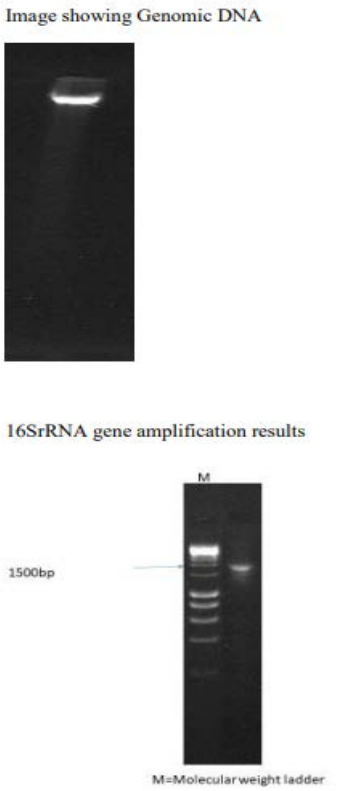

Fig. 3a: Image showing genomic DNA of *Bacillus safensis* LRF3X.

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CACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCG
GTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAG
GCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGG
AAACTGGGGAAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTG
AAATGCGTAGAGATGT

Fig.3b: Gene sequence of *Bacillus safensis* LRF3X

Optimization of Culture Parameters for Keratinase Production by Bacillus safensis LRF3X

Effect of pH on keratinase production.

The effect of pH on keratinase productionby*Bacillus safensis* LRF3Xis seen in Fig. 4. Keratinase production by this bacteria increased at pH 7(33.3U/ml) after which the enzyme production declined with increase in pH. Optimum keratinase production was obtained at pH of 7.0 whereas the least was obtained at pH 3.0 (12.0U/ml). The increase in keratinase production at pH 7 could be that the accessibility of the raw feathers for degradation by the bacteria was greater at that pH. This result is an indication that keratinase production by *Bacillus safensis* LRF3Xis more in alkaline environment than in the acidic range and extreme alkaline environments. The alkaline environment has been reported to make feathers more accessible for degradation by keratinase from microorganisms. The report is in agreement with the results by (Revathi *et al.,* 2013; Kanchana *et al.* 2013*;* Sahoo *et al.,* 2010) with maximum enzyme production at pH 7 with alkaliphilic bacteria.

*Effect of temperature on keratinase production***:**

The effect of temperature for keratinase production such as 20°C, 30°C, 40°C, 50°C and 60°C was studied. The highest keratinase production by *Bacillus safensis* LRF3X was observed at 30°C (36.6 U/ml) as seen in fig.5. Minimal production was seen at 60^{o}C with an activity of about 12.3U/ml. This is an indication that *Bacillus safensis* LRF3X is a mesophilic bacterium. This results is in line with previous reports which shows *Bacillus* sp. (Sandeep *et al.,* 2017; Suntornsuk *et al*., 2003), *Lysobacter* sp. (Allpress *et al*., 2002), and *Stenotrophomonas* sp. D-1 (Williams *et al*., 1990), showed optimum temperature for growth and keratinolytic enzyme production ranging from 20 °C to 40 °C.

Effect of substrate concentrations

In this study, the effect of substrate concentration on the production of keratinase by *Bacillus safensis* LRF3X was determined. The substrate used was feathers. Substrate concentration range of 0.1, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, to 3.5% was studied as shown in (Fig. 6). According to the results, the highest keratinase production was obtained at 1% feather concentration (38.6U/ml) while substrate concentration above 1% i.e. from 1.5% - 3.5% showed a decreasing trend in keratinase production. This decreasing trend in keratinase production above 1% feather concentration is due to substrate repression on keratinase production. A Higher substrate concentration may also have increased the medium viscosity which can result in oxygen limitation for the bacterial growth. The results of this study were found in accordance with the previous studies for keratinase production. Cheng *et al*. (1995) also reported that 1% feather powder gave the highest keratinase activity for *B. licheniformis* PWD-1. Brandelli and Riffle (2005) also indicated that the production of keratinase by *Chryseobacterium* sp. was repressed by high quantity of keratin substrate in the production medium.

Effect of incubation period

The effect of incubation period for keratinase production from *Bacillus safensis* LRF3X was studied. It was observed that the maximum enzyme production of was attained at 192 h of incubation period as shown in fig 7. Incubation beyond the optimum time showed a rapid decline in the enzyme yield, as compared to maximum (38.3 U/ml) at 192hrs. An increase in the enzyme production from 0 h towards 192 h was observed. After 192 h of incubation a decrease in the trend of enzyme activity at 216 h was observed with minimum (32.8 U/ml). The optimum incubation period in this study was found similar to the results of

(Lin and Yin, 2010) who observed maximum keratinase production after 72 h. Saibabu *et al*. (2013) reported maximum extracellular alkaline keratinase production after 72 h when *B. megaterium* was grown in the feather meal medium.

Effect of inorganic and organic carbon sources on keratinase production:

Result of effect of different carbon sources on enzyme production by *Bacillus safensis* LRF3X is shown Fig.8. In this study, highest keratinase production was observed with galactose (33.6U/ml) followed by fructose (31.0U/ml) while the least was noticed when glucose (14.0U/ml) was used. However, the bacterial isolate was able to utilize the different inorganic carbon sources (feathers, Hooves and hair in decreasing order) for keratinase production. From the results, feathers gave a higher activity of (38.85U/ml). The crude keratinase from *Pseudomonas stutzeri* K4 has been reported to show high substrate specificity for keratin and chicken feathers, whereas low specificity for collagen, casein and hair (Chaturvedi *et al., 2014*).Other researchers working with keratinase have reported various carbon sources optima. Sivakumar *et al*. (2012) reported optimum production with mannitol and starch, respectively for *Bacillus* sp. and *B. thuringiensis*. Ramnani and Gupta (2006) reported that in optimization of medium for keratinase production by *Bacillus subtilis* RGI, glucose and peptone were found to have positive effects. Usually glucose has negative effects on microbial proteinase (keratinase included) production. For example, the keratinase produced by strain *Aspergillus fumigatus* (Santos *et al*., 1996). *Thermoactinomycetus candidus* and *Stenotrophomonas* sp. (Yamamura *et al*., 2002) were partially inhibited by glucose.

Effect of inorganic and organic nitrogen sources on keratinase production

Feather basal medium supplemented with 1% casein as additonal nitrogen showed maximum keratinase production of 24.2 U/ml by *Bacillus safensis* LRF3X (Fig. 10). This was closely followed by yeast extract and the least enzyme production was observed when sodium nitrate was used. Venkata *et al*. (2013) recorded maximum keratinase production with 0.1% yeast extract for *B. megaterium*, *Bacillus* sp., *B. licheniformis* KMBVP and *B. megaterium* while Sivakumar *et al*. (2012) reported optimum production with peptone for *B. thuringiensis*. Kainoor and Naik (2010) reported that in the presence of two different substrates, one which is structurally more compact and resistant (feather) and other which is more accessible and small protein supplement, the bacteria may preferentially use the latter. This would explain the comparative lower enhancement of keratinase activity measured in the presence of external nitrogen sources. Effect of organic nitrogen sources were also screened(fig.11). From the study, maximum keratinase production was seen in the medium supplemented with Defatted nut (37.5U/ml) followed by Bambara nut powder (36.8U/ml) and the least keratinase production was Soybean powder. Lakshmi *et al*.(2013) reported that among the organic nitrogen sources tested, soybean meal was found to be the best nitrogen source for *Bacillussubtilis* (212 KU/mL), whereas the maximum yield for *Bacillus cereus* (207 KU/mL) was obtained with groundnut cake supplementation. Supplementation of groundnut cake was also observed to enhance the production of the alkaline protease as well as keratinase in *Bacillus* sp. (Wang and Shih, 1999).

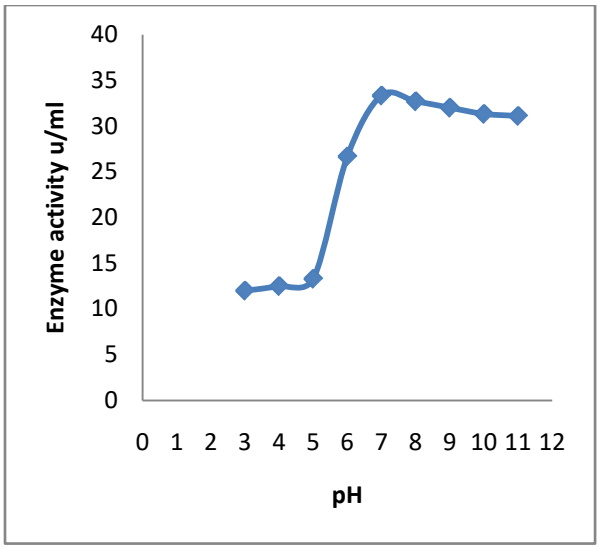

Fig. 4: Effect of different pH on keratinase production by *B. safensis* LRF3X

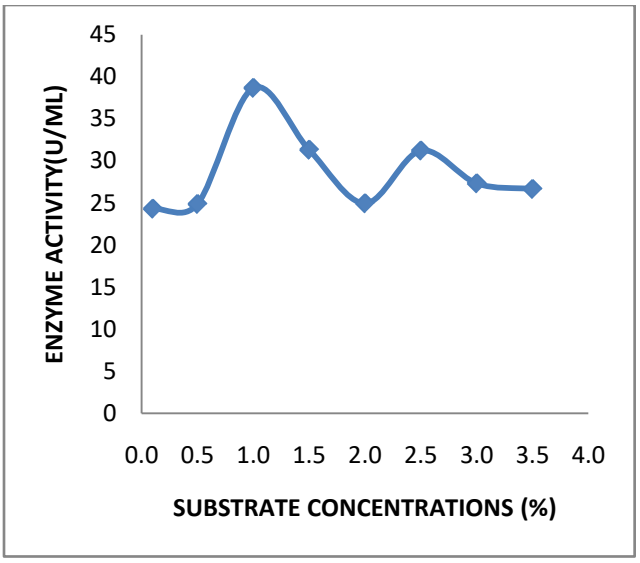

Fig.6: Effect of different substrate concentrations on keratinase production by *B. safensis* LRF3X

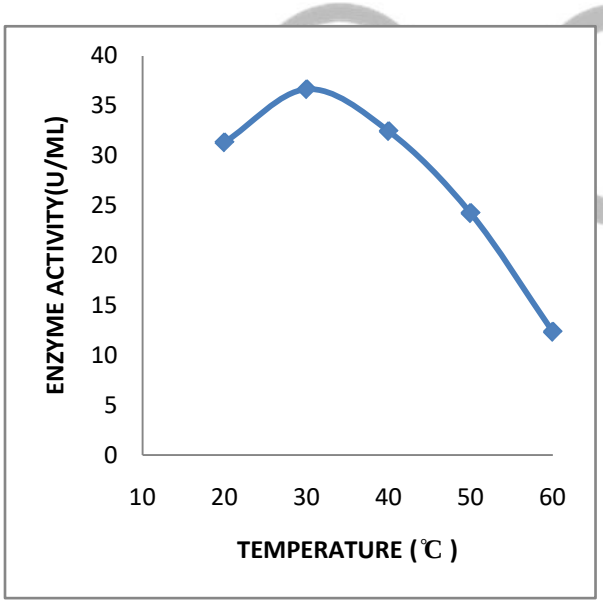

Fig.5: Effect of different temperatures on keratinase activity by *B.safensis*

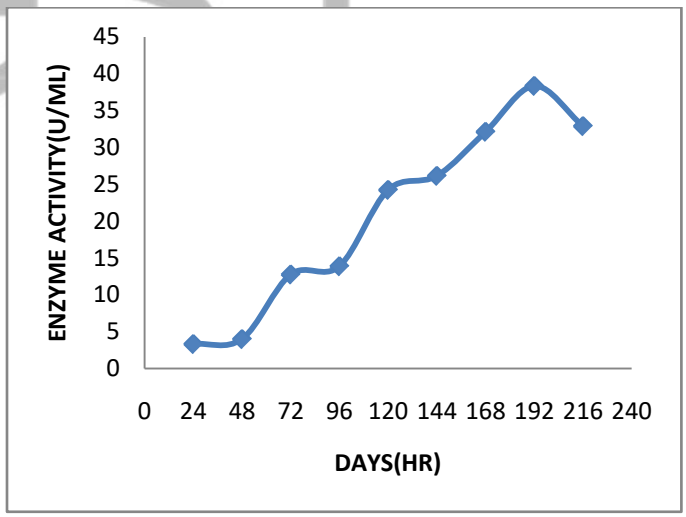

Fig. 7: **Effect of different incubation periods on keratinase production by *B. safensis* LRF3X**

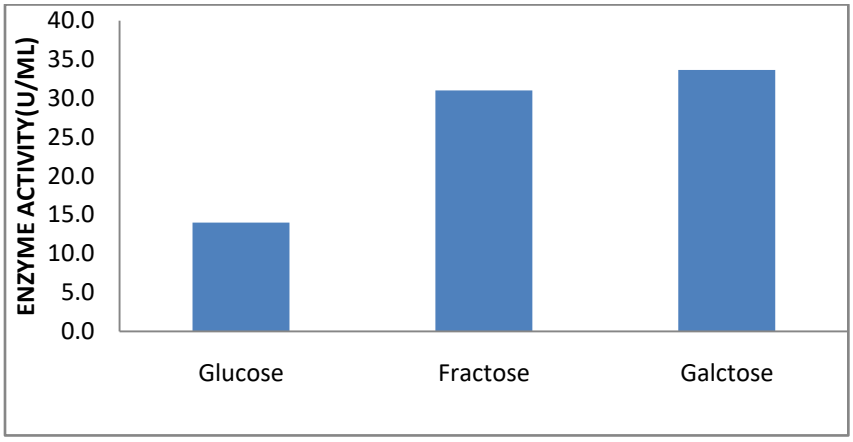

FIG.8: Effect of inorganic carbon sources on keratinase activity by *B.safensis*

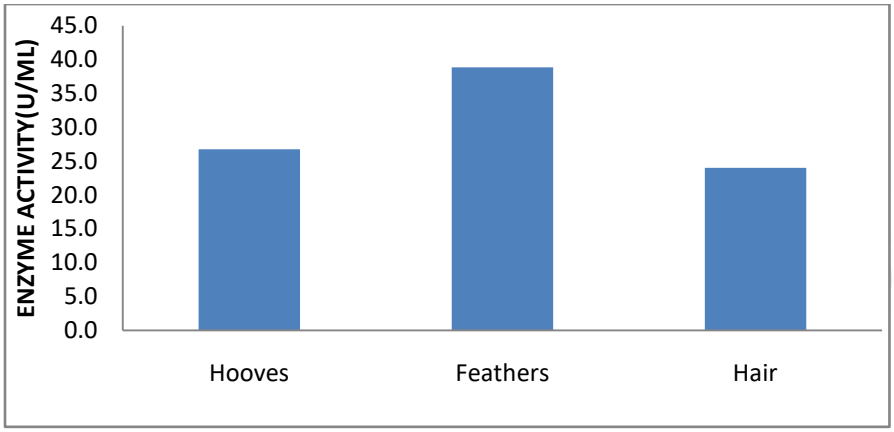

FIG.9: Effect of organic carbon sources on keratinase activity by *B.safensis*

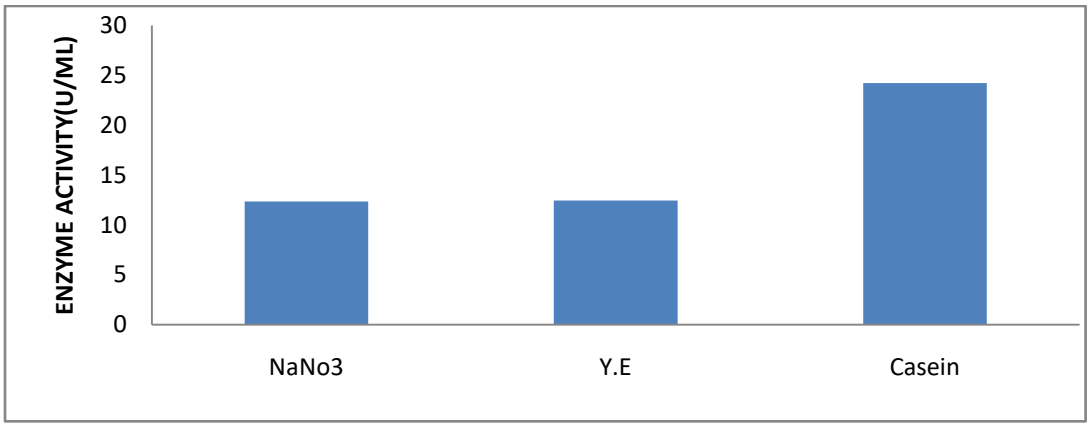

Fig.10: Effect of Inorganic nitrogen sources on keratinase production by *B. safensis* LRF3X

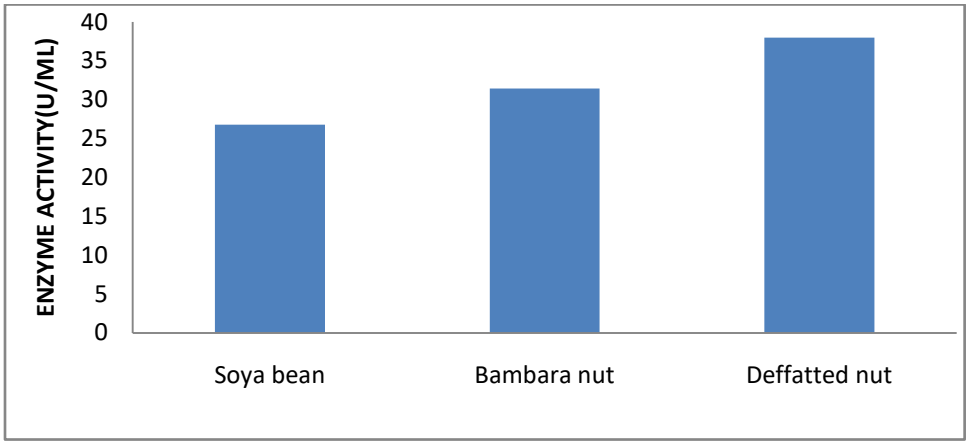

Fig.11: Effect of organic nitrogen sources on keratinase production by *B. safensis* LRF3X

Conclusion

The results obtained from this study showed that keratinase produced from *Bacillus safensis* LRF3Xwhen adequately optimized with the above optimum parameters could be very useful in decomposition of keratin-wastes (feather), recycling to poultry feeds and could also find applications in leather, pharmaceutical and cosmetics industries.

REFERENCES

Agbaje, L., Isiaka, A., Adelerea, B. and Evariste, B. G. (2015). *Bacillus safensis* LAU 13: a new source of keratinase and its multi-functional biocatalytic applications. *Biotechnology & Biotechnological Equipment*. 29(1): 5463.

Agrahari, S. (2013). Production of extracellular keratinase enzymes from *Bacillus pumilis* SN3 isolated from soil sample of Ghazipur poultry waste site. *Int J Sustainable Dev Green Econ*. 2:26.

Agrahari, S. and Wadhwa, N. (2010). Degradation of chicken feather a poultry waste product by keratinolytic bacteria isolated from dumping site at Ghazipur poultry processing plant, *Int. J. Poult. Sci.*, 9**(5),** 482-489.

Allpress, J.D., Mountain, G. and Gowland, P.C. (2002). Production, purification and characterization of an extracellular keratinase from *Lysobacter* NCIMB 9497. *Lett. Applied Microbiol.*, 34: 337-342.

Anbu, P., Gopinath, S.C.B., Hilda, A., Lakshmipriya, T. and Annadurai, G. (2005). Purification of keratinase from poultry farm isolate *Scopulariopsis brevicaulis* and statistical optimization of enzyme activity. *Enzym Microb Technol*. 36:639647.

Brandelli, A. and Riffel, A. (2005) Production of an extracellular keratinase from *Chryseobacterium* sp. growing on raw feathers, *Electr. J. Biotechnol.*, 8**(1),** 35-42.

Chaturvedi, V., Bhange, K., Bhatt, R. and Verma, P. (2014). Production of keratinases using chicken feathers as substrate by a

novel multifunctional strain of *Pseudomonas stutzeri* and its dehairing application. *Biocatalysis Agric Biotechnol*. 3:176174.

Cheng, S.W., Hu, H.M. and Shen, S.W. (1995). Production and characterization of keratinase of a feather degrading *Bacillus licheniformis* PWD-l. *Biosci. Biotechnol. Biochem*., 59: 2239-2243.

Dipak, K. S., Suman, K.H. and Arpan, D.K. (2015). Keratinase production by*B. weihenstephanensis* PKD5 in solid-state fermentation and its milk clotting potential. *Indian J. of Biotech.* 14:200-207.

Gupta, R. and Ramnani, P.(2006). Microbial keratinases and their prospective applications: an overview, *Appl. Microbiol. Biotechnol*., 70: 21-33.

Harison, M. and Sandeep, S. (2014). Degradation of keratinous waste products by keratinolytic bacteria isolated from soil. *International Journal of Engineering and Computer Science*. 3(**8**):7588-7595.

Jeevana, P. Lakshmi, C.H., Kumari, M. Chitturi, and. Lakshmi, V. V. (2013). Efficient Degradation of Feather by Keratinase Producing *Bacillus* sp. *International Journal of Microbiology*. Volume 7 pages http://dx.doi.org/10.1155/20

Kanchana, R. and Mesta, D. (2013). Native feather degradation by a keratinophilic fungus. *Int J ChemTech Res*. 5 (6):29472954.

Kainoor, P.S. and Naik, G.R. (2010). Production and charecterization of feather degrading keratinase from *Bacillus* sp. JB 99, *Indian J. Biotechnol.*,**9**, 384-390

Lateef, A., Oloke, J.K., Gueguim, E.B., Sobowale, B.O., Ajao, S.O. and Bello, B.Y. (2010). Keratinolytic activities of a new feather degrading isolate of *Bacillus cereus* LAU08 isolated from Nigerian soil. *International Journal of Biodeterioration and Biodegradation*. 64:162–165.

Lin, H.H. and Yin, L.J., (2010). Feather meal and rice husk enhanced keratinase production by *Bacillus licheniformis* YJ4 and characterization of produced keratinases, *J. Mar. Sci. Technol.*, 18(**3**), 458-465.

Mohamed, A.H., Bakry, M.H., Amro, A.A. and Serour, A.E. (2015). Production and characterization of keratinolytic protease from new wool-degrading *Bacillus* species isolated from Egyptian ecosystem. *BioMed Research International*. 14. Article ID: 175012.

Mabrouk, M.E.M. (2008). Feather degradation by a new keratinolytic *Streptomyces* sp. MS-2. *World J Microbiol Biotechnol*. 24, 2331–2338.

McKittrick, J., Chen, P.Y., Bodde, S.G., Yang, W., Novitskaya, E.E. and Meyers, M.A. (2012). The structure, functions, and mechanical properties of keratin. *JOM*. 64:449–468.

Revathi, K., Shaifali, S., Mohd, A.K. and Suneetha, V. (2013). A potential strain of keratinolytic bacteria VIT RSAS2 from Katpadi and its pharmacological benefits. *Int J Pharm Sci Rev.* 20:8992.

Riffel, A., Ortolan, S. and Brandelli, A. (2003). Dehairing activity of extracellular proteases produced by keratinolytic bacteria. *J. Chem. Technol. Biotechnol*., 78: 855-859.

Santos, R.M.D.B., Firmino, A.A., Desa, C. and Felix, C.R.(1996). Keratinolytic activity of *Aspergillus fumigates*. *World J.Microbiol*, 33: 364-370.

Saibabu, F.N. and Sunil, S.M. (2013). Isolation, partial purification and

characterization of keratinase from *Bacillus megaterium. International Research Journal of Biological Sciences.* 2(2):13-20.

Sandeep, S., Harison, M.G., Ebenezer, J., Rubina, L. and Ramteke, P.W. (2017). Optimization of fermentative production of keratinase by *B. subtilis* SI in submerged state fermentation using feather wastes. *Int. curr. Microbial.App. Sci.* 6(2):1499-1510.

Sangali, S. and Brandelli, A. (2000). Feather keratin hydrolysis by a *Vibrio* sp. strain kri. *J. Applied Microbiol.*, 89: 735-743.

Sahoo, D.K., Das, A., Thatoi, H., Mondal, K.C. and Mohapatra, P.K.D. (2012). Keratinase production and biodegradation of whole chicken feather keratin by a newly isolated bacterium under submerged fermentation. *Appl Biochem Biotechnol*. 167:10401051.

Sivakumar T, Shankar T, Vijayabaskar P. and Ramasubramaman, V. (2012). Optimization for keratinase enzymes production using *Bacillus thuringiensis*. *Academic Journal of Plant of Sciences*.5(3):102-109.

Suntornsuk, W., Tongjun, J., Onnim, P., Oyama, H., Ratanakanokchai, K., Kusamran,T. and Oda, K. (2004). Purification and characterisation of keratinase from a thermotolerant feather-degrading bacterium, *World J. Microbiol. Biotechnol.,***21**, 1111-1117.

Suntornsuk, W. and Suntornsuk, L. (2003). Feather degradation by *Bacillus* sp. FK 46 in submerged cultivation. *Bioresour. Technol*., 86: 239-243.

Sreenivasa, N., Gireesh, B.K. and Vidyasagar, G.M. (2013). Purification and characterization of keratinase from hair-degrading *Streptomyces albus. International Journal of Bioassays*. 2(3): 559-604.

Tamura, K., Strecher, G., Peterson, D., Filipska, A. and Kumar, S. (2013). MEGA6: Molecular evolutionary genetics analysis version 6.0. *Molecular Biology Evolution*. 30: 2725-2729.

Tiwary, E. and Gupta, R. (2013). Rapid conversion of chicken feather to feather meal using dimeric keratinase from *Bacillus licheniformis* ER-15. *J Bioprocess Biotechnol*. 2:123. doi: 10.4172/2155-9821.1000123. [30].

Tork, S.E., Shahein, Y.E., El-Hakim, A.E., Abdel-Aty, A.M. and Aly, M.M. (2013). Production and characterization of thermostable metallo-keratinase from newly isolated *Bacillus subtilis* NRC 3. *Int J Biol Macromol*. 55:169175.

Venkata, S., Francois, N.N. and Sunil, S.M. (2013). Isolation, partial purification and characterization of keratinase from *Bacillus megaterium. International Research Journal of Biological Sciences.*2(2):13-20.

Wang, J.J. and Shih, J.C.H. (1999). "Fermentation production of keratinase from Bacillus licheniformis PWD-1 and a recombinant B. subtilis FDB-29".*Journal of Industrial Microbiology and Biotechnology*. vol. 22, no. 6, pp. 608–616.

Williams, C. M., Richter, C. S., Mackenzie, J. R. and Jason, C. H. (1990). Isolation, identification and characterization of feather degrading bacterium. *Applied Env. Microbiol*.1:1509-1515. ` .

Yamamura, S., Ramachandra, S. and Yokoyama, K.(2002). Characterization of a New KeratinDegrading Isolated from Deer Fur Bacterium. *J. Biosci. Bioeng*. 93, 595–600.