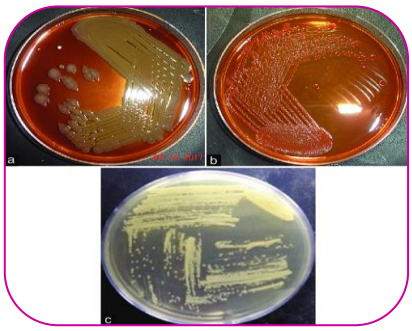
REVIEW OF RESEARCH
ISSN: 2249-894X
IMPACT FACTOR : 5.7631(UIF)

SCREENING AND PARTIAL 16 S rRNA GENE SEQUENCING OF KERATINASE PRODUCING *Stenotrophomonasmaltophilia* 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 meal 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 (150 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 meal medium pH 8. Maximum keratinase production was demonstrated by isolate number Kar05 (41U/ml) at 30°C on shaker condition (150rpm) after 4 days of incubation. Promising isolate was identified as Stenotrophomonasmaltophila KARUNA5 by morphological, cultural, biochemical and 16S rRNA sequence analysis and was submitted to NCBI (Accession no: LC271188).

KEY WORDS: *Keratinase, StenotrophomonasmaltophilaKARUNA5*

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 *etal*., 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; Kornillowicz*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 are important because of their various industrial applications. Bacterial strains belonging to genera Pseudomonas spp., Vibrio spp., Chryseobacterium spp., Xanthomonas spp., Fervidobacterium spp., Stenotrophomonas sp., Micrococcus

spp., Nesterenkonia, Arthrobacter, Clostridium, Caldicoprobacter, Bacillus and Kytococcuss arekeratinase 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, AspergillusCladosporium*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 dermatophyticAscomycetous fungi such as *Arthroderma sp*., *Microsporum 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

Eighty soil samples were collected from the regular feather and poultry waste dumping sites in Kolhapur district in clean dry plastic containers.

Enrichment: One gram of soil sample was added into 10 ml of sterile phosphate buffer saline solution (pH 7.2), mixed well and allowed to stand for 30 min. From this 2 ml of supernatant was inoculated in 250 ml flask containing100ml of Whole feather meal medium [(g/l):0.5 g of NH_4Cl, 0.5 g of NaCl, 0.3 g of K_2HPO_4, 0.4 g of KH_2PO_4, 0.1 g of $MgCl_2{\cdot}6H_2O$, 0.1 g of yeast extract 1 g of defatted whole chicken feathers] pH 8. These flasks were incubated at 30°C on rotary shaker (120rpm) till visible degradation or disappearance of the feathers observed (Agrahari and Wadhwa, 2010).

Primary screening of keratinolytic Bacteria : Primary screening of keratinolytic bacteria was carried out with Skimmed Milk agar medium [Peptone, 5.0; yeast extract, 3.0; dextrose, 1.0; skim milk Powder, 10.0, agar 15.0 and pH was maintained at 7.2] (BrandelliandsRiffel, 2006)

0.1 ml of the enriched sample was streaked on Skimmed milk agar plates (SMA) incubated at 30°C for 24 h to observe clear zone of proteolytic activity around the colony (Saha*etal.*, 2010).

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.

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

Keratinase assay:

Preparation of crude enzyme extract: Cultures were grown on sterile Luria Bertani broth on rotary shaker incubator (120 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 meal medium and incubated at 30°C on rotary shaker incubator (120rpm) for 5 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 50^0C. 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 (A594 nm) of 0.01 after reaction at 50^0C for 1 hr with keratin azure (Bressollier *etal.,* 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, M J 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.

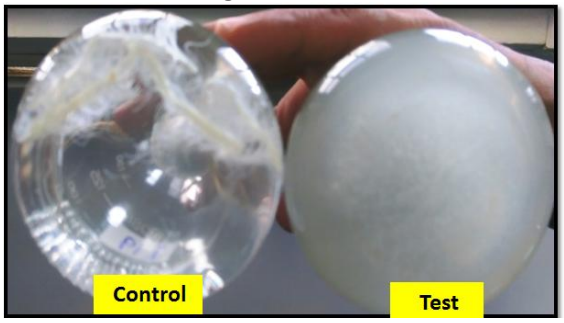

Figure 1: Enrichment medium showing visible feather degradation

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 meal medium was used for enrichment of keratinase producers(Ramya*et al.,* 2014; Shah, 2015).

Keratinase production ability of isolates was confirmed byproduction of caseinaseon 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.

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 basal broth for 5 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 fifthday 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 byKar05 isolate and Kar 01 and Kar 09 showed lowest % of feather degradation(Table 2).

Table 2: Percentage (%) of feather degradation demonstrated by the different isolates

Isolate designation	Extents of feather Degradation	% of feather degradation On 5th Day
Kar/01	--	04
Kar/02	+	20
Kar/03	++	45
Kar/04	++	47
Kar/05	+++	69
Kar/06	+++	61
Kar/07	++	52
Kar/08	+	30
Kar/09	--	05
Kar/10	+	11

++ = Degradation of feather vanes

+++ = Extensive degradation of feather vanes plus slight degradation of feather shaft.

++++ = Extensive degradation of feather vanes and shaft.

Keratiase assay: The keratinolytic activity was determined by using keratin azure (Sigma–Aldrich) as the insoluble substrate (Bressollier *etal.*, 1999). All 8 isolates produced keratinase enzyme (Table 3) but Kar05showed maximum production of keratinase enzyme after 4 days of incubation.Kar 05 was selected as a promising isolate for further studies.

Table 3: Production of Keratinase enzyme by different isolates

Name of the isolate	Enzyme activity U/ml (1st Day)	Enzyme activity U/ml (2nd Day)	Enzyme activity U/ml (3rd Day)	Enzyme activity U/ml (4th Day)	Enzyme activity U/ml (5th Day)
Kar/02	0	3	7	7	08
Kar/03	0	2	7	11	10
Kar/04	2	3	6	9	12
Kar/05	23	38	39	41	41
Kar/06	0	4	4	4	05
Kar/07	8	8	10	11	10
Kar/08	8	19	36	38	27
Kar/10	3	3	2	8	14

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 *Sternotrophomonasmaltophila* strain Al-Khrj-5 (GenbankAcc. No:KY123858.1) showing 96% 16S rRNA gene sequence identity represented the closest phylogenetic neighbour of the strain *Sternotrophomonasmaltophila*KARUNA05. The topologies of thepresent 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 *Stenotrophomonasmaltophilia Strain* KARUNA05 was deposited at GeneBank (accession no. LC271188).

Keratinase production by several bacteria such as *Stenotrophomonasmaltophilia* strain S-1,*Stenotrophomonasmaltophilia* N4, *Stenotrophomonasmaltophilia* K279a, *Stenotrophomonasmaltophilia* KB13,*Stenotrophomonas sp. Strain* Norja-1, *Stenotrophomonasmaltophilia* DHHJ and *Stenotrophomonasmaltophilia*YArckwere previously reported (Miyaji *et al*., 2005;Kuraneand Attar, 2017; Shah and Vaidya, 2017; Cao *et al.,* 2009). Many related other gram negative bacteria such as *Pseudomonasaeroginosa,* Pseudomomanas sp., MS21, *Fervidobacteriumpennavorans*, Microbacterium sp. kr10, Burkholderia,Chryseobacterium, Microbacterium species,*Chryseobacterium*sp. And *Serratia*sp. HPC 1383 were also demonstrated keratinoltyicacivity(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, Kytococcussedentarius(*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 re[ported 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).

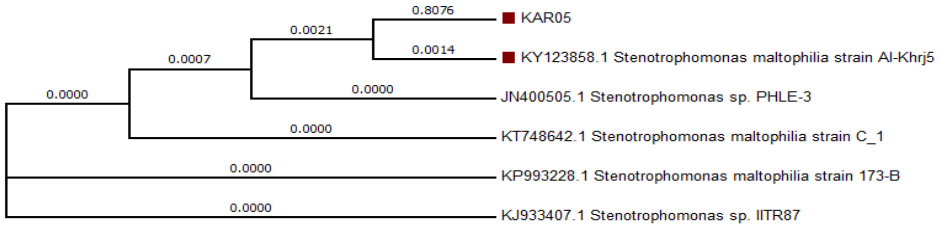

Figure 2: The phylogenetic tree constructed from the sequence data by the Neighbor-joining

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.

REFERENCES:

Agrahari, S., &Wadhwa, N. (2010). Degradation of Chicken Feather a Poultry Waste Product by Keratinolytic Bacteria Isolated from Dumping Site at Ghazipur Poultry Processing Plant, *International Journal of poultry Sciences*9(5):482-489.

Azza Abdel fattah AM, Nashy E SHA, Sabiel E TA, Hussien MM. and Attia AS. (2015). Novel Keratinolytic Activity of *Cyberlindnerafabianii*Nrc3 Aza as A Plant Growth Promoting Agent (PGPA). *Int J ApplSciBiotechnol*, 3(4):609-618.

Azza M. Abdel-fattah (2013). Novel keratinase from marine Nocardiopsisdassonvillei NRC2aza exhibiting remarkable hide dehairing. *Egyptian Pharmaceutical Journal* 12:142–147.

Brandelli A, Daroit DJ and Riffel A (2010). Biochemical features of microbial keratinases and their production and applications. *Appl Microbial Biotechnol*. (85): 1735- 1750.

Brandelli A. Riffel A (2005).Production of an extracellular keratinase from *Chryseobacterium*sp. growing on raw feathers *Process Biotechnology* 8(1): 1-6. 33

Bressollier P., Letourneau, F., Urdaci, M. and Verneuil, B. (1999) Purification and characterization of a keratinolytic serine proteinase from Streptomyces albidoflavus. *Applied and Environmental Microbiology*(65): 2570–2576).

Gioppo NMR, Moreira Gasparin F, Costa AM, Alexanddrio AM, Souza CGM and Peralta RM (2009). Influence of the carbon and nitrogen sources on keratinase production by *Myrotheciumverrucariain*submerged and solid state cultures. *J Ind Microbial Biotech* (36): 705–11.

Gousterova A, Braikova D, Goshev I, Christov P, Tishinov K, Vasileva-Tonkova E, Haertle T, Nedkov P (2005). Degra- dation of keratin and collagen containing wastes by newly isolated thermoactinomycetes or by alkaline hydrolysis. *LettApplMicrobiol*40(5):335–340.

Gupta, R., Ramnani, P. (2006). Microbial keratinase and their prospective application: an overview. *ApplMicrobiolBiotechnol*. 70(1):21–33.

Huang Y, Busk PK, Lange L (2015). Production and Characterization of Keratinolytic Proteases Produced by *Onygenacorvina*. *Fungal GenomBiol*(5) 1:1-7.

Karthikeyan, R., Balaji, S. and Sehgal, P. K. (2007). Industrial application of keratins – A review. *J. Sci. Ind. Research.* (66): 710-715.

Kaul S &Sumbali G, Keratinolysis by poultry farm soil fungi, Mycopathologia, 139 (1997) 137-140., Mohamedin A H, Isolation, identification and some cultural conditions of a protease-producing thermophilic Streptomyces strain grown on chicken feather as substrate, *IntBiodeteriorBiodegrad,*43 (139): 13-21.

Kim K. (2007). Purification and Characterization of a Keratinase from a Feather-Degrading. *Mycobiology*35(4):219–225.

Korniłłowicz, Kowalska T, Bohacz J.(2011). Biodegradation of keratin waste: Theory and practical aspects. *Waste Manag*. (31):1689-1701.

Kumar CG, Takagi H. Microbial alkaline proteases: from a bioindustrial viewpoint. *Biotechnol Adv*. 1999;17(7):561-594.

Kurane, A. B., & Attar, Y. C. (2017).Screening and Isolation of Keratinase Producing Micro Organisms, *5*(V), 489–495.

Martinez DA, Oliver BG, Gräser Y, Goldberg JM, Li W, et al. (2012) Comparative genome analysis of Trichophytonrubrum and related dermatophytes reveals candidate genes involved in infection. *mBio*3: 00259-0021.

Mazotto A. M, LageCedrola SM. and Lins U. (2010). Keratinolytic activity of *Bacillus subtilis*AMR using human hair. *LettApplMicrobiol*. 50(1):89–96.

Miyaji, T., Otta, Y., Shibata, T., Mitsui, K., Nakagawa, T., Watanabe, Tomizuka, N. (2005). Purification and characterization of extracellular alkaline serine protease from *Stenotrophomonasmaltophilia* strain S-1. *Letters in Applied Microbiology*, *41*(3): 253–257.

Nayaka, S., &Babu, K. G. (2014).Isolation, Identification and Characterization of Keratin degrading Streptomyces albus. *International Journal of Current Microbiology and Applied Sciences*, *3*(10): 419–431.

Patience N, Abigail O, Ponchang W. and Deborah A. (2015). Keratinolytic activity of *Cladosporium*and *Trichoderma species* isolated from barbers ‟ landfill. *Biosci IJ*. 6655:104–115.

Saha S, Dhanasekaran D. Isolation and Screening of KeratinolyticActinobacteria form Keratin Waste Dumped Soil in Tiruchirappalli and Nammakkal , Tamil Nadu , India. *Cur Research J BiolSci*2(2): 124-131.

Sahoo DK, Halder SK, Das A, Jana A, Paul T. and Thatoi H. (2015). Keratinase production by *Bacillus weihenstephanensis*PKD5 in solid state fermentation and its milk clotting potential. *Indian journal of biotechnology*, (14): 200–207.

Sahoo K D, Das A, Thatoi H, Mondal KC and Mohapatra PKD (2012). Keratinase production and biodegradation of whole chicken feather by a newly isolated bacterium under submerged fermentation *ApplBiochemBiotechnol*. 167:1040-1051.

Saibabu. V, and Niyongabo N. (2013): Isolation Partial purification and characterization of keratinase from *Bacillus megaterium*. *Int Res J of Biol Sci.* 2(2):13-20.

Shah M. (2015). A novel feather degrading Acinetobacter sp. PD 12 isolated from feather waste dumping site in Mumbai *European Academic Research* 3(1):757–773.

Shah Malay and Vaidya Rajnish (2017). Investigation of environmental parameters affecting feather degradation and keratinase production by Stenotrophomonasmaltophilia K279a. (2017) *Int. J. of Life Sciences*A8: 2320-7817.

TamilKani, P., Subha, K., Madhanraj, P., Senthilkumar, G., Panneerselvam, A. 2012. Degradation of chicken feathers by *Leuconostoc*sp.And*Pseudomonas microphilus. Eur. J.* 3(10):419-431.

Thanikaivelan P. Jonnalagadda. and Rao R (2004). Progress and recent trends in biotechnological methods for leather processing. *Trends in biotechnology.* (22) 4:181-188.

Thatheyus, A. J., &Ramya, D. (2015). Biodegradation of Poultry Feathers using a Novel Bacterial Isolate P seudomonasaeruginosa, *1*(1): 25–30.

Tork, S. E., Shahein, Y. E., El-Hakim, A. E., Abdel-Aty, A. M., and Aly, M. M. (2016). Purification and partial characterization of serine-metallokeratinase from a newly isolated Bacillus pumilus NRC21. *Int. J. Biol. Macromol*. (86) 189–196.

Uttangi, V., & Aruna, K. (2018). Optimization of Production and Partial Characterization of Keratinase Produced by *Bacillus thuringiensis* strain Bt407 Isolated from Poultry Soil, *Int.J.Curr.Microbiol.App.Sci* (2018) 7(4): 596-626.

Vermelho AB, Mazotto AM, de Melo ACN, et al. (2010). Identification of a Candida parapsilosis strain producing extracellular serine peptidase with keratinolytic activity. *Mycopathologia*. 169(1):57–65.

Vesela M and Friedrich J (2009). Amino Acid and Soluble Protein Cocktail from Waste Keratin Hydrolysed by a Fungal Keratinase of *Paecilomycsemarquandii. Biotechnology and Bioprocess Engineering*. (14):84-90.