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### RESEARCH ARTICLE

## SCREENING AND PARTIAL 16 S rRNA GENE SEQUENCING OF KERATINASE PRODUCING *Stenotrophomonas maltophilia* KARUNA5 ISOLATED FROM POULTRY WASTE

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#### Abstract

Keratinase is a protease which degrades the insoluble protein keratin that is largely found in feather waste. Fifty-seven soil samples were collected from a feather and poultry waste dumping sites. All these soil samples were subjected for enrichment in Whole feather medium containing 1% chicken feather as a sole source of Carbon, nitrogen and energy with pH 8.0 and incubated at 30°C on shaker condition (100 rpm) for 7 days. Out of 57 flasks only 21 showed visual feather degradation. Enriched sample from each flask was isolated on skimmed milk agar plates. Only 10 isolates demonstrated proteolytic activity by showing zone of clearance around the colony on skimmed milk agar medium. The above 10 bacterial isolates (Kar:01-12) were assayed for keratinase enzyme by growing them in Whole feather medium pH 8. Maximum keratinase production was demonstrated by isolate number Kar05 (35.6 U/ml) at 30°C on shaker condition (100rpm) after 7<sup>th</sup> days of incubation. Promising isolate was identified as *Stenotrophomonas maltophilia* KARUNA5 by morphological, cultural, biochemical and 16S rRNA sequence analysis and was submitted to NCBI (Accession no: LC271188).

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#### Introduction: -

Keratinase [E.C.3.4.21/24/99.11] belong to group of proteolytic enzymes which have ability to hydrolyze insoluble protein keratin more efficiently than other proteases. Due to the strength and stability of keratin, very few microorganisms are able to degrade keratin and utilize it as carbon, nitrogen and sulphur source. Keratinase is an extracellular enzyme used for the bio degradation of keratin. Some microbes have been reported to produce keratinase in the presence of keratin substrate. Keratinase attacks the disulfide bond of keratin to degrade it. (Sahoo et al., 2012) Keratinases from microorganisms have attracted a great deal of attention in the last couple of decades, particularly due to their multitude of industrial, agricultural and medical applications such as in the animal feed and supplement, fertilizers, detergents, leather and pharmaceutical industries (Uttangi and Aruna, 2018; Thanikaivelan et al., 2004; Gupta and Ramnani, 2006; Karthikeyan et al., 2007; Brandelli and Riffel, 2005; Brandelli et al., 2010; Kornilowicz et al., 2011). Alkaline proteases like keratinase enzymes have been obtained from various bacterial, Actinomycetes and fungal strains. Among all the microbial sources of keratinase, bacterial keratinase is important because of their various industrial applications. Bacterial strains belonging to genera *Pseudomonas* spp., *Vibrio* spp.,

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Chryseobacterium spp., Xanthomonas spp., Fervidobacterium spp., Stenotrophomonas sp., Micrococcus spp., Nesterenkonia, Arthrobacter, Clostridium, Caldicoprobacter, Bacillus and Kytococuss are keratinase producers (Uttangi and Aruna, 2018). Actinomycetes Genera include Streptomyces, Microbispora, Nocardia and Streptomyces (Kumar and Takagi, 1999; Kaul and Sumbali, 1997; Gushterova *et al.*, 2005; Azza, 2013). Fungal genera which have potential to produce keratinases include Paecilomyces, Myrothecium, Aspergillus Cladosporium and Trichoderma and Candida (Veselá and Friedich., 2009; Gioppo *et al.*, 2009; Kim, 2007; Patience *et al.*, 2015; Huang *et al.*, 2015; Vermelho *et al.*, 2010, Uttangi and Aruna, 2018). There are many reports where fungal keratinase enzymes are derived primarily from dermatophytic Ascomycetous fungi such as Arthroderma sp., Microsporium sp. and Trichophyton sp., (Burmter *et al.*, 2011; Martinez *et al.*, 2012). The current work deals with screening of keratinase producer from various soil samples collected from the feather and poultry waste dumping sites. Most promising keratinase producer was selected on the basis of maximum keratinase production and was identified.

## Material And Methods: -

### Collection of samples

For Enrichment of microorganism's soil samples were collected from the local spots, include Poultry farm, Chicken shop, Slaughter house, Leather industry and Municipal dumping ground of Kolhapur, Sindhudurg, Sangli and Mumbai Districts. Some samples were also collected from natural environmental sites which includes Hot spring water, Forest and Agricultural wastes, and soil from marine shores.

### Enrichment:

One gram of soil sample or one ml of effluent was weighed/measured and added to 10 mL of sterile Phosphate Buffered Saline solution (PBS; pH 7.2). It was mixed well and allowed to stand for 30 min. From this mixture, 2mL of supernatant was inoculated in 100mL of sterile Whole feather medium [Composition in g/L: NH<sub>4</sub>Cl (0.5), NaCl (0.5), K<sub>2</sub>HPO<sub>4</sub> (0.3), KH<sub>2</sub>PO<sub>4</sub> (0.4), MgCl<sub>2</sub>·6H<sub>2</sub>O (0.1), yeast extract (0.1), and defatted whole chicken feathers (10), pH 7.4] for enrichment of keratinase producers (Kim *et al.*, 2001). Defatting of the feather pieces was done by soaking them in a mixture of chloroform: methanol (1:1) for 2 days followed by chloroform: acetone: methanol (4:1:3) for another 2 days. The solvent was replaced every day. After treatment with the defatting mixture, the feathers were washed several times with tap water to eliminate the residual solvent. They were dried for 24h in an oven at 50°C The flasks were incubated at 30°C under shaker conditions (100 rpm) and monitored for visible degradation or complete decomposition of the feathers. The time taken for degradation of feathers, in different flasks, was also noted for further studies. The flasks showing partial or complete degradation of feathers were considered as an enriched sample for keratinolytic microorganisms (Agrahari and Wadhwa, 2010).

### Screening of keratinolytic Bacteria:

The isolated single colonies were screened for their ability to produce protease by observing a clear zone on skimmed milk agar (SMA) plates around the spot-inoculated cultures (Riffel and Brandelli, 2006). Individual component of skimmed milk agar medium [Composition g/L]: peptone (5.0), yeast extract (3.0), dextrose (1.0), milk powder / Milk (10.0), agar (20.0), pH 7.2] was weighed using an electronic balance (Contech, India). The pH of the medium was adjusted using a pH meter (ELICO, India) and sterilized in an autoclave (except milk powder/Milk). After sterilization, the medium was cooled to around 45°C and sterile skimmed milk (10 ml) was added to it. The medium was mixed thoroughly and poured in sterile petri-plates. The isolated colonies from Feather meal medium were spot-inoculated on SMA medium and incubated at 30°C for 24h. The colonies showing >11mm zone of clearance were considered to have a proteolytic activity (Tork *et al.*, 2010). For qualitative detection of culture were grown individually in 100 ml Erlenmeyer's flask with 60 ml Whole feather Medium and incubated at 30 °C for 24 hrs on shekar 100 rpm condition for 7 days. Loopfull culture from each flask streak on SMA and observed for zone clearance around growth which was indicative of protease potential (Lin *et al.*, 1992; Ramya *et al.*, 2014).

### Determination of Percent degradation feather:

The 10 isolates were tested for their keratin degrading ability. 100 ml of whole feather meal medium with pre-weighed feather pieces was autoclaved at 121°C for 15 minutes. A 1ml of inoculums with O.D. of 0.1 was inoculated into respective medium. Un-inoculated flask was maintained as control. These flasks were incubated at 30°C for 7 days. The percentage of degradation of feather by the isolates was determined using the following formula: (Nayaka and Babu, 2014). Initial weight of feathers before degradation and final weight of feathers after degradation were taken for calculation of percentage of feather degradation.

The following formula was used for calculation.

$$\text{Percentage of Weight loss (\%)} = \frac{\text{Initial weight} - \text{Final weight}}{\text{Initial weight}} \times 100$$

#### **Keratinase assay: Preparation of crude enzyme extract:**

Cultures were grown on sterile Luria Bertani broth on rotary shaker incubator (100 rpm) at 30°C for 24 hrs. The broth was centrifuged at 3000 rpm for 20 min. The cell pellet was washed and resuspended in phosphate buffer saline pH 7.2. Washed cells (2 ml) were inoculated in 100ml of Whole Feather medium and incubated at 30°C on rotary shaker incubator (100 rpm) for 7 days. After every 24 hrs of incubation an aliquot of the broth was centrifuged and the supernatant containing the enzyme extract was assayed by Keratin azure assay (Saibabu and Niyongabo, 2013)

#### **Keratinase Azure assay:**

The keratinolytic activity was determined by using keratin azure (Sigma–Aldrich) as the insoluble substrate (Bressollier *et al.*, 1999). Aliquots of 500 µL of the enzyme samples were incubated in a solution of 10 mg of keratin azure in 500 µL of 20 mM Glycine NaOH Buffer of pH 9.6 for 1 h at 50°C. Subsequently, these were centrifuged at 5,000× g for 20 min and the absorbance of the supernatant was determined at 594nm (Beckmann DU-640 spectrophotometer). Control samples were prepared in a similar manner except that the enzyme was replaced by the buffer. The assays were conducted in triplicate. One enzymatic unit was defined as the amount of enzyme that resulted in an increase in absorbance at 594 nm (A 594 nm) of 0.01 after reaction at 50°C for 1 hr with keratin azure (Bressollier *et al.*, 1999)

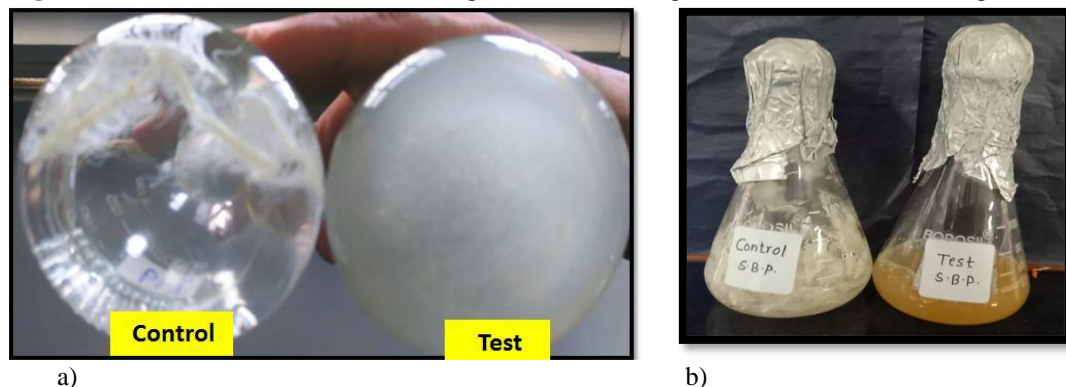
#### **PCR amplification of the 16S rDNA and sequence determination:**

A PCR was performed in order to amplify the 16S ribosomal DNA (rDNA) of the Kar05 strain. The primers used 16S Forward primer: 5' AGA GTT TGA TCC TGG CTC AG 3' and 16S Reverse primer: 5' AAG GAG GTG ATC CAG CCG CA 3' this primer pair has been shown to amplify the 1500 nucleotides in 16S rDNA from a wide variety of bacterial taxa. The PCR was performed as previously described by using a DNA thermal cycler (PTC 100, MJ Research, and Water Town, MA). The DNA sequencing was done using 50 ng PCR products having 8 µl of ready reaction mix (BDT v 3.0, Applied Bio-systems, Foster City, CA). The sequencing was carried out in ABI prism 3100 Genetic Analyzer (Applied Bio-systems). The sequences were checked against the microbial nucleotide databases using BLASTN search algorithm. Results and discussion:

#### **Sample collection, Enrichment:**

Primary screening was employed to obtain potential bacterial isolates capable of producing keratinase enzyme using feather (keratin substrate) as sole carbon and nitrogen source. Presence of the keratinase rich substrate like poultry feathers induces production of the keratinase (Mazotto *et al.*, 2010). In current study fifty-seven soil samples from different Feather and poultry waste dumping sites from Kolhapur were successfully enriched in Whole feather medium out of which 21 flasks showed visible feather degradation as shown in figure 1.

**Figure 1: - a)** Enrichment medium showing visible feather degradation. **b)** Flask shows degradation



a)

b)

The enriched samples were streaked on feather meal basal medium agar plates and 12 different isolates Kar1, Kar2, Kar3, Kar5, Kar6, Kar7, Kar8, Kar9, Kar10, Kar11 and Kar12 were obtained. There were reports of isolating keratinase producing microorganisms from poultry soil, poultry wastes, poultry farm, poultry processing industry, feather and hair dumping sites and barbers' landfill (Gioppo *et al.*, 2009; Sahoo *et al.*, 2015; Shah, 2015). There are other studies where whole feather medium was used for enrichment of keratinase producers (Ramya *et al.*, 2014; Shah, 2015). Keratinase production ability of isolates was confirmed by production of caseinase on agar plates containing skimmed milk (Tork *et al.*, 2010; Ramya *et al.*, 2014). In the present study, all the 12 isolates (Kar 01- Kar 12) were subjected to primary screening on skimmed milk agar plates. Except Kar11 and Kar12 all the other isolates showed the clear zone of caseinase which was due to hydrolysis of casein (Skim milk powder) as shown in table 1. Zone of clearance i.e., protease activity around the colony on Skimmed milk agar plate suggested that these isolates might possess keratinase activity as well. Similar findings were reported by many researchers (Nayaka and Babu, 2014; Agrahari and Wadhwa, 2010, Tamilkani *et al.*, 2015).

**Table 1:** - Proteolytic activity of different isolates on Skimmed Milk agar.

Isolate designation	Proteolytic Activity
Kar/01	+
Kar/02	+
Kar/03	+
Kar/04	+
Kar/05	+
Kar/06	+
Kar/07	+
Kar/08	+
Kar/09	+
Kar/10	+
Kar/11	-
Kar/12	-



(+) = zone of proteolysis observed  
 (-) = Zone proteolysis not observed.

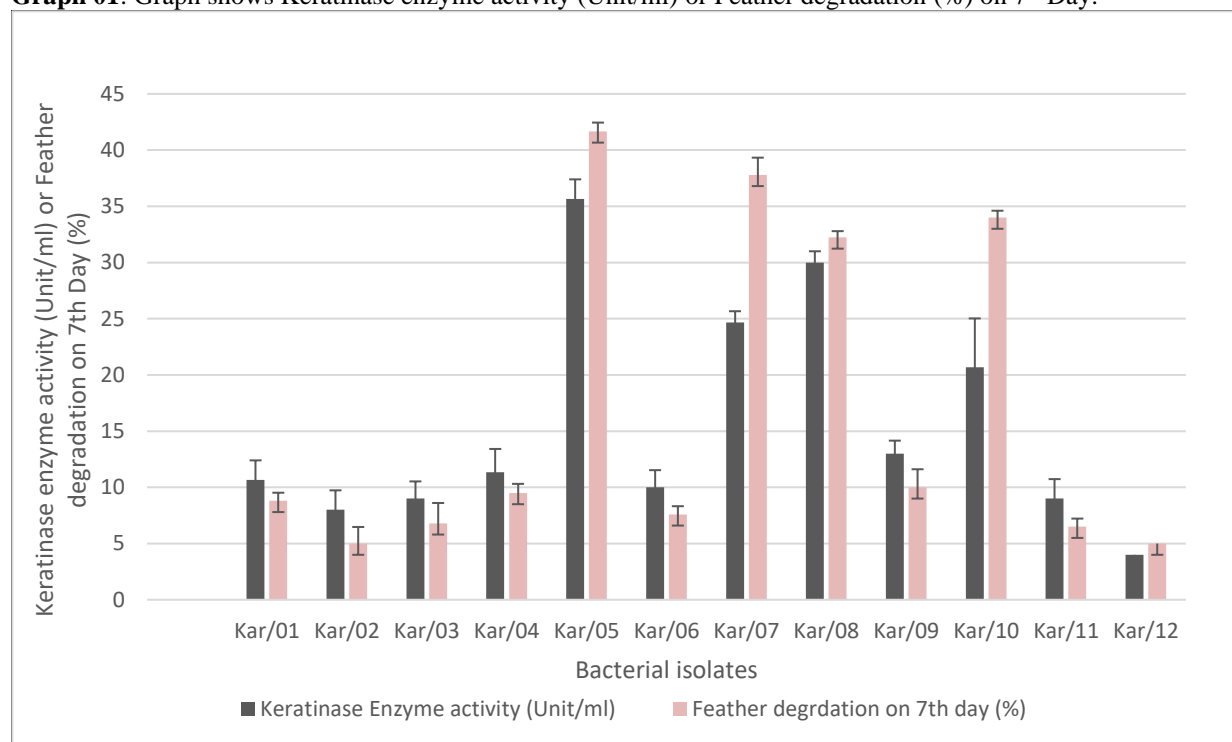
**Image:1** Isolate Kar05 grown on Nutrient agar medium

### Secondary screening:

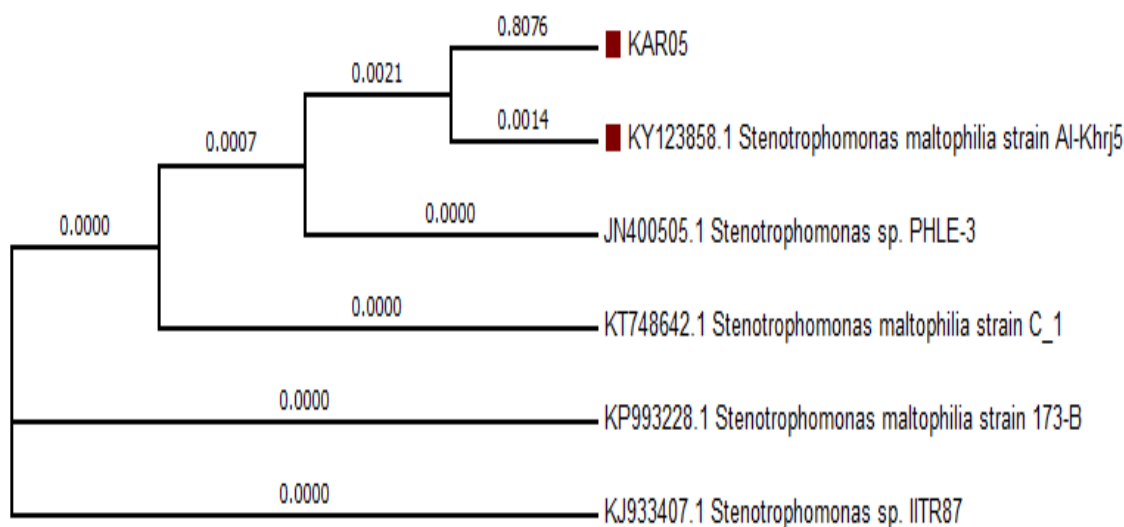
The above 10 isolates which were showing proteolytic activity were checked for the feather degradation as it is indicator of keratinase production and these isolates were grown on whole feather medium for 7 days. The capability of these isolates to produce maximum keratinase in the shortest period of time was studied. Out of 10 bacterial isolates, the isolate Kar05 was found to show maximum % of feather degradation on fifth day where the whole feather basal medium was completely turbid, suggesting disintegration and degradation of the feathers in the medium. The highest % of feather degradation was demonstrated by Kar05 isolate and Kar 02 and Kar 12 showed lowest % of feather degradation (Graph 1).

### Keratiase assay:

The keratinolytic activity was determined by using keratin azure (Sigma–Aldrich) as the insoluble substrate (Bressollieretal., 1999). All 8 isolates produced keratinase enzyme (Graph 1) but Kar05 showed maximum production of keratinase enzyme after 4 days of incubation. Kar 05 was selected as a promising isolate for further studies. (Image 01)

**Graph 01:** Graph shows Keratinase enzyme activity (Unit/ml) or Feather degradation (%) on 7<sup>th</sup> Day.**Identification of promising isolate:**

The 16S rRNA gene based phylogenetic analysis demonstrated 90–95% sequence similarity of Kar05 with other species of the genus *Sternotrophomonas*, which suggested that the bacterium under study belongs to the genus *Sternotrophomonas*. The phylogenetic tree constructed from the sequence data by the Neighbor-joining method (Figure 2) which showed the detailed evolutionary relationships between the strain Kar05 and other closely related species of the genus *Sternotrophomonas*. The strain *Sternotrophomonas maltophilia* strain Al-Khrj-5 (GenbankAcc. No:KY123858.1) showing 96% 16S rRNA gene sequence identity represented the closest phylogenetic neighbour of the strain *Sternotrophomonas maltophilia* KARUNA05. The topologies of the present isolate estimated from the distance-based methods (Neighbor-joining and UPGMA) and the maximum-likelihood and parsimony analyses were essentially consistent. The nucleotide sequence of *Sternotrophomonas maltophilia* Strain KARUNA05 was deposited at GeneBank (accession no. LC271188.1). Keratinase production by several bacteria such as *Sternotrophomonas maltophilia* strain S-1, *Sternotrophomonas maltophilia* N4, *Sternotrophomonas maltophilia* K279a, *Sternotrophomonas maltophilia* KB13, *Sternotrophomonas* sp. Strain Norja-1, *Sternotrophomonas maltophilia* DHHJ and *Sternotrophomonas maltophilia* YArck were previously reported (Miyaji *et al.*, 2005; Kurane and Attar, 2017; Shah and Vaidya, 2017; Cao *et al.*, 2009). Many related other gram-negative bacteria such as *Pseudomonas aeruginosa*, *Pseudomonas* sp., MS21, *Fervidobacterium pennavorans*, *Microbacterium* sp. kr10, *Burkholderia*, *Chryseobacterium*, *Microbacterium* species, *Chryseobacterium* sp. And *Serratia* sp. HPC 1383 were also demonstrated keratinolytic activity (Fredrich *et al.*, 1996; Tork *et al.*, 2010; Thatheys and Ramya, 2015; Brandelli and Riffel, 2005; Khardenavis *et al.*, 2009; Laba *et al.*, 2015). *Micrococcus luteus*, *Kytococcus sedentarius* (Uttangi and Aruna, 2018). Also there are gram positive bacteria which have also shown to produce keratinase such as *B. licheniformis* and *B. subtilis*, *B. subtilis* strain, KD-N2, *Bacillus* sp. JB 99, *B. amyloliquefaciens* MA20, *B. subtilis* MA21, *Bacillus thuringiensis* strain Bt407, *Bacillus subtilis* S14 are reported to be keratinolytic bacteria. (Macedo *et al.*, 2005). Similarly, Keratinase producing bacterial strains of *Bacillus thuringiensis* were also isolated from the chicken feather dumping site (Uttangi and Aruna, 2018; Sivakumar *et al.*, 2012; Shanker *et al.*, 2014).



**Figure 2:-** The phylogenetic tree constructed from the sequence data by the Neighbor-joining.

Bacterial isolate capable of producing keratinases is a possible alternative to convert poultry waste into low-cost amino acids and peptides which are beneficial in the animal food stuff and agricultural use (Sahoo *et al.*, 2012). Also the several fungi and Actinomycetes are reported to have keratinase production ability but still bacterial keratinase have more importance because of their tolerance to broad range of pH, different salts and temperature which make them significant in industrial process.

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