved. 16S rDNA sequence of the isolate under this study (AB20) was aligned with these sequences using ClustalX (version 2.0.9)[22]. All sequences were manually edited and using Unrooted phylogenetic tree was constructed using MEGA523. The final alignments consisted of 1286 bp.

Production medium

Selected isolates were grow in production medium (containing g/L) NaCl 0.5; K_2HPO_4 0.1; KH_2PO_4 0.6, $MgCl_2.6H_2O$ 1, Yeast extract 0.1, Feather meal 10, pH 7.5. 0.3 ml of bacterial suspension was inoculated in 30 ml production medium and incubated at 30°C for 5 days at 120 rpm in a shaker incubator (Remi)[24]. After incubation, the broth was centrifuged at 10,000 rpm at 4°C for 10 min. Supernatant was used as crude enzyme to determine keratinolytic ability as described by Saber *et al*.,[25].

Keratinase assay

The keratinase assay mixture consisted of 0.2 ml of crude enzyme, 0.8 ml Tris HCL buffer (pH 8) containing keratin (0.1%) and incubated at 45°C for 30 min. The reaction was terminated by adding 1ml 5% Trichloroacetic acid (TCA). After centrifugation at 1000 rpm for 10 min, the absorbance of the supernatant was determined at 280 nm against a control. The reaction mixture without crude enzyme was used as control[26]. One unit of keratinolytic activity was defined as an amount of tyrosine release at 280 nm per ml per minute under the conditions described above [27].

Measurement of soluble protein

The soluble protein released was measured using Lowery method and bovine serum albumin was used as standard protein[28].

Degradation of feathers

The ability of the isolates to degrade native poultry feathers was determined as illustrated by Kani *et al*.,[12]. Weight loss of feather was compared to control and calculated as percentage.

RESULTS AND DISCUSSION

The FTIR spectrum (Fig 1a and 1b) of feather meal powder showed presence of similar functional groups as that of keratin powder (Hi-media). The bands at 3000 to 4000 cm^{-1} suggest presence of hydroxyl group and amino groups, 3128 to 2700 cm^{-1} carboxyl groups, peak at 2875 cm-1suggest aldehyde group, 2360 to 2339 cm^{-1} cyanide group and 1500 to 1550 cm^{-1} indicates existence of aromatic ring.

From 25 soil samples by using primary and secondary screening approaches a total of 82 bacterial isolates were obtained, amongst which 39 showed zone of clearance above 20 mm as measure of proteolytic activity. When these were screened for feather degrading properties, 17 isolates exhibited feather degradation ability. The zone of clearance for proteolytic and keratinolytic activity is shown in Fig 2 and screening profile is depicted in Fig 3. The isolate AB 20 showed highest feather degrading potential within 6 days.

The keratinase activity of potential isolate AB 20 was found to be 52.82U/ml and feather degradation was observed as 87.3% (Fig. 4). The strain *Pseudomonas sp.* MS 21 was reported to produce keratinase activity 43 U/ml[29], another strain *Stenotrophomonas maltopila* R13 screened from soil was reported to be producing 14.96 U/ml in un optimized basal medium[30] whereas isolate reported by Liang *et al* [31] *Stenotrophomonas maltopila* DHHJ produced Keratinase 20.46 U/ml. Compared to earlier findings, the present isolate

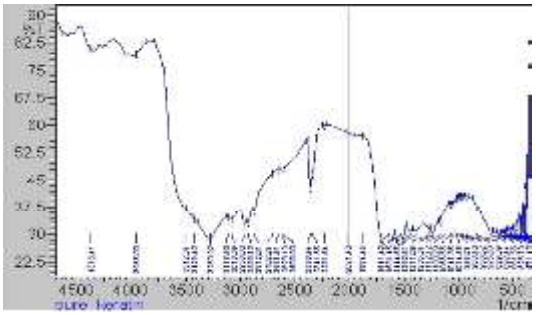


Fig. 1a. FTIR spectrum of pure keratin powder (Hi-media)

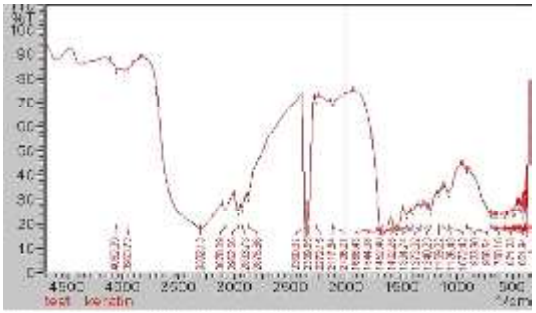
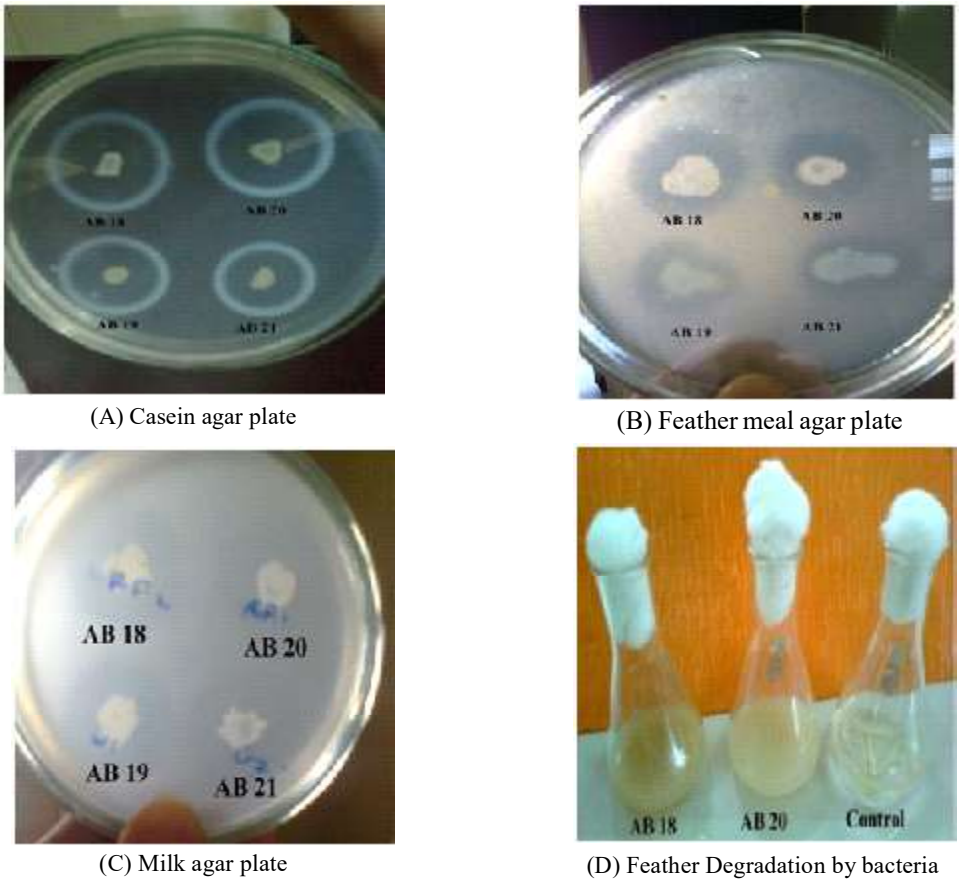


Fig. 1b. FTIR spectrum of test keratin powder (Feather meal)



(A) Casein agar plate (B) Feather meal agar plate

(C) Milk agar plate (D) Feather Degradation by bacteria

Fig. 2. Primary and secondary screening for keratinolytic bacteria
a. Zone of proteolytic activity; b. Zone of keratinolytic activity; c. Zone of proteolytic activity

exhibits superior kerationlytic potential even in un optimized conditions.

Primary identification of the potential isolate by morphological and biochemical characteristics reveled that it is short rod, Gram negative bacterium which is facultative, motile, strong catalalase and oxidase positive. Further molecular identification by Gene Phylogeny using 16S rRNA gene sequence and neighbor-joining method (Fig.5) showed strong clustering of AB20 with *Pseudomonas beteli* (AB021406) while *Pseudomonas geniculata* (AB021404), *Pseudomonas hibiscicola* (AB021405) and *Stenotrophomonas maltophila* (AB008509) are the nearest number[32] suggested that though these three *Pseudomonas* species were reported in this genus, it need to reclassify. They further suggested including theme in genus *Stenotrophomonas*. Hence this isolates AB20 was identified and given name as *Stenotrophomonas sp.* The sequence of 16S rRNA gene of the strain AB20 is available under the GeneBank accession number *Stenotrophomonas* sp. KF201645.

Similar approach for screening of keratinase producing bacterial strains from soil samples was used by Tork *et al.*[29] showing dominance of *Pseudomonas sp.* Also screening of *Stenotrophomonas sp.* from poultry feather decomposed soils is reported by several researchers[11,33]. Similar isolation of *Stenotrophomonas maltophila* R13 is repoted by Jeong *et al.*[30].

In view of the literature study, the present investigation reports isolation of potential bacterial strain *Stenotrophomonas* sp. (AB20) KF201645 with comparatively good ability of keratinase

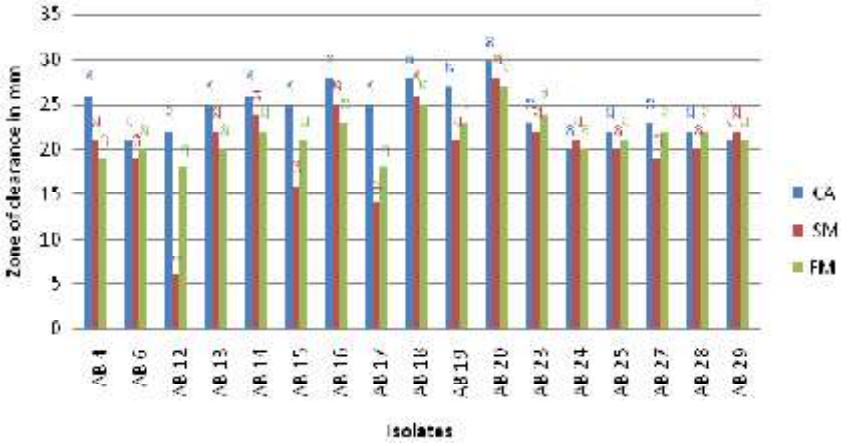


Fig. 3. Screening of protease and keratianse producers
CA: Casein agar, SM: Skim milked agar, FM: Feather meal agar.

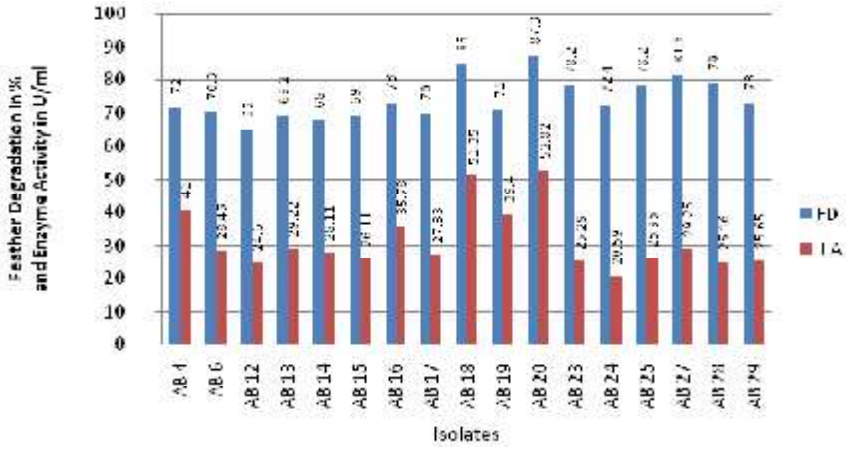


Fig. 4. Percentage of feather degradation and Keratinase activity
FD: Feather degradation, EA: Enzyme activity

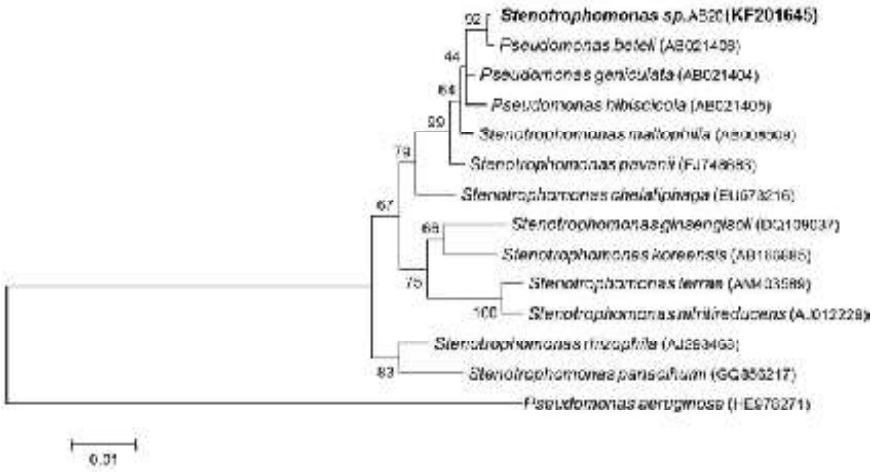


Fig 5. Phylogenetic analysis of AB20

production 52.82 U/ml. Considering the potential of isolate AB 20, it can be further investigated and explored for their enzymatic potential and biotechnological applications.

CONCLUSION

In conclusion, this study reports screening and isolation of keratinolytic bacterial strain *Stenotrophomonas* AB 20 from feather waste dumped soil sample belonging to North Maharashtra region. It showed maximum keratinase activity 52.82 U/ml and has ability to degrade feathers within 6 days up to 87.83%. In view of the results obtained, the keratinolytic isolate can be further explored for feather waste management, feather meal production, leather processing steps and pharmaceutical application.

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