Journal of Applied Sciences

ISSN 1812-5654

science alert

ANSInet
an open access publisher
http://ansinet.com

OPEN ACCESS Journal of Applied Sciences

ISSN 1812-5654
DOI: 10.3923/jas.2019.789.796

Research Article
Production and Purification of Keratinase Enzyme from *Serratia* sp. Isolated from Poultry Wastes

[1]Veerapura Narayanappa Yogananda Murthy, [2]Veerapura Narayanappa Murulidhara and [1]Mahesh Mariswamy

[1]Department of Biotechnology, Azyme Biosciences Pvt. Ltd., #1188/20, 3rd Floor, 26th Main, Opposite Raagigudda Temple Arch, 9th Block, Jayanagar, Bengaluru, Karnataka, India
[2]Department of Botany, Government First Grade College of Arts, Science and Commerce, Sira, Karnataka, India

Abstract

Background and Objectives: Keratins comprise largest complex family of cytoskeletal filament proteins found in human hairs, chicken feathers and wool. Biotechnical applications might augment the human efforts to degrade these keratins which pose a problem to environment. Present study was aimed to enumerate the potential microbial sources for production and purification of keratinase followed by characterization. **Materials and Methods:** Poultry waste samples (n = 20) collected and used for the isolation of organism through serial dilution. Keratin broth media employed to assess the activity using azocasein as substrate by spectrophotometry. Biochemical and morphological identifications done and confirmed as *Serratia* sp. by 16S rRNA sequencing. To purify the enzyme, ammonium sulphate precipitation, dialysis, ion exchange and gel filtration chromatography were carried out followed by SDS-PAGE based molecular weight determination. **Results:** Out of 12 organisms, one showed increased keratinase activity. The activity and yield were increased 6.4 and 16.25%, respectively followed by purification. Enzyme activity was maximum at pH 7 and temperature 37°C with keratin azure as substrate. Molecular weight of purified enzyme was shown to have 50 kDa as determined by SDS-PAGE. **Conclusion:** It is revealed that *Serratia* sp. could be a potential microbial source can be harnessed for alleviation of environmental pollution and feather waste utilization.

Key words: Chromatography, gel filtration, keratinase, SDS-PAGE, poultry waste

Citation: Veerapura Narayanappa Yogananda Murthy, Veerapura Narayanappa Murulidhara and Mahesh Mariswamy, 2019. Production and purification of keratinase enzyme from *Serratia* sp. isolated from poultry wastes. J. Applied Sci., 19: 789-796.

Corresponding Author: Mahesh Mariswamy, Azyme Biosciences Pvt. Ltd., #1188/20, 3rd Floor, 26th Main, Opposite Raagigudda Temple Arch, 9th Block, Jayanagar, Bengaluru, Karnataka, India Tel: +91 9379215771

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Keratin is an insoluble protein present in nature mainly in the form of feathers, wool, hooves, scales, nails (hard keratins) and stratum corneum (soft keratins)[1,2]. Proteins belong to the scleroprotein group are compounds that are extremely resistant to the action of physical, chemical and biological agents. They have high mechanical stability and high resistant to the degradation of keratin due to the presence of their disulfide bonds, hydrogen bonds, salt linkages and cross linkings[3,4]. Goat hair, sheep wool and buffalo horn exhibited lower response to Keratinolytic hydrolysis[5]. Keratin comprising materials are profuse in nature but have inadequate uses in practices, meanwhile they are insoluble and resistant to degradation by the common proteolytic enzyme keratinase wastes characterize a substance of valuable protein and amino acid[6]. Hair is mainly composed of 90% of keratin protein and 1-9% of lipids[7,8].

Human hair is a material considered useless in most societies and therefore is found in municipal waste streams in almost the entire world. Due to slow degradation, it stays in the dumps/waste streams for long occupying large volumes of space. Burning of human hair or the waste piles containing them is a practice observed in many parts of the world produces foul odour and toxic gases, such as; ammonia, carbonyl sulphides, hydrogen sulphides, sulphur dioxide, phenols, nitriles, pyrroles and pyridines[9]. Open dumps of hair generate hair dust which causes discomfort to people near them and if inhaled in large amounts can result in several respiratory problems. The best way to address such problems is to develop systems which utilize the waste material as a resource. Other promising applications have been associated with keratinolytic enzymes, including elimination of keratin in acne, depilation process, preparation of vaccine for dermatophytosis therapy, pharmaceutical enhancement of the nail treatment and degradation of prion and prion-like proteins[10].

A number of keratinolytic micro-organisms have been reported including species of fungi like *Microsporum*[11], *Aspergillus*, *Bacillus*[12], *Streptomyces*[13] and other *Actinomycetes* which are capable of degrading hair. Microbial keratinase have become biotechnologically important in the recent decade, particularly due to their potential in numerous industrial applications, such as; in the animal feeds, fertilizers, detergents, leather, glues, films and pharmaceutical industries[14]. Therefore; in this study, it was aimed to isolate that novel strain Gram-negative bacteria *Serratia* sp. for the production and characterization of keratinase enzyme by degrading poultry waste and also intellects the effect of mutagens and metal ions. Produced keratinase enzyme established the significant feather degrading capabilities, demonstrating prospective of the isolate in various industrial processes to produce novel bioproducts.

MATERIALS AND METHODS

Chemicals: The LB Agar (Himedia India), Azocasein (Sigma aldrich India) Fc reagent (SD fine India) and all other reagents were of analytical grade. The molecular mass SDS–PAGE standards were obtained from Bangalore Genei.

Collection and isolation of bacteria from poultry waste: The experiment was conducted for a period of 10 months between July, 2017-March, 2018. Poultry waste samples (n = 20) were collected from three different areas of Bengaluru. Organism was isolated by the suspension culture method; 1 g of sample was mixed in 1% of 10 mL saline solution by vertexer and allowed for sedimentation. About 0.1 mL of inoculum was pipetted into sterile Petri plates and casein agar was poured in order to get proteolytic organisms followed by swirling. Plates were incubated at 35°C for 24 h colonies of distinct morphological characters cultured on LB agar slant to obtain pure isolates.

Screening of strain for keratinase activity: Keratinolytic activity was tested by bioassay method[15] using Azo casein substrate for every 24 h up to 5 days. Quantitative estimation of proteins was determined in the culture supernatants using Bovine Serum Albumin (BSA) as the standard[16]. Readings were carried out in a spectrophotometer at 660 nm.

Effect of pH on keratinase production: About 50 mL of minimal broth with 1% feather was added in different conical flaks and broth was set for different pH such as; 4, 5, 6, 7, 8, 9 and 10 using acetic acid and sodium hydroxide. Conical flasks were autoclaved and inoculated with the organism. After 72 h of incubation, the enzyme activity was measured.

Effect of temperature on keratinase production: About 50 mL of minimal broth with 1% feather was prepared in different conical flasks and pH of each conical flask was adjusted to 7. Conical flasks were autoclaved and inoculated with the organism. The flasks were inoculated at different temperature like 20, 25, 30, 35, 40, 45, and 50°C. After 72 h of incubation, the enzyme activity was measured.

Effect of different metal ions on keratinase production: About 50 mL of minimal broth with 1% of feather was prepared in five different conical flask and 10 mg of each metal ions like cupric sulphate, cadmium sulphate, manganese chloride, zinc sulphate and mercuric chloride were used. Media was autoclaved, organism was inoculated to each conical flask and incubated at 35°C for 72 h. Afterwards, keratinolytic activity was determined by the assay method and different concentration of metal ion was tested for the highest activity producing metal ion.

Effect of UV radiation on the activity of organism: The lawn culture was prepared on five different LB agar plates and exposed to UV radiation of 15 watts with distance 50 cm at different time intervals such as 5, 10, 15, 20 and 25 min. Then the plates were incubated at 35°C for 48 h. Pure cultures were isolated from each plate and sub-cultured on LB agar media. About 50 mL of Keratinase broth was prepared in five different conical flasks and autoclaved. Then each flask is inoculated with pure cultures isolated from UV mutated plates. Conical flasks were incubated at 35°C for 72 h. Then keratinolytic activity was determined by the assay method. Highest activity produced UV mutated organism was subjected to different distance such as 50, 40, 30, 20 and 10 cm UV exposure.

Purification and characterization of keratinase enzyme: About 200 mL keratinase broth was prepared for the production of enzyme. Cell free media was used for the purification by three step method like ammonium sulphate precipitation, dialysis, ion exchange and gel filtration chromatography.

Molecular weight determination: Polyacrylamide gel electrophoresis (PAGE) in presence of Sodium Dodecyl Sulphate (SDS) was prepared and carried out[17]. About 40 µL of the sample was mixed with 40 µL of gel loading dye, 50 µL of sample was loaded into wells. The necessary electrical connections are made and the voltage was adjusted to 50 mv. Electrophoresis was made to run for 75-80% of the length of the gel slab. Gel was stained with coomassie blue for 8 h and destined to observe the bands.

Enzyme characterization by kinetic study: During this process, the optimum enzyme activity was determined by different pH, temperature, substrate concentration and incubation time.

Statistical analysis: The experiment was conducted in triplicates and the results were analyzed statistically with the use of one-way analysis of variance (ANOVA) software. The means were compared for significance at $p \leq 0.05$.

RESULTS

Selection of bacteria: All the seven strains grew well on skim milk agar media. As the strain 1 from soil sample 1 (0.6 cm), strain 2 from soil sample 2 (0.4 cm) and strain 3 (0.4 cm) from soil sample 3 has shown the highest clear zone, they were subjected for keratin hydrolysis test. During this process, the organism strain 1, strain 2 and strain 3 were inoculated in minimal agar broth containing feather as source for keratinase production. Keratinolytic activity was measured for every 24 h up to 96 h by assay method. In all the strains, strain 1 showing highest result at 72 h of incubation (28.312 U mL^{-1}) as presented in Table 1. Hence it is selected for the process of enzyme production.

Identification of the strain: The colonies observed on LB agar were circular, entire margin, slightly raised, smooth surfaced and showed negative result for Gram-staining. By performing the various morphological and biochemical tests, one isolate was grouped under the genus *Serratia*. In the 16S rRNA sequencing and phylogenetic investigation (Fig. 1), the bacterial strain is identified as *Serratia* sp.

Effect of pH on keratinase production: The media was set for different pH and keratinolytic activity was analyzed for each pH after 72 h of incubation. Results revealed that organism showed 21.217 U mL^{-1} of maximum activity at neutral pH 7 (Fig. 2a).

Effect of temperature on keratinase production: The optimum temperature for keratinase production was recorded. Results showed that maximum activity (21.567 U mL^{-1}) of keratinase production was recorded at 40°C (Fig. 2b). *Serratia* sp. produce the enzyme in wide range of temperatures is an example for the production of keratinase enzyme.

Effect of metal ions on keratinase production: The enzyme activity was highest in presence of $MgCl_2$ (26.163 U mL^{-1}) and decreased in presence of $HgCl_2$ (2.344 U mL^{-1}) (Fig. 2c).

UV mutation: Keratinase production of the mutated strain at 20 min showed highest activity (41.514 U mL^{-1}). As compared

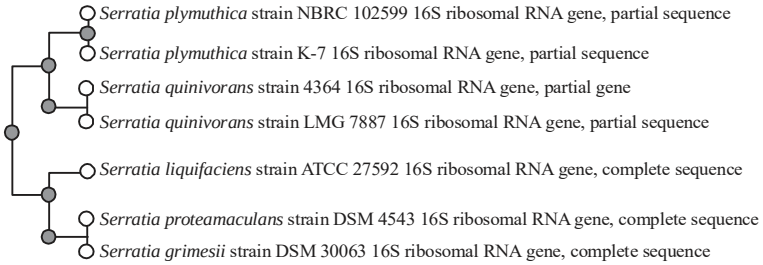

Fig. 1: Phylogenetic analysis of 16S rRNA gene sequence for organism identification

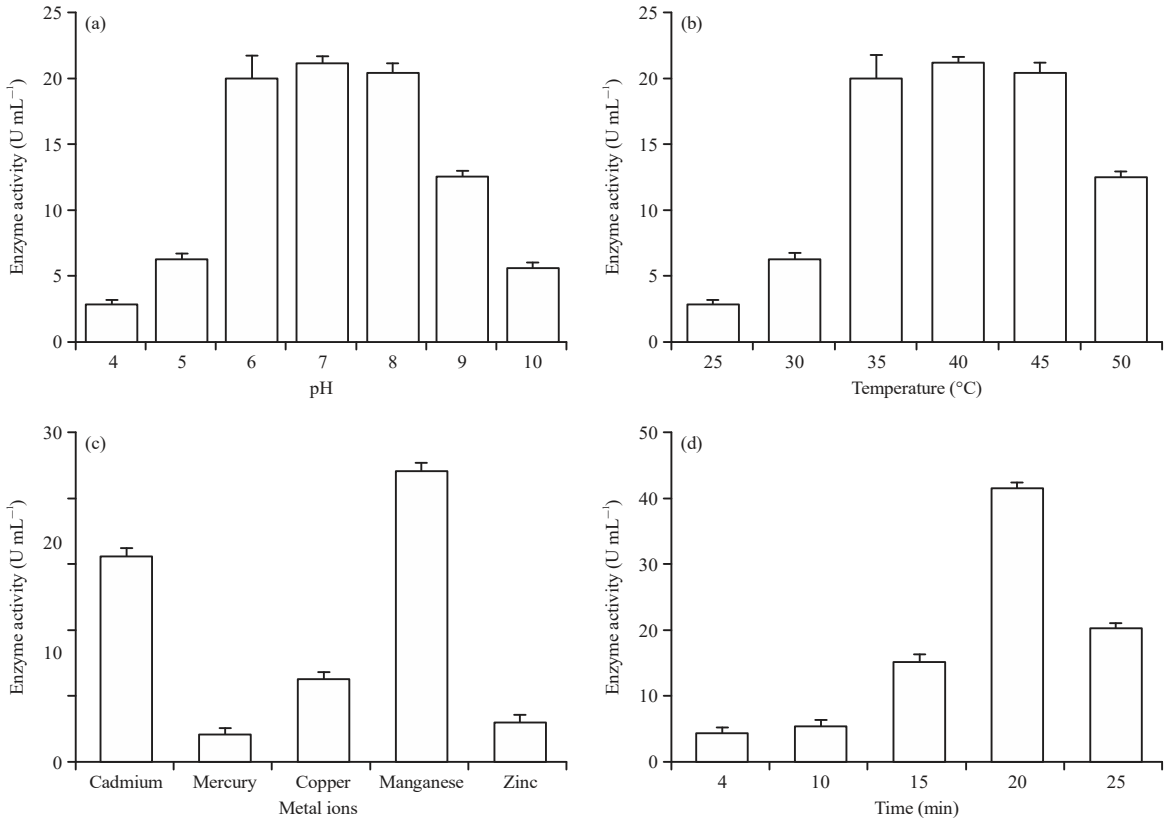

Fig. 2(a-d): (a) Effect of pH on keratinase enzyme production (b) Effect of temperature on keratinase enzyme production (c) Effect of different metal ions on keratinase enzyme production (d) Effect of time on keratinase enzyme production.

Table 1: Production of enzyme from selected bacterial strains

	Enzyme activity (U mL^{-1})		
Organism	24 h	72 h	96 h
Strain 1	20.15	28.312	24.60
Strain 2	16.19	23.76	21.78
Strain 3	17.85	24.78	26.27

Table 2: Fold purification of keratinase enzyme

Samples	Activity (U mL^{-1})	Specific activity (U mg^{-1})	Purification (fold)	Yield (%)
Crude	17.592	24.433	1.000	100.00
Salt ppt.	22.656	43.906	1.796	71.677
Dialysis	21.084	42.00	1.719	69.722
Ion exchange	17.286	54.702	2.239	43.89
Gel filtration	18.596	158.632	6.492	16.25

to control organism, UV exposed organism showed 48.8% more keratinolytic activity. Hence the strain is selected for production of enzyme (Fig. 2d).

Purification of keratinase enzyme: It is shown in Table 2 that the crude enzyme exhibited 24.433 U mg^{-1} of activity, for ammonium sulphate saturated enzyme activity was increased to 43.906 U mg^{-1} and enzyme activity after dialysis was 42.00 U mg^{-1}. After ion exchange chromatography, specific activity was 54.702 U mg^{-1} and for gel filtration enzyme activity was highest at 16th elution showing 18.596 U mL^{-1} (Fig. 3a) and specific activity

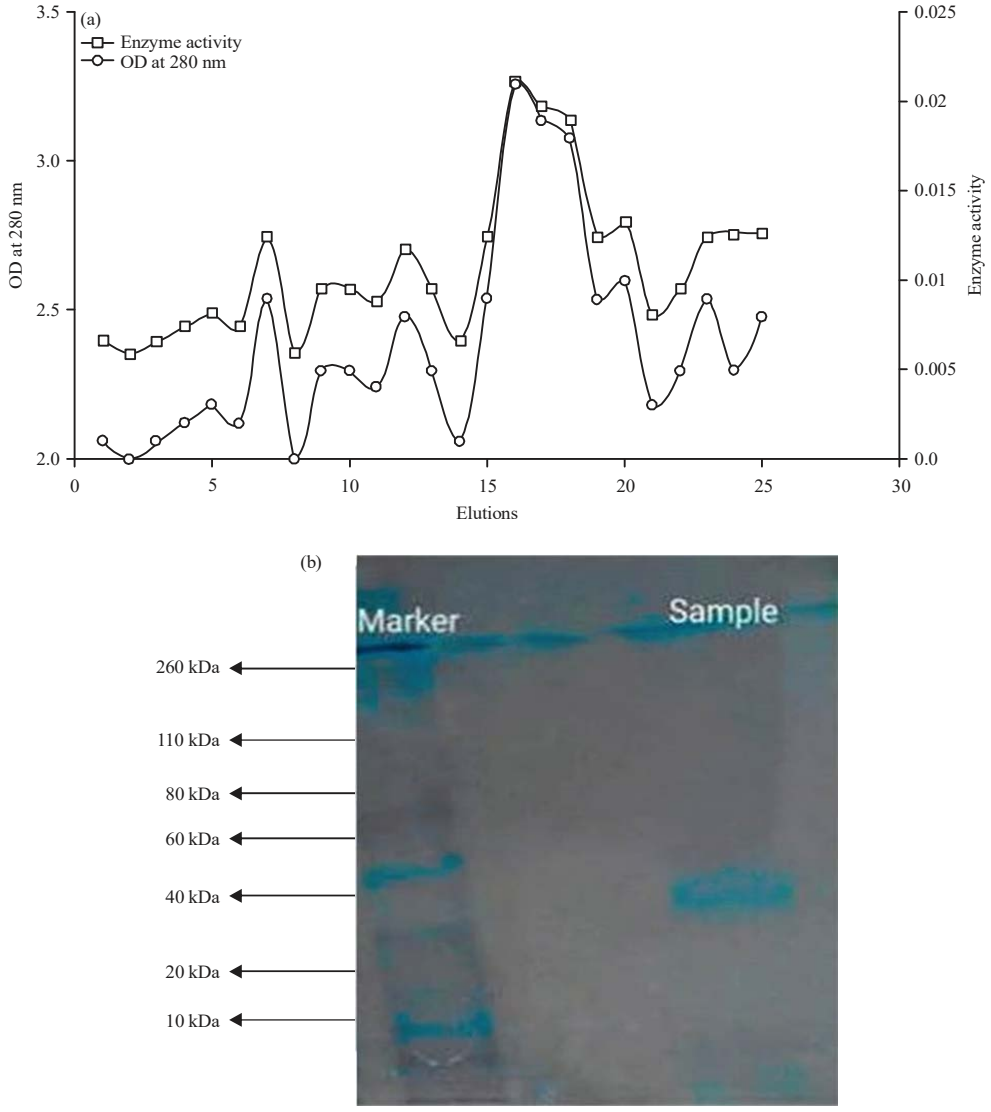

Fig. 3(a-b): (a) Keratinase enzyme purification by gel filtration chromatography (b) SDS-PAGE of the purified keratinase enzyme and marker proteins, number beside the gel are molecular mass of enzyme bands expressed in kDa

158.632 U mg^{-1}. Over all fold purification achieved was 6.492 and yield percentage was 16.25 as shown in Table 2.

Molecular weight of the keratinase enzyme: SDS-PAGE for the purified samples was carried out to distinguish the molecular weight of the keratinase enzyme 50 kDa (Fig. 3b).

Enzyme kinetics

Effect of pH: Keratinase activity was assayed at different pH by preparing buffers with different pH. Results revealed that the organism showed highest activity at pH 7.0 (Fig. 4a).

Effect of temperature: Keratinase production is assayed by incubating the enzyme at different temperatures like 25, 35, 45 and 55°C. The enzyme was active within the temperature range of 37-80°C. Keratinolytic activity increases gradually from 37°C to reach the optimum at 80°C (Fig. 4b) and showed highest activity at 35°C.

Effect of substrate concentration: Keratinase production was assayed at various substrate concentrations such as 0.5, 1.0, 1.5, 2.0, 2.5 and 3 mg at 37°C (Fig. 4c). Minimum keratinase activity (36.627 U mL^{-1}) was recorded in presence of 0.5 mg substrate concentration and the activity increased with the increase in substrate concentration. Keratinase

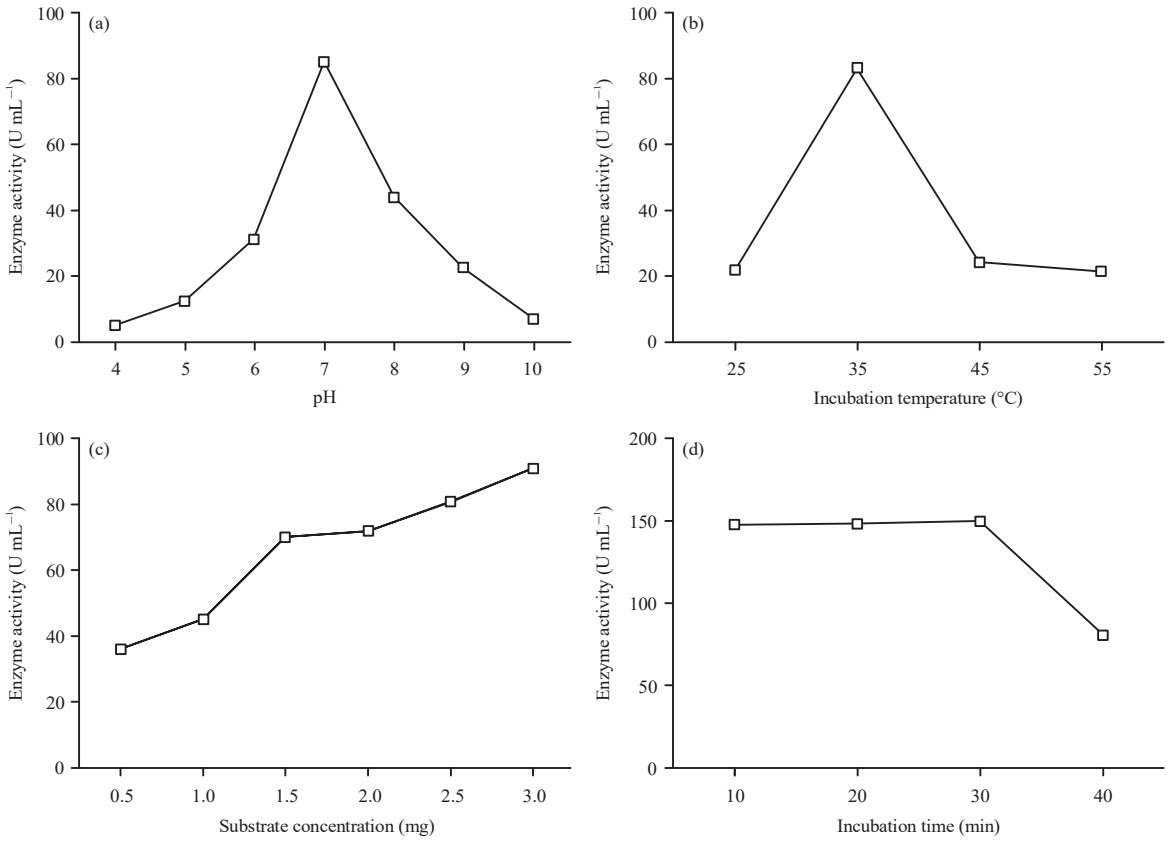

Fig. 4(a-d): (a) Effect of pH (b) Incubation temperature (c) Substrate concentration (d) Incubation time on keratinase enzyme activity
Results are the mean of three experiments

activity was found highest at 1.5 mg substrate concentration, (Substrate concentration was slightly increasing from 1.5-3 mg. Because of this, it has been considered 1.5 mg is the good concentration).

Effect of incubation time: In order to investigate thermal stability of the enzyme, incubate for 10, 20, 30 and 40 min at 37°C and then the residual activity was measured. Keratinase activity determined at incubation period of 10, 20 and 30 min was 153.091 U mL^{-1} and it decreased after 40 min of incubation (81.937 U mL^{-1}) (Fig. 4d).

DISCUSSION

In the present study, a novel organism Gram-negative bacteria *Serratia* sp. is isolated and UV mutation exposure of 20 min on an organism is significant for the production of enzyme. Keratinolytic activity of organisms increased with the increase in time. However, the rates of increase in enzyme activity were not similar in pattern for all the isolates[18]. Twelve pure cultures were isolated from three different samples, among the 12 and 7 isolated strains showed clear zones on skimmed milk agar plates since casein was hydrolyzed by the extracellular proteolytic enzyme secreted by the isolated strains[19]. Different organisms showed maximum enzyme production at different pH levels. For example, *Bacillus cereus*, *Bacillus subtilis* and *Bacillus pumilus* produced maximum enzyme at pH levels of 7.0, 5.9 and 5.6, respectively[20]. In earlier intelligences, bacteria with feather degrading ability were grown at wide range of pH. *Baciluss cereus* at pH 9 *Bacillus megaterium*[21] exhibited maximum activity[22] at pH 7 and it is observed that keratinase production by *Bacillus subtilis* KD-N2 in a hair medium[23] at pH 6. Different studies showed that *Bacillus cereus* is capable of growing at 40°C with optimum temperature for keratinase enzyme production[24] is at 30°C. *Bacillus* sp. produced maximum keratinase[12] at 45-50°C and *Arthobacter* sp. NFH 5 is produced keratinase enzyme[25] at 37°C. Metal ions are helpful to grow the micro-organisms as an essential micronutrient, not much of the work has been done for the production of keratinase enzyme by using metal ions. Many divalent metal ions increased the enzyme activity such as;

Ca^{+2}, Mg^{+2}, Co^{+2} and Mn^{+2}. However; in the presence of $PbCl_2$, $FeCl_3$, $ZnCl_2$ and $HgCl_2$, keratinase activity was decreased[26]. Stimulation of keratinase in presence of metal ion like Mn^{+2} was due to salt formation or an ion bridge which maintain the confirmation of enzyme-substrate complex[27]. Inhibition of keratinase activity by metal ions may be attributed to the formation of bridges between metal monohydroxide and catalytic ions at the active site[28].

Keratinolytic activity was determined for UV mutated strains at different time intervals (Fig. 2d). According to the previous results, it was observed that, the UV mutated strains produced highest keratinase activity[29]. Molecular weight of the keratinase enzyme was assessed by relating the electrophoretic flexibility of the enzyme with electrophoretic mobilities of marker proteins[30]. Molecular weight was 60 kDa for *Bacillus* species, they are of medium size, such as 33 kDa for *B. licheniformis*, 25.4 kDa for *B. subtilis* and 24 kDa for *B. paeudofirmis*[31-33]. The effect of pH on keratinolytic activity of keratinase enzyme showed that, the enzyme will be active over a broad range of pH of 5-10 with more than 50% activity occurred[19] between pH 7-10. Temperature effect revealed that, more than 50% of activity obtained[19] between 45 and 70°C. As the substrate concentration increased, the enzyme activity also increases[34]. Keratinase activity is same till a specific time of incubation and then starts decreasing[30]. For the future benefit, the present research work is concentrating on UV exposure on an organism for increasing the production. This method is highly significant and useful for the large scale industrial production to fulfil the present needs.

CONCLUSION

From the present result it is concluded that, one feather degrading bacteria was isolated from poultry waste dumping soil. Identification and characterization exhibited that this novel bacteria belongs to *Serratia* genus and found to have proteolytic (especially Keratinolytic) activity and henceforth might be used for production of Keratinase enzyme at large scale manufacture. The enzymes might be beneficial in progressions where keratin must be hydrolyzed. It is the first report describing the production and purification of Keratinase enzyme from *Serratia* sp. isolated from poultry waste. In addition to that, it enhances the production of Keratinase enzyme by UV mutation on *Serratia* sp. It leads to the large scale production of Keratinase enzyme to meet the present day needs in leather processing, pharmaceutical and cosmetic industries.

SIGNIFICANCE STATEMENT

This study discovered the novel organism Gram-negative bacteria *Serratia* sp., UV exposure for 20 min which significantly increase the production of enzyme nearly 48.8% that can be beneficial for large scale production and this study will help the researchers to uncover the critical areas of mutation that many researchers were not able to explore.

REFERENCES

1. Friedrich, J., H. Gradisar, D. Mandin and J.P. Chaumont, 1999. Screening fungi for synthesis of keratinolytic enzymes. Lett. Applied Microbiol., 28: 127-130.
2. Kornillowicz-Kowalska, T. and J. Bohacz, 2011. Biodegradation of keratin waste: Theory and practical aspects. Waste Manage., 31: 1689-1701.
3. Kaluzewska, M., K. Wawrzkiewicz and J. Lobarzewski, 1991. Microscopic examination of keratin substrates subjected to the action of the enzymes of *Streptomyces fradiae*. Int. Biodeterior., 27: 11-26.
4. Kornillowicz-Kowalska, T., 1997. Studies on the decomposition of keratin wastes by saprotrophic microfungi. II. Sulphur and nitrogen balance. Acta Mycol., 32: 81-93.
5. Saber, W.I.A., M.M. El-Metwally and M.S. El-Hersh, 2010. Keratinase production and biodegradation of some keratinous wastes by *Alternaria tenuissima* and *Aspergillus nidulans*. Res. J. Microbiol., 5: 21-35.
6. Jayalakshmi, T., P. Krishnamoorthy, G.R. Kumar and P. Sivamani, 2010. Isolation and screening of a feather-degrading keratinolytic actinomycetes from *Actinomyces* sp. J. Am. Sci., 6: 45-48.
7. Kajiura, Y., S. Watanabe, T. Itou, K. Nakamura and A. Iida *et al*., 2006. Structural analysis of human hair single fibres by scanning microbeam SAXS. J. Struct. Biol., 155: 438-444.
8. Villa, A.L.V., M.R.S. Aragao, E.P.D. Santos, A.M. Mazotto, R.B. Zingali, E.P. de Souza and A.B. Vermelho, 2013. Feather keratin hydrolysates obtained from microbial keratinases: Effect on hair fiber. BMC Biotechnol., Vol. 13. 10.1186/1472-6750-13-15.
9. Brebu, M. and I. Spiridon, 2011. Thermal degradation of keratin waste. J. Anal. Applied Pyrolysis, 91: 288-295.
10. Cai, C.G., B.G. Lou and X.D. Zheng, 2008. Keratinase production and keratin degradation by a mutant strain of *Bacillus subtilis*. J. Zhejiang Univ. Sci. B, 9: 60-67.
11. Essien, J., A. Umoh, E. Akpan, S. Eduok and A. Umoiyoho, 2009. Growth, keratinolytic proteinase activity and thermotolerance of dermatophytes associated with alopecia in Uyo, Nigeria. Acta Microbiol. Immunol. Hung., 56: 61-69.

12. Lakshmi, P.J., C.M.K. Chitturi and V.V. Lakshmi, 2013. Efficient degradation of feather by keratinase producing *Bacillus* sp. Int. J. Microbiol., Vol. 2013. 10.1155/2013/608321
13. Nayaka, S. and G.M. Vidyasagar, 2013. Purification and characterization of keratinase from native feather-degrading *Streptomyces albus*. Int. J. Dev. Res., 3: 34-39.
14. Rao, K.V.B. and R. Arthi, 2012. Screening of keratinolytic bacteria isolated from feather and hair dumping sites of Tamilnadu. Asian J. Biochem. Pharmaceut. Res., 2: 113-126.
15. Grzywnowicz, G., J. Lobarzewski, K. Wawrzkiewicz and T. Wolski, 1989. Comparative characterization of proteolytic enzymes from *Trichophyton gallinae* and *Trichophyton verrucosum*. J. Med. Vet. Mycol., 27: 319-328.
16. Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall, 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem., 193: 265-275.
17. Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227: 680-685.
18. Govinden, G. and D. Puchooa, 2012. Isolation and characterization of feather degrading bacteria from Mauritian soil. Afr. J. Biotechnol., 11: 13591-13600.
19. Pandian, S., J. Sundaram and P. Panchatcharam, 2012. Isolation, identification and characterization of feather degrading bacteria. Eur. J. Exp. Biol., 2: 274-282.
20. Kunert, J., 1992. Effect of reducing agents on proteolytic and keratinolytic activity of enzymes of *Microsporum gypseum*. Mycoses, 35: 343-348.
21. Nilegaonkar, S.S., V.P. Zambare, P.P. Kanekar, P.K. Dhakephalkar and S.S. Sarnaik, 2007. Production and partial characterization of dehairing protease from *Bacillus cereus* MCM B-326. Bioresour. Technol., 98: 1238-1245.
22. Suntornsuk, W., J. Tongjun, P. Onnim, H. Oyama, K. Ratanakanokchai, T. Kusamran and K. Oda, 2005. Purification and characterisation of keratinase from a thermotolerant feather-degrading bacterium. World. J. Microbiol. Biotechnol., 21: 1111-1117.
23. Cai, C. and X. Zheng, 2009. Medium optimization for keratinase production in hair substrate by a new *Bacillus subtilis* KD-N2 using response surface methodology. J. Ind. Microbiol. Biotechnol., 36: 875-883.
24. Williams, C.M. and J.C.H. Shih, 1989. Enumeration of some microbial groups in thermophilic poultry waste digesters and enrichment of a feather-degrading culture. J. Applied Bacteriol., 67: 25-35.
25. Barman, N.C., F.T. Zohora, K.C. Das, M.G. Mowla, N.A. Banu, M. Salimullah and A. Hashem, 2017. Production, partial optimization and characterization of keratinase enzyme by *Arthrobacter* sp. NFH5 isolated from soil samples. AMB Express, Vol. 7. 10.1186/s13568-017-0462-6
26. Riessen, S. and G. Antranikian, 2001. Isolation of *Thermoanaerobacter keratinophilus* sp. nov., a novel thermophilic, anaerobic bacterium with keratinolytic activity. Extremophiles, 5: 399-408.
27. Balaji, S., M.S. Kumar, R. Karthikeyan, R. Kumar, S. Kirubanandan, R. Sridhar and P.K. Sehgal, 2008. Purification and characterization of an extracellular keratinase from a hornmeal-degrading *Bacillus subtilis* MTCC (9102). World J. Microbiol. Biotechnol., 24: 2741-2745.
28. Sivakumar, T., T. Shankar and V. Ramasubramanian, 2012. Purification properties of *Bacillus thuringiensis* TS2 keratinase enzyme. Am.-Eurasian J. Agric. Environ. Sci., 12: 1553-1557.
29. Zhang, X., 2012. Applying the mutation of *Bacillus subtilis* and the optimization of feather fermentation medium to improve *Keratinase* activity. Adv. Biol. Chem., 2: 64-69.
30. Cai, C.G., J.S. Chen, J.J. Qi, Y. Yin and X.D. Zheng, 2008. Purification and characterization of keratinase from a new *Bacillus subtilis* strain. J. Zhejiang Univ. Sci. B, 9: 713-720.
31. Gessesse, A., R. Hatti-Kaul, B.A. Gashe and B. Mattiasson, 2003. Novel alkaline proteases from alkaliphilic bacteria grown on chicken feather. Enzyme Microb. Technol., 32: 519-524.
32. Lin, X., D.W. Kelemen, E.S. Miller and J.C.H. Shih, 1995. Nucleotide sequence and expression of *kerA*, the gene encoding a keratinolytic protease of *Bacillus licheniformis* PWD-1. Applied Environ. Microbiol., 61: 1469-1474.
33. Kojima, M., M. Kanai, M. Tominaga, S. Kitazume, A. Inoube and K. Horikoshi, 2006. Isolation and characterization of a feather-degrading enzyme from *Bacillus pseudofirmus* FA30-01. Extremophiles, 10: 229-235.
34. Sivakumar, T., T. Shankar, V. Thangapandian and V. Ramasubramanian, 2013. Optimization of cultural condition for keratinase production using *Bacillus cereus* TS1. Insight Microbiol., 3: 1-8.