



Production and optimization of keratinase enzyme by *Bacillus safensis* LRF3X isolated from feather dump sites.

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Abstract

This study investigated the isolation, identification and optimization conditions on keratinase production by *Bacillus safensis* LRF3X using raw feather. A total of twelve (12) bacterial isolates were isolated from different feather dumping sites. Out of the 12 isolates screened, only one isolate with the highest zone of inhibition on feather meal agar was selected. The isolate was morphologically and biochemically characterized based on Bergey's manual of determinative bacteriology as well as molecular characterization by sequencing 16S rRNA gene. In order to improve keratinase production by the selected isolate, culture medium and different cultural parameters were individually optimized. The following cultural parameters: pH, temperature, nitrogen sources (inorganic and organic), carbon sources (inorganic and organic), substrate concentrations and incubation period were evaluated for the production of keratinase by the isolate. From this study, the highest keratinase producing isolate, was found to be *Bacillus safensis* LRF3X. The highest enzyme production (38.33U/ml) was obtained at 192h after incubation. The optimum pH and temperature for the production of keratinase *Bacillus safensis* LRF3X were pH 7.0 and 30°C, respectively. The best carbon sources (organic and inorganic) were feathers and galactose at concentration of 1%, respectively. The best nitrogen sources (organic and inorganic) were defatted nut and casein at concentration of 1%, respectively. The highest activity (38.66U/ml) was observed at 1% feather concentration. The results obtained from this study showed that keratinase produced from *Bacillus safensis* LRF3X 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.

Key words: keratinase, *Bacillus safensis*, feathers, optimization.

Introduction

In order to meet the increasing demand of chicken, the number of poultry processing and slaughter houses are on the rise. As a result, lots of feathers are accumulated worldwide. These keratinous substances found in feathers are also structural component of skin, hair, horns, hooves, cloves, nails, beaks, reptilian osteoderm, etc (McKittrick *et al.*, 2012). Keratin renders animals more robust against both abiotic stress and biotic attacks. Since microbial degradation of keratin is not widespread in nature, keratin can serve as an efficient defense even against microbial attack. Keratin is truly recalcitrant. Unfortunately, these keratinous substances are not easily degradable so they are often dumped or burnt thus, creating potential environmental pollutions (Dipak *et al.*, 2015). This keratinous waste could have great potential as a source of protein and amino acids for animal feed. Because of the shortage of protein in feed, waste poultry feather are already added to feedstuff as a dietary protein supplement. However, the current processes using physical and chemical treatments to obtain feather meal are unfriendly to environment and also destroy amino acids, yielding a product with poor digestibility and variable nutrient quality (Mabrouk, 2008; Sangali and Brandelli, 2000).

Fortunately, keratinases from microorganisms and these keratinolytic microorganisms could solve these problems effectively when they are used to process these wastes to products. Keratinase is an extracellular enzyme used for biodegradation of keratin. Microbe produce keratinase in the presence of keratin. Many microorganisms like *Bacillus* spp., *Burkholdreia*, *Chryseobacterium*, *Pseudomonas*, *Microbacterium* spp. etc have ability to produce keratinase enzyme.

(Anbu *et al.*, 2004; Riffel *et al.*, 2003). This enzyme is proteolytic in nature. It is serine protease. Use of keratinase or keratinolytic microorganisms can be an alternative for recycling of keratinous waste to produce cheap and supplementary protein for poultry feeds as well as organic fertilizers, dehairing processes in leather industry and in clearing obstructions in sewage system during waste water treatment (Mohamed *et al.*, 2014). The aim of this study is to isolate Keratinase producing microorganisms, identify them and optimize the enzyme production.

Materials and Methods

Sample Collection

Soil samples of about 2-6cm deep were collected with sterile container from different feather dump sites in Enugu State, Nigeria and taken to the microbiology Lab.

Processing Of The Keratinolytic Substrates

Raw Feathers were used as the substrates. The raw feathers were washed extensively with water. The feather was oven dried at 70°C for 48 h. The dried feathers were milled using a manual grinder to fine powder which served as a sole source of carbon and nitrogen.

Isolation of Keratinase producing microorganisms

One gram of each soil sample was transferred into 9 ml sterile distilled water. The sample was serially diluted. Screening of the isolates was done by streaking cultures of the isolates on basal feather meal agar plates using the methods of Agrahari and Wadhwa, 2010 with a slight modification (g/l :NH₄Cl 0.5, NaCl 0.5, K₂HPO₄ 0.3, KH₂PO₄ 0.3, MgSO₄ 0.1, yeast extract 0.1, feather powder 10, agar 12, pH 7.5). The cultures were incubated for 2-5 days for colony formation. Keratin

degradation was indicated by the formation of clear zone of hydrolysis around the colonies. Strains which showed maximum zones of clearance were selected for further studies.

Degradation of Chicken Feather by Keratinase Producers

For studying the biodegradation of keratinous material, keratinous wastes (chicken feathers 1% w/v) were added to the feather meal medium (g/l :NH₄Cl 0.5, NaCl 0.5, K₂HPO₄ 0.3, KH₂PO₄ 0.3, MgSO₄ 0.1, yeast extract 0.1, pH 7.5) as a sole source of carbon and nitrogen. After inoculation of organism, flask was incubated at room temperature on rotary shaker at 160 rpm for 2-7days. The percentage of keratinous waste degradation was then determined (Harison and Singh, 2014).

Determination of Degree of Degradation (DD)

The residual feather was washed, dried and weighed to calculate DD by using following formular.

$$DD(\%) = \frac{(TF-RF) \times 100}{TF}$$

Where, TF is total feather and RF is residual feather.

Identification of keratinase producing bacteria

Morphological and a range of biochemical tests were performed in order to identify the isolate. The isolate was identified based on morphological, biochemical characteristics as described in the Bergey's manual of systematic bacteriology and by 16S rRNA gene sequencing.

Primers set used to amplify 16S rRNA sequence were **27f** (5'-AGA GTT TGA TCC TGG CTG AG-3') and **1492r** (5'-GGC TAC CTT GTT ACG ACT T-3') as forward and reverse primer-respectively in a PCR thermal cycler (iCycler 170-8740, USA). The thermal cycling program was initial denaturation at 95 °C for 5 min, followed by 30 cycles, denaturation at 95 °C for 30 s, annealing at 56 °C for 30 s, extension at 72 °C for 90 s and the final extension at 72 °C for 10 min. The amplified DNA was visualized by gel electrophoresis. The 16S rDNA sequence was analyzed and compared with other deposited sequences in the Genbank. Neighbor-joining phylogenetic tree was constructed based on the 16S rRNA sequences using MEGA6 (Tamura *et al.*, 2013).

Keratinase Enzyme production

The keratinase enzyme production was carried out in the basal medium by using shaker incubator. The composition of the medium was (g/l): feathers (10 g), NH₄Cl 0.5, NaCl 0.5, K₂HPO₄ 0.3, KH₂PO₄ 0.3, MgSo₄ 0.1, yeast extract 0.1, (Rajesh *et al.* 2014). Fermentation was carried out with 1% (v/v) inoculum at 30 °C for 7 days at 150 rpm. After incubation, the broth was centrifuged and the supernatant was used to assay for keratinase activity.

Determination of keratinase activity

One milliliter of 1% (keratin) feather powder was mixed with 0.2 ml of phosphate buffer (pH 8.0) and 0.5 ml of enzyme solution in test tubes. The solution was incubated for 30 min at 30°C. After incubation, the reaction was terminated by addition of 2 ml of 10% trichloroacetic acid (TCA). The untreated keratin (feather

powder) precipitate was removed by centrifugation at 10000 rpm for 10 min. One milliliter (1 ml) of the supernatant was mixed with 5 ml of 4.2% sodium carbonate (Na_2CO_3) and 0.5 ml of Folin Ciocalteu phenol reagent. The reaction mixture was precipitated by standing in ice for 15 min and insoluble precipitate was removed by centrifugation at 10000 rpm for 10 min. Absorbance of the supernatant was read at 660 nm. A control assay, without the enzyme in the reaction mixture was done and used as the blank in all spectrophotometric measurements. All assays were done in duplicate. One unit of keratinase activity was defined as the amount of enzyme that released one microgram of tyrosine per min under standard assay conditions (Sreenivasa *et al.*, 2013).

Optimization of cultural conditions for keratinase production

The production of keratinase by bacterial inoculums was studied by considering the medium compositions and cultural conditions. All the experiments were carried out in triplicate and the mean values were presented.

Effects of Incubation period on keratinase production

The effect of incubation period on keratinase production was examined by carrying out fermentation using feather meal medium ((g/l : NH_4Cl 0.5, NaCl 0.5, K_2HPO_4 0.3, KH_2PO_4 0.3, MgSO_4 0.1, yeast extract 0.1, and whole feather 1.0) up to 24h to 168h separately. Fermentation was carried out with 1% (v/v) inoculum at 30 °C for 7 days at 150 rpm. After incubation, the broth was centrifuged and the supernatant was used to assay for keratinase activity.

Effects of Temperature and pH on Keratinase Production

To determine the suitable temperature for enzyme production, the feather meal medium were incubated from 20 to 60 °C (20, 30,40,50,60 °C) temperature and for determination of optimum pH for keratinase production experiments were carried out from 3 to 11 (3, 4, 5, 6, 7, 8, 9, 10, 11) separately. Fermentation was carried out with 1% (v/v) inoculum at 30 °C for 7 days at 160 rpm.

Effects of organic and inorganic carbon sources on keratinase production

Various carbon sources such as hair, feathers, hooves (organic sources) and glucose, galactose, fructose (inorganic sources) were used (10 g/l) as supplements separately for the production of keratinase. Fermentation was carried out with 1% inoculum at 30 °C for 7 days at 160 rpm.

Effects of organic and inorganic nitrogen sources on keratinase production

The keratinase production by the isolated bacterium strain was also optimized by supplementing different organic and inorganic nitrogen sources individually. The organic nitrogen sources such as defatted nuts, bambara nut powder and soybeans powder were used as 1% concentration as well as yeast extract, NaNO_3 and casein, were used 1% concentration as inorganic nitrogen sources in the media. Fermentation was carried out with 1% (v/v) inoculum at 30 °C for 7 days at 160 rpm.

Effect of substrate concentrations on keratinase production

Feather basal medium was prepared with different substrate concentrations (%): 0.1, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, and 3.5. Fermentation was carried out with 1% (v/v)

inoculum at 30 °C for 7 days at 160 rpm. After incubation, the broth was centrifuged and the supernatant was used to assay for keratinase activity.

Results and Discussion

Isolation, Screening and Identification of Keratinase Producing Bacteria.

A total of 12 different bacteria isolates showed hydrolysis of keratin as seen in table 1. The percentage degradation of the feather was also calculated as shown in table 2. Isolate A8 which had the best hydrolysis as seen in fig.2 and 3 was selected and identified as *Bacillus safensis* LRF3X (fig.3) and table 3 using morphological, biochemical and molecular methods. There are previous reports on the isolation of keratinase producing strains of *B. subtilis*, *B. licheniformis*, *B. pumilus*, *B. cereus*, *B. halodurans* and *B. weihenstephanensis*, [Agrahari, 2013; Tiwary and Gupta, 2013; Tork *et al.*, 2013; Lateef *et*

al., 2010]. Agbaje *et al.* (2015) was the first to report *Bacillus safensis* as a keratinase producer

Determination of Degree of Degradation of Chicken Feather by Keratinase Producers

Among the 12 isolates, A5 isolate demonstrated highest feather degrading activity (100%) after 6 days incubation where degradation of all feather barbules and almost all feather rachises were observed (Table-2). This result is in line with reports of Agbaje *et al.*, (2015). There are reports of complete or partial degradation of chicken feathers by bacteria in the range of 4 to 10 days (Agrahari, 2013; Tiwary and Gupta, 2013; Tork *et al.*, 2013; Lateef *et al.*, 2010). These results were an indication that this bacterium could be useful in the biotechnological management of poultry feathers through efficient biodegradation.

Table 1: Table showing measurements of hydrolysis on Feather Meal Agar.

s/n	Isolates	Clear zone of hydrolysis (mm)
A1	<i>Bacillus licheniformis</i>	12
A2	<i>Bacillus cereus</i>	18
A3	<i>Pseudomonas sp.</i>	10
A4	<i>Bacillus cereus</i>	16
A5	<i>Bacillus safensis</i>	24
A6	<i>Micrococcus sp.</i>	8
A7	<i>Bacillus subtilis</i>	12
A8	<i>Bacillus licheniformis</i>	16
A9	<i>Bacillus cereus</i>	19
A10	<i>Micrococcus sp.</i>	10
A11	<i>Bacillus cereus</i>	20
A12	<i>Bacillus subtilis</i>	10

Table 2: Percentage degradation of keratin by isolates.

s/n	Isolates	Total feather (g)	Residual feather(g)	% degradation
A1	<i>Bacillus licheniformis</i>	1.0	0.4	60
A2	<i>Bacillus cereus</i>	1.0	0.2	80
A3	<i>Pseudomonas sp.</i>	1.0	0.5	50
A4	<i>Bacillus cereus</i>	1.0	0.4	60
A5	<i>Bacillus safensis</i>	1.0	0.0	100
A6	<i>Micrococcus sp.</i>	1.0	0.6	40
A7	<i>Bacillus subtilis</i>	1.0	0.5	50
A8	<i>Bacillus licheniformis</i>	1.0	0.3	70
A9	<i>Bacillus cereus</i>	1.0	0.2	80
A10	<i>Micrococcus sp.</i>	1.0	0.5	50
A11	<i>Bacillus cereus</i>	1.0	0.15	85
A12	<i>Bacillus subtilis</i>	1.0	0.6	40

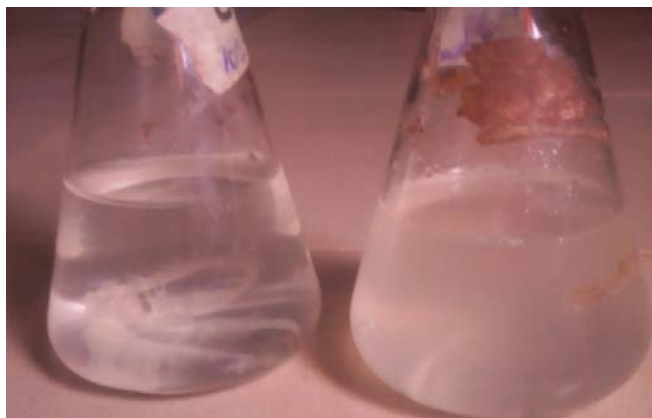


Fig.1: Image showing complete degradation of feathers by *B. safensis*LRF3X(A5) in feather meal broth



Figure 2: Image showing *Bacillus safensis* LRF3X(A5) hydrolysis on Feather meal agar

Image showing Genomic DNA



16SrRNA gene amplification results

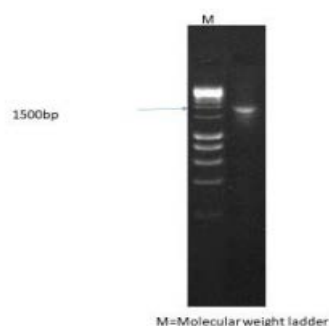


Fig. 3a: Image showing genomic DNA of *Bacillus safensis* LRF3X.

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GGGGTTAGGTATATGCTTGCCGGGCGCGGAGGGCGGGAGCTTGCTCTTTATAGTT
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AAACTGGGGAAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTG
AAATGCGTAGAGATGT
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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 production by *Bacillus safensis* LRF3X is 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* LRF3X is 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°C with an activity of about 12.3U/ml. This is an indication that *Bacillus safensis* LRF3X is a mesophilic bacterium. This result 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 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). *Thermoactinomyces 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 additional 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 *Bacillus subtilis* (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).

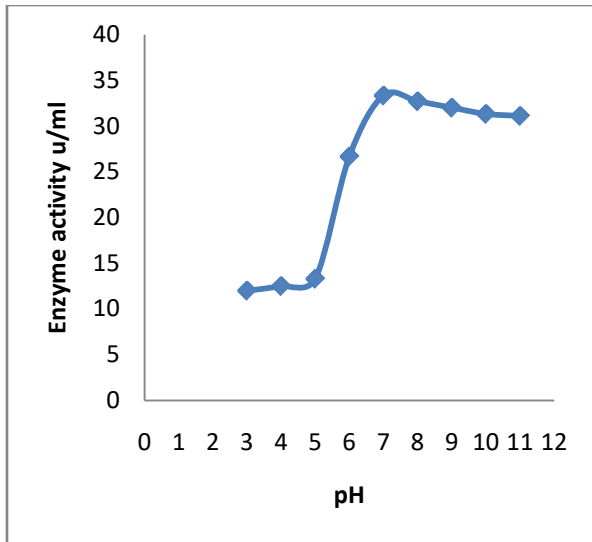


Fig. 4: Effect of different pH on keratinase production by *B. safensis* LRF3X

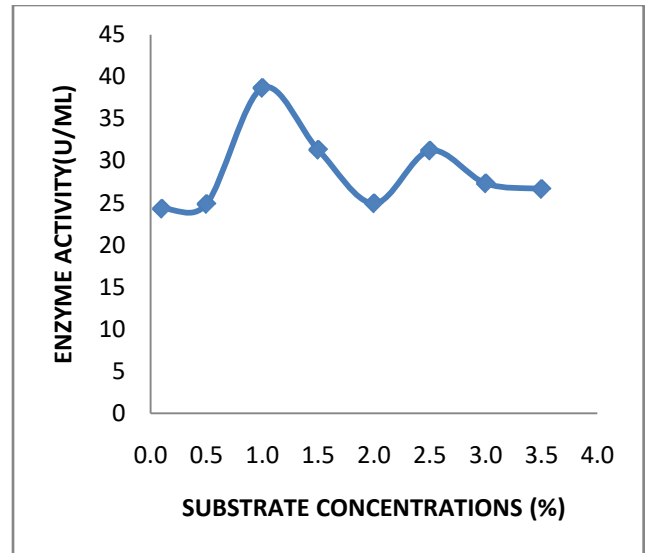


Fig.6: Effect of different substrate concentrations on keratinase production by *B. safensis* LRF3X

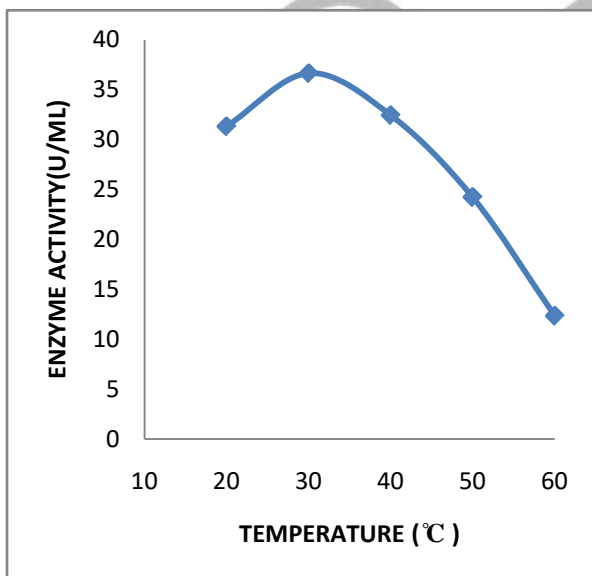


Fig.5: Effect of different temperatures on keratinase activity by *B. safensis*

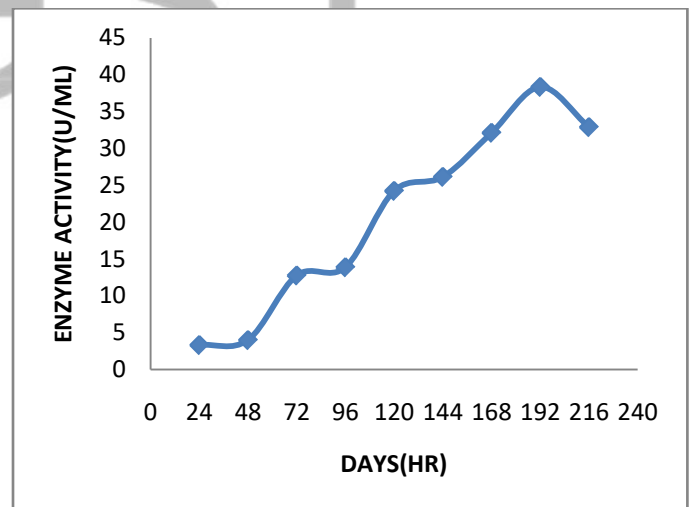


Fig. 7: Effect of different incubation periods on keratinase production by *B. safensis* LRF3X

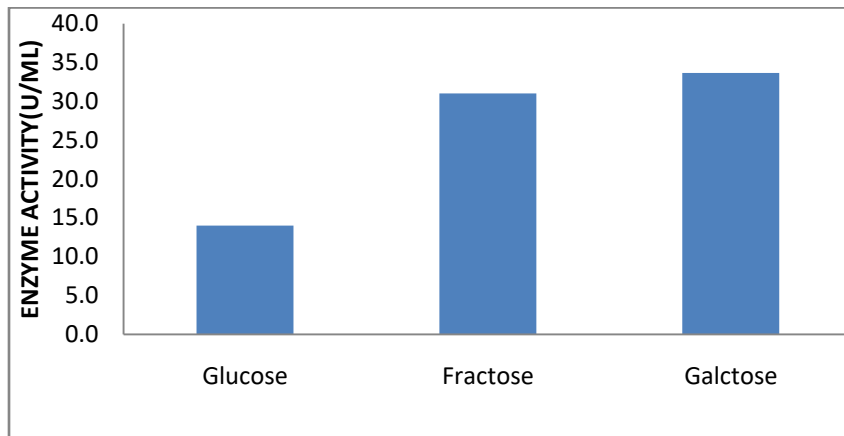


FIG.8: Effect of inorganic carbon sources on keratinase activity by *B.safensis*

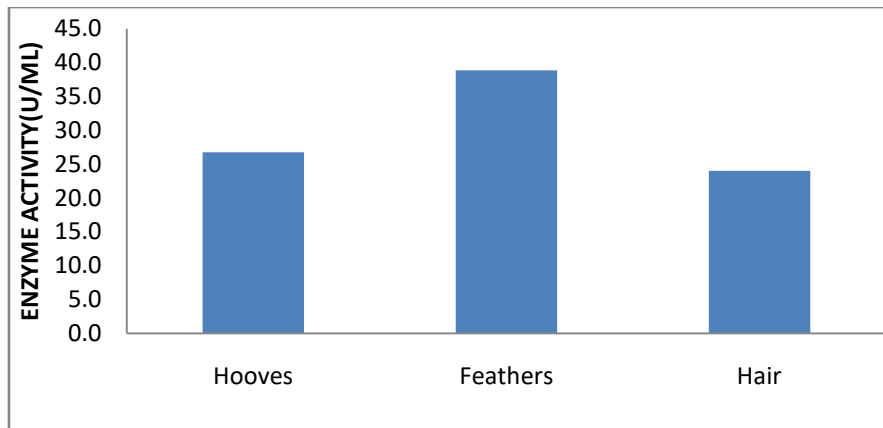


FIG.9: Effect of organic carbon sources on keratinase activity by *B.safensis*

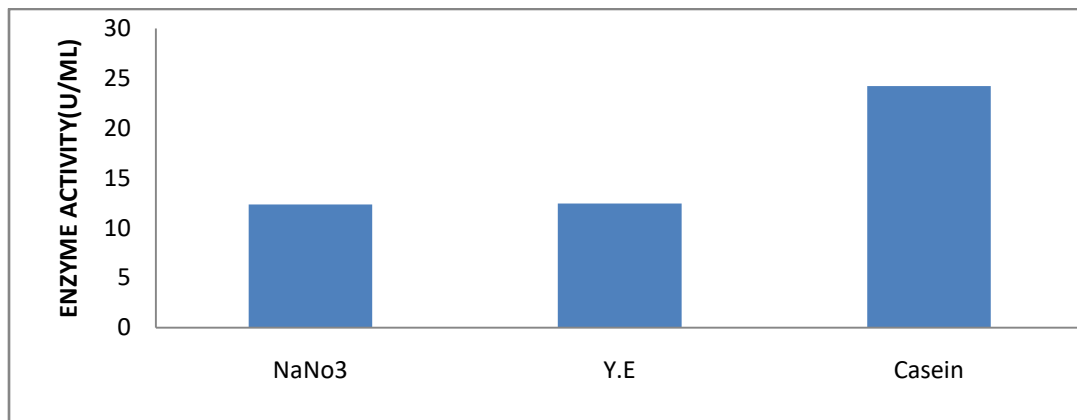


Fig.10: Effect of Inorganic nitrogen sources on keratinase production by *B. safensis* LRF3X

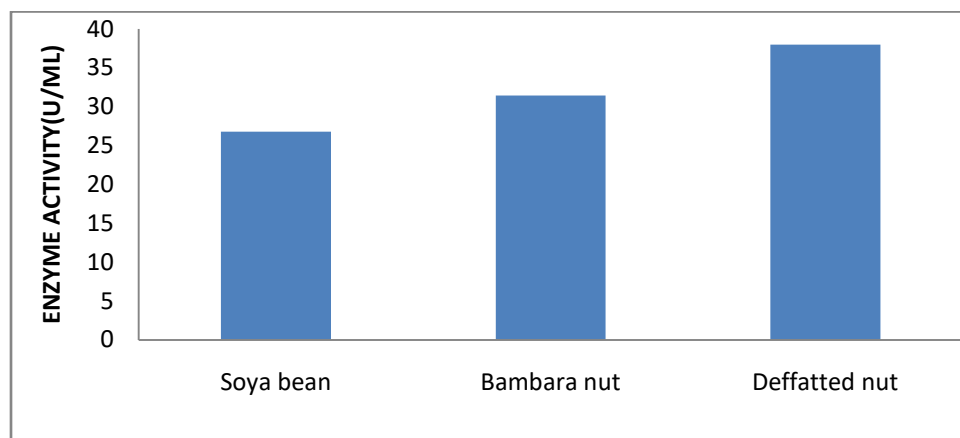


Fig.11: Effect of organic nitrogen sources on keratinase production by *B. safensis* LRF3X

Conclusion

The results obtained from this study showed that keratinase produced from *Bacillus safensis* LRF3X when 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., Lakshmi priya, 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-1. *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 keratin 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., Filipaska, 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.