Journal of Applied Life Sciences International

11(3): 1-9, 2017; Article no.JALSI.33165
ISSN: 2394-1103

Purification and Some Properties of Keratinase from *Bacillus licheniformis* Strain NBRC 14206

Francis Sopuruchukwu Ire[1*] and Amarachi Chisom Onyenama[1]

[1]Department of Microbiology, Faculty of Science, University of Port Harcourt, P.M.B.5323, Choba, Port Harcourt, Nigeria.

Authors' contributions

This work was carried out in collaboration between both authors. Author FSI designed the study and performed the statistical analysis, author ACO wrote the protocol, managed the analyses of the study and wrote the first draft of the manuscript. Author FSI managed the literature searches and wrote the final manuscript. Both authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JALSI/2017/33165
Editor(s):
(1) Purnachandra Nagaraju Ganji, Department of Hematology and Medical Oncology, Emory University School of Medicine, USA.
(2) Vasil Simeonov, Laboratory of Chemometrics and Environmetrics, University of Sofia "St. Kliment Okhridski", Bulgaria.
Reviewers:
(1) Muhammad Irfan, University of Sargodha, Sargodha, Pakistan.
(2) Alya Sellami Kamoun, University of Sfax, Tunisia.
(3) Ionica Mihaela Iancu, Banat University of Agronomical Sciences and Veterinary Medicine Timisoara, Romania.
Complete Peer review History: http://www.sciencedomain.org/review-history/18943

Original Research Article

Received 31st March 2017
Accepted 28th April 2017
Published 6th May 2017

ABSTRACT

Keratinases are extracellular enzyme used for the biodegradation of keratin and hydrolyze both native and denatured keratin. They have potential roles in biotechnological processes involving keratin containing wastes from poultry and leather industries. This study investigated the purification and characterization of a keratinase from *Bacillus licheniformis* strain. The enzyme was purified using ammonium sulphate precipitation, carboxyl methyl cellulose and gel filtration on Sephadex G75. The purified keratinase was characterized by assessing the influence of various physicochemical parameters (temperature, pH, metal ions, inhibitors and substrate concentrations) on the activity and stability of the enzyme. The crude enzyme was purified using three purification steps (ammonium sulphate, carboxyl methyl cellulose and Sephadex G75 filtration) with 10-fold purification and 3.46% yield. The result obtained revealed that the optimum pH of activity and stability were at pH 9.0 while optimum temperature was 40ºC. The purified keratinase was stable at 40ºC. The enzyme activity was slightly stimulated by Ca^{2+}, Zn^{2+} and Fe^{2+} while EDTA had the highest stimulatory effect on the purified keratinase. The enzyme was strongly inhibited by H_20_2, PMSF and Hg in decreasing order suggesting that it belongs to the family of serine proteases.

**Corresponding author: E-mail: frankire4@yahoo.com, francis.ire@uniport.edu.ng;*

Results obtained from this study suggest the keratinase produced from *B. licheniformis* strain as promising candidate in enzymatic feather degradation and could find application in leather, pharmaceutical and cosmetics industries.

Keywords: Bacillus licheniformis; chicken feather; Keratinase; purification; characterization.

1. INTRODUCTION

Poultry farms have been known to produce huge quantities of feather waste which may pose serious disposal and environmental pollution challenges due to poor waste management system [1]. The global feather waste from the poultry processing industry has been reported to reach 8.5 million tons annually [2]. Feathers consist of β-keratin rich protein and are highly resistant to hydrolysis [3]. Also, other keratinous proteins like hair, nail, wools, horn etc which contain α-keratin are insoluble and highly resistant to action of weak acids, alkalis and organic solvents or hydrolysis by common proteolytic enzymes due to its extensive cross-linking by disulfide bonds, hydrogen bonding, and hydrophobic interaction [4]. Keratinous wastes represent a source of valuable proteins and amino acids and could find application as a fodder additive for animals or source of nitrogen [5].

Keratinases have potential roles in biotechnological processes involving keratin containing wastes from poultry and leather industries. Keratinolytic enzymes have wide range potential industrial applications in food and feed in leather and fertilizer industries, in production of biohydrogen, in hydrolysis of prion proteins, in medicine and cosmetics for drug delivery, for silver recovery from X-ray film and also as detergent additives [3,5,6]. In the industrial production scale, keratinases with high feather degradation ability have been reported mainly from *Bacillus* strains [2,3,5] and other microorganisms [3,7,8]. We reported earlier the secretion and optimization of keratinase production by *B. lichenformis* isolated from feather dumping local sites [9]. In this present study, we report the purification and characterization of keratinase from *B. lichenformis*.

2. MATERIALS AND METHODS

2.1 Source of Inoculum

Soil samples from feather - dumping site was used for the study. The soil samples used for the study were collected from Rumuodumanya market feather dumping site, Sunny poultry farm, Port Harcourt, and Mile 3 market feather dumping site Port Harcourt, Rivers State, Nigeria. All the soil samples collected was collected from a depth of 2-6 cm using spade in a specimen bag and was transported to Industrial Microbiology laboratory of University of Port Harcourt, Rivers State Nigeria.

2.2 Processing of Keratinolytic Substrate

The substrate used for the study is raw feather. The raw substrate was collected from different locations and include (i) Rumuodomanya Market feather dumping site (ii) Sunny poultry farm (iii) Mile 3 market feather dumping site. The feather was prepared as previously described [9].

2.3 Isolation of Keratinolytic Bacteria

One gram of each soil sample was transferred into 9 ml sterile physiological water. The sample was serially diluted to 10^{-7} dilutions and 0.1 ml of each dilution was aseptically transferred to plates containing basal feather medium by spread plate techniques. The basal medium comprised of (g/L): NH_4Cl 5.0, NaCl 5.0, K_2HPO_4 3.0, KH_2PO_4 3.0, $MgCl_2.6H_2O$ 1.0, yeast extract 1.0, feather powder 10, agar 12, pH 9.0. Inoculated plates were incubated at 30ºC for 2-5 days.

2.4 Production of Crude Keratinase

The fermentation was performed in 250 ml Erlenmeyer flask containing 100 ml of the feather basal medium with 1% feather powder as the sole source of carbon. The pH of the feather basal medium was adjusted to 9.0 and sterilized at 121ºC for 15 min at 15 psi. One millilitre of isolate culture was inoculated into the medium and incubated at 40ºC. After incubation, 10 ml of the fermentation broth was aseptically collected from the fermentation flask and centrifuged at 10000 revolutions per minute. The clear supernatant was used for enzyme purification.

2.5 Determination of Enzyme Assay

One millimetre 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 minutes at 30ºC. 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 millimetre (1 ml) of the supernatant was mixed with 5 ml of 4.2% sodium carbonate (Na_2CO_3) and 0.5 ml of Folin Ciocalteau phenol reagent (Folin Ciocalteau phenol reagent was prepared by diluting 1ml with 3 ml of distilled water). 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 minute under standard assay conditions [10].

2.6 Purification of Keratinase

2.6.1 Ammonium sulphate precipitation

Crude enzyme was purified according to the method of Tork et al. [11] with some modifications. Crude extract obtained from the isolate was centrifuged at 5000 rpm at 4ºC for 15 min to increase clarity. After which solid crystals (40 g) of ammonium sulphate were added to the crude enzyme until it was 70% saturated. Mixtures were incubated at 40ºC for 15 min. The filtrates were obtained by centrifugation at 5000 rpm at 4ºC for 15 min. The obtained precipitates/pellets were suspended in a 10 ml of 0.2 M phosphate buffer (pH 5.5) and 0.2 M Tris-HCl buffer (pH 8.0) for keratinase.

2.6.2 Dialysis

The solution was subjected to a process of dialysis. The precipitate was desalted using 10 cm dialysis bags by rinsing against the same buffer. One end of the dialysis bag was tied tightly and the formed precipitate was placed into the bags, the other end of the dialysis bag was tightly tied to prevent any leakage and thereafter, the dialysis bags were suspended in beakers each containing the same buffer was made up to, so as to remove low molecular weight substance, ions and other impurities that interfere with the enzyme activity.

2.6.3 Carboxyl methyl cellulose and Sephadex G75 chromatography

The dialyzed enzyme fraction (50.5 ml) was further purified by carboxyl methyl cellulose and Sephadex G-75 chromatography (Sigma, USA). The column was packed or loaded to the height of 120 cm with internal diameter of 2.0 cm; samples were loaded onto the column and eluted with phosphate buffer pH 5.5. The flow rate was maintained at 0.5 ml/min and 15 fractions of 1 ml each were collected. The enzyme activity and protein content were determined for each separate fraction as earlier described. The active fractions were pooled and concentrated. Thereafter, the enzyme was applied on Sephadex G75 column (1.6 × 100 cm). The column was equilibrated with 0.2 M phosphate buffer pH 5.5 and elution was carried out at 4ºC at a flow rate of 0.5 ml/min. Fifteen fractions (3 ml) were collected. Protein content and enzyme were determined as earlier explained.

2.7 Characterization of Purified Keratinase

2.7.1 Effect of temperature on purified enzyme activity and stability

The effect of temperature of the purified enzyme was determined by incubating the purified enzyme solution in the reaction mixture containing 1 ml of 1% keratin in phosphate buffer pH 9. The enzyme mixture was incubated at varying range of temperature of 20 to 70ºC. After incubation, the purified enzyme mixture was cooled. The temperature stability of the enzyme was determined by incubating pure enzyme with 0.2 M phosphate buffer without keratin substrate. Thereafter, residual activity of the enzyme was assessed.

2.7.2 Effect of pH on purified enzyme activity and stability

The effect of pH on enzyme activity was determined by incubating pure enzyme in a reaction mixture with 1 ml of 1% keratin in 0.2 M phosphate buffer solution. The pH of enzyme mixture was adjusted to varying pH of 3 to 14 by drop wise addition of either 1 N NaOH or 1 N HCl and incubated at 40ºC. The pH stability of the enzyme was measured by incubating pure enzyme with 0.2 M phosphate buffer with varying pH of 3 to 14 without keratin and residual activity determined as described earlier.

2.7.3 Effect of metal ions on purified enzyme activity

The effect of various metal ions (Fe^{2+}, Al^{2+}, Zn^{2+}, Ca^{2+} and Hg^{2+}) on the purified keratinase activity were determined. The enzyme was pre-incubated with the individual metal salts (10 mM) at pH 9.0 in phosphate buffer at 40ºC for 10 min. Thereafter, the residual enzyme activities were quantified using 1 ml of 1% keratin as described earlier. Reaction mixture without any metal ion served as control.

2.7.4 Effect of metal ions on purified enzyme stability

The stability of the enzyme was measured by incubating 2.0 ml of pure enzyme with 0.2 M phosphate buffer pH 9.0 with various metal ions: Fe^{2+}, Al^{2+}, Zn^{2+}, Ca^{2+} and Hg^{2+} for 30 min. Thereafter, residual enzyme activity was determined as described above.

2.7.5 Effect of inhibitors on purified enzyme activity

The effect of inhibitors on enzyme activity was evaluated by using various inhibitors: EDTA, PMSF, SDS, KCN and H_2O_2. The purified enzyme was pre-incubated with 2 mM of each of the inhibitors at pH 9.0 in phosphate buffer at temperature of 40ºC for 10 min. The enzyme activity was then determined at 40ºC by using 1.0 ml of 1% keratin in 0.2 M phosphate buffer (pH 9.0) as substrate. The residual activities were estimated under standard assay conditions. Reaction mixture without any inhibitor served as control.

2.7.6 Effect of substrate concentration on purified enzyme activity

The effect of substrate concentration on enzyme activity was determined using various substrate concentration levels (%): 0.5, 1, 2, 3, 4 and 5. Each reaction mixture contained 1 ml of 1% keratin with 2.0 ml of phosphate buffer pH 9.0 and was inoculated with 2 ml of purified enzyme solution and incubated at 40ºC for 15 min.

2.8 Statistical Analysis

All experiments were carried out in triplicates. ANOVA were used for Statistical analysis and Statistical significance was at $\alpha = 0.05$.

3. RESULTS AND DISCUSSION

3.1 Purification of Crude Enzyme

Purification of keratinase from raw feather was carried out by subjecting the crude enzyme to ammonium sulphate precipitation, dialysis and two types of column Chromatography (Carboxyl methyl cellulose and Sephadex G75). Crude enzyme extract exhibited 29.2033 U/mg specific activity, ammonium sulphate precipitation had 29.1974 U/mg specific activity, carboxyl methyl cellulose gave 161.4407 U/mg activity while Sephadex G75 exhibited 297.5 U/mg specific activity. Overall purification fold achieved in this study is 10.19 with 3.46% yield and specific activity 297.5 U/mg. Similar purification protocols were reported by [11] for *Pseudomonas* sp.,

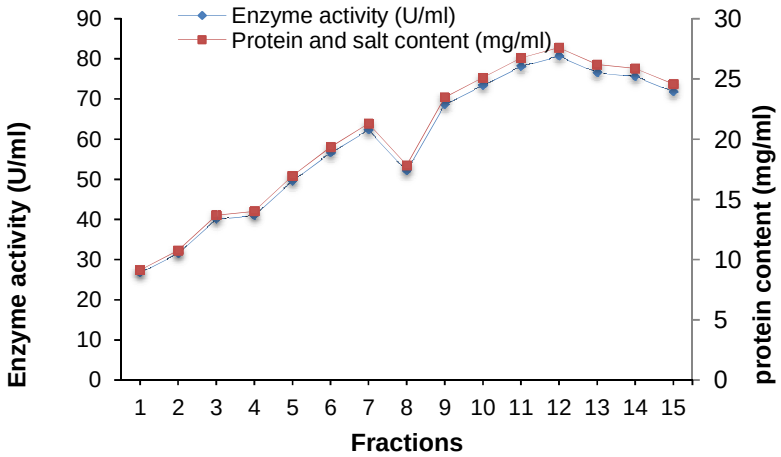

Fig. 1. Elution profile on Carboxyl methyl cellulose (CMC)

MS21. Nandini et al. [12] reported 113.45 fold purification with 3.8% yield and 2486.9 IU/mg specific activity with *Streptomyces minutiscleroticus* DNA. The elution pattern of carboxyl methyl cellulose (Fig. 1) and Sephadex G75 (Fig. 2) purification of keratinase from feather showed that the optimum enzyme was eluted in latter fraction in tube number 12. The peaks of enzyme activities were found along with the highest protein eluted samples in both carboxyl methyl cellulose and Sephadex G75 chromatography. Selvam et al. [13] recorded elution of peak enzyme activity and protein with early fractions.

3.2 Characterization of Purified Enzyme

3.2.1 Effect of temperature on purified enzyme activity and stability

The effect of temperature on purified enzyme activity and stability by *Bacillus licheniformis* indicated that maximum enzyme activity and stability occurred at temperature of 40ºC (Fig. 3). Maximum temperature activity of 40ºC is in consistent with the report of [5,10,14,15] for *Bacillus subtilis*, *Streptomyces albus,* and *Streptomyces* sp, respectively. Several researchers have reported various optimum temperatures for keratinase activity from various organisms. Prasad et al. [7] recorded optimum temperature of 30ºC with *Bacillus* sp., Thoomatti and Peramachi [16] reported 50ºC with *Aspergillus parasitics* while Rajput et al. [17] recorded 60ºC The thermo-stability decreased as temperatures increased above 40ºC and this observation is similar to the report of [15] for *Streptomyces* but differs with the findings of [18] who obtained an optimum thermo-stability of 55ºC for *Clostridium* sporogenes. Other researchers had reported temperature of 60ºC for keratinase from *Bacillus licheniformis* H62 and *Streptomyces gulbargensis* [19,20] whereas thermo-stability of 50-60ºC was observed with keratinase of *Bacillus licheniformis* k-19 [21]. The result showed that the purified keratinase had a better thermo tolerant and this could offer economic advantage for the bacteria strain and the enzyme, mainly in detergent industry [15] and feather waste utilization [14].

3.2.2 Effect of pH on purified enzyme activity and stability

Fig. 4 shows the effect of pH on the activity and stability of purified enzyme. The result indicated that the enzyme exhibited different levels of activity and stability at different pH values. Optimum activity and stability was obtained at pH 9.0. This result is consistent with previous findings for most feather degrading bacteria [20,21,22]. This finding is in contrast with some previous reports for other organisms for optima keratinase activity: pH 7 for *Streptomyces albus* [10], pH 6 for *Aspergillus parasiticus* [16], pH 6-10 for *Bacillus licheniformis* K-19 [21].

3.2.3 Effect of metal Ions on purified enzyme activity and stability

The effect of various metal ions on purified enzyme activity and stability is shown in Table 2.

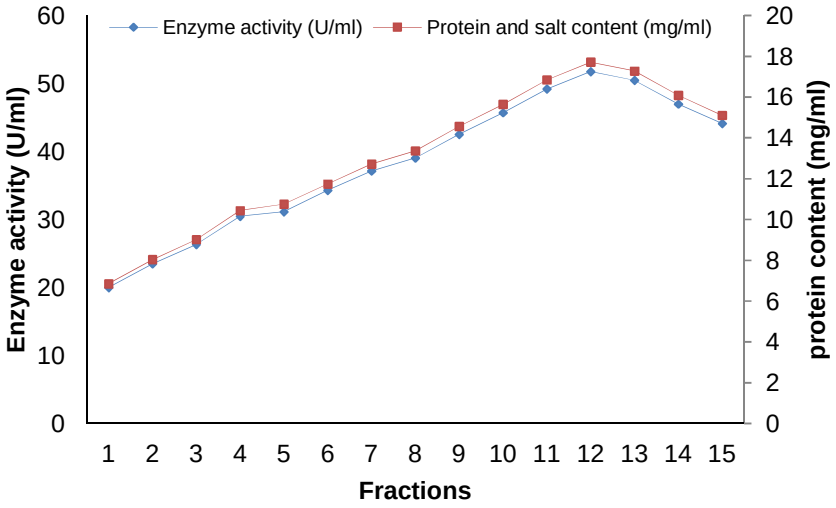

Fig. 2. Elution profile of gel filtration on sephadex G75

Table 1. Summary of the purification of keratinase from *Bacillus licheniformis*

S/No.	Purification step	Protein volume (ml)	Total protein (mg)	Total activity (U/mg)	Specific activity (U/mg)	Yield (%)	Fold
1	Crude extract	100	470.7	13746	29.203	100	1
2	Ammonium sulphate (70%)	71.3	170.55	4979.59	29.197	50.98	1
3	CM-cellulose	50.5	5.959	962.03	161.44	13.86	5.5
4	Sephadex G75	29.6	0.474	140.90	297.5	3.46	10.19

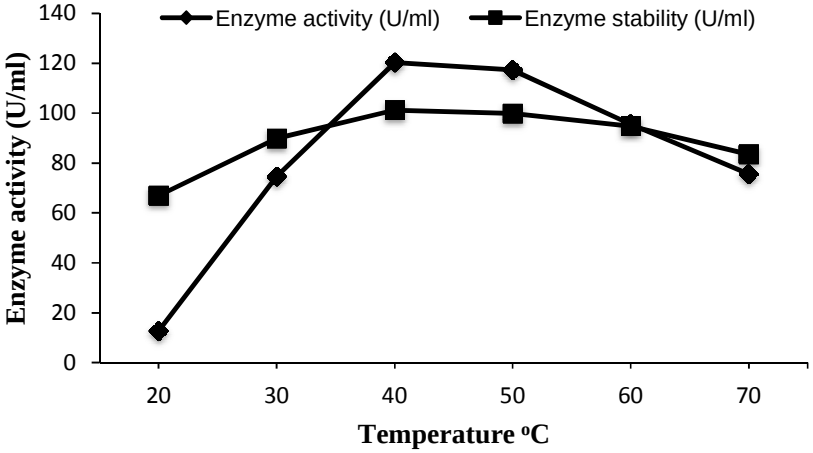

Fig. 3. Effect of temperature on enzyme activity and stability by *Bacillus licheniformis*

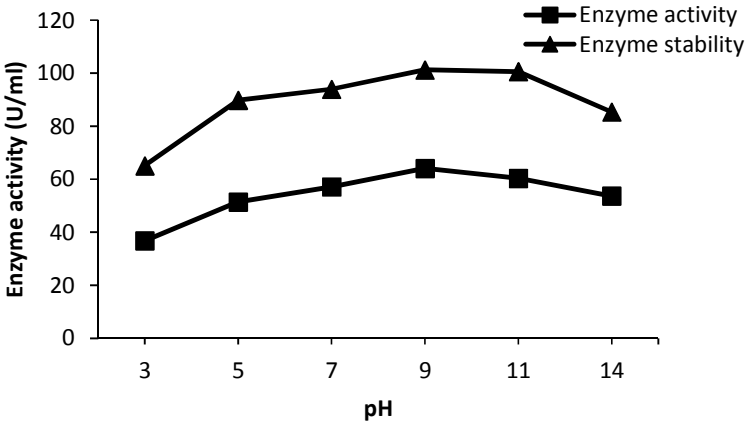

Fig. 4. Effect of pH on enzyme activity and stability by *Bacillus licheniformis*

Mercury ion (Hg^{2+}) had the highest inhibitory effect on enzyme activity (95.85%) whereas calcium (Ca^{2+}) exhibited the highest stimulatory effect of about 82% on enzyme activity followed by Zn^{2+} (74% activation). Fe^{2+} slightly (7%) enhanced enzyme activity. Previous finding had reported decrease in keratinase activity in the presence of Zn^{2+} and Hg^{2+} [22,23,24,25]. Complete inhibition (0% relative activity) has been obtained with Hg^{2+} and Ag^{2+} on keratinase from *Bacillus licheniformis* K-19 [21]. Keratinase inhibition by Hg^{2+} could suggest that a free cysteine is present at or near the active site. Thus, suggesting that inhibition by Hg^{2+} is not just related to binding of the thiol groups but may be as a result of an interaction with tryptophan

residues or with the carbonyl group of amino acids in the enzyme [22].

3.2.4 Effect of inhibitors and feather concentrations on enzyme activity

Effect of inhibitors on keratinase activity was studied and presented in Table 3. Result obtained revealed that keratinase activity was most stimulated in the presence of EDTA (126%). Hydrogen peroxide (H_2O_2), PMSF, SDS and KCN inhibited keratinase activity, with H_2O_2 and PSMF been the most potent inhibitors. About than 97.7% and 98.6% of the initial activity was lost in the presence of PMSF and H_2O_2, respectively. PMSF and H_2O_2 strong inhibition showed that the inhibitors completely inactivated the activity of enzyme, an indication of the existence of serine residues at the active site of the keratinase which suggested the enzyme to be a serine protease. The activation of keratinase from *B. licheniformis* by EDTA indicated that the purified keratinase is a metallo-enzyme and metal ion dependent for maximum activity [10]. This result is consistent with the report of [23,24]. PMSF also inhibited keratinase extracted from *Bacillus licheniformis* [25], *Streptomyces albus* [10]. Similar result has been reported by [16] for *Aspergillus parasiticus*, [22] for *Bacillus megaterium* and [10] for *Streptomyces albus* JB 99. Our result is equally in agreement with Cai et al. [25] who reported partial inactivation by PMSF and activation by ethylene diamine tetraacetic acid (EDTA) had positive on the keratinase activity of a new *B. subtilis* strain. The result of this study is not in agreement with Han et al. [26] who reported that keratinolytic activity from *Pseudomonas aeruginosa* C11 was strongly inhibited by EDTA.

3.2.5 Effect of feather concentrations on enzyme activity

The effect of substrate (feather) concentration on purified enzyme activity is shown in Fig. 5. Optimum enzyme activity was observed with 1% feather concentration after which the enzyme activity declined. The result revealed that increase in substrate concentration beyond 1% inhibited the activity enzyme.

Table 2. Effect of metal ions on purified keratinase activity

Metal salt	Concentration (mM)	Relative activity (%)
None	0	100
Iron sulphate	10	107.5
Aluminium chloride	10	72.1
Zinc sulphate	10	174.5
Calcium chloride	10	182.4
Mercuric chloride	10	4.2

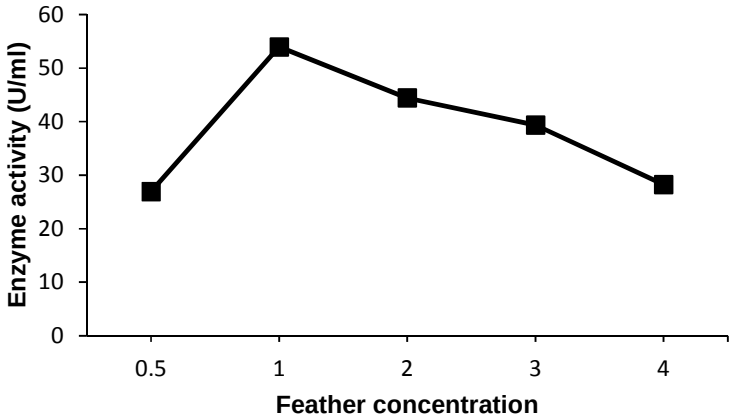

Fig. 5. Effect of feather concentrations on keratinase activity by *Bacillus licheniformis*

Table 3. Effect of some inhibitors on purified keratinase activity

Inhibitor	Concentration (mM)	Relative activity (%)
None	0	100
EDTA	2	126
SDS	2	3.7
H_2O_2	2	1.4
PMSF	2	2.3
KCN	2	6.5

KCN – Potassium cyanide, PMSF – Phenyl methyl sulfonyl fluoride, ‘
EDTA - Ethylene diamine tetraacetic acid, SDS - Sodium dodecyl sulphate

4. CONCLUSION

The results obtained in this current result showed that crude keratinase from *Bacillus licheniformis* was purified to homogeneity by three steps purification using ammonium sulphate precipitation, carboxyl methyl cellulose and gel filtration on Sephadex G75. The purification fold and percentage yield obtained were 10-fold and 3.46% with specific activity of 297.5 U/mg. The purified enzyme activity was optimum at pH 9.0 and temperature 40ºC and was stable at 40ºC. The enzyme activity was slightly stimulated by Ca^{2+}, Zn^{2+} and Fe^{2+} and significantly ($P < 0.05$) activated by EDTA. H_20_2, PMSF and Hg inhibited the purified enzyme in decreasing order. The results of the study suggest that the keratinolytic enzyme from the *B. licheniformis* strain belongs to alkaline serine protease family. *B. licheniformis* strain could be a potential candidate for the degradation of feather keratin and also in biotechnological applications.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Cao, L. Tan, H. Liu Y, Xue X, Zhou S. Characterization of a new keratinolytic *Trichoderma atroviride* strain F6 that completely degrades native chicken feather. Letter of Applied Microbiology. 2008;46:389-394.
2. Fellahi S, Chibani A, Feuk‐Lagerstedt E, Taherzadeh MJ. Identification of two new keratinolytic proteases from a *Bacillus pumilus* strain using protein analysis and gene sequencing. AMB Expr. 2016;6:42.
3. Huang Y, Busk PK, Lange L. Production and characterization of keratinolytic proteases produced by *Onygena corvina*. Fungal Genom Biol. 2015;5:119.
4. Riffel A, Daroit DJ, Brandelli A. Nutritional regulation of protease production by the feather-degrading bacterium Chryseo-bacterium sp. kr6. New Biotechnol. 2011;28:153-157.
5. Jani SA, Heta R, Patel H, Darji D, Rathod A, Pal S. Production and characterization of keratinolytic protease from *Streptomyces* sp. Int. J. Curr. Microbiol. App. Sci. 2015;4(5):962-975.
6. Cheng, Shu-Wen, Hu H, Shen S, Takagi H, Asano M, Tsai Y. Production and Characterization of Keratinase of a Feather-degrading *Bacillus licheniformis* PWD-l Biosci. Biotech. Biochem. 1995;59(12):2239-2243.
7. Prasad HV, Kumar G, Karthik L, Rao B. Screening of extracellular producing bacteria from feather processing areas in Vellore, Tamil Nadu, India. Journal of Scientific Research. 2010;2(3):559-565.
8. Yuhong H, Peter KB, Lene L. Production and characterization of keratinolytic proteases produced by *Onygena corvine.* Fungal Genomics & Biology. 2014;5:1.
9. Ire FS, Onyenema AH. Effects of some cultural conditions on keratinase production by *Bacillus licheniformis* strain NBRC 14206. Journal of Advances in Biology & Biotechnology; 2017. (In Press)
10. Sreenivasa N, Gireesh BK, Vidyasagar GM. Purification and characterization of keratinase from hair-degrading *Streptomyces albus.* International Journal of Bioassays. 2013;2(3):559-604.
11. Tork S, Aly MM, Nawar L. Biochemical and molecular characterization of a new local keratinase producing *Pseudomonas* sp. MS21. Asian Journal of Biotechnology. 2010;2(1):13.
12. Nandini A, Madhusudhan DN, Dayanand A. Enhanced production, purification and

characterization of alkaline keratinase from *Streptomyces minutiscleroticus* DNA38. Int. J. Adv. Res. Biol. Sci. 2015;2(7):161–170.
13. Selvam K, Vishnupriya B. Biochemical and molecular characterization of microbial keratinase and its remarkable applications. International Journal of Pharmaceutical and Biological Archives. 2012;3(2):267-275.
14. Jian-Yu M, Lian-Lian X, Xiu-Juan Z. Isolation of a feather-degrading *Bacillus subtilis* strain from alimentary tract of grebes. African Journal of Microbiology Research. 2013;7(21):2550-2559.
15. Daniel MT, Maria LG. Production and partial characterization of keratinase produced by a microorganism isolated from poultry processing plant wastewater. African Journal of Biotechnology. 2008;7(3):296-300.
16. Thoomatti SA, Peramachi P. Production and characterization of keratinolytic protease(s) from fungus, *Aspergillus parasiticus.* International Journal of Research in Biological Science. 2012;2(2): 87-93.
17. Rajput R, Sharma R, Gupta R. Biochemical Characterization of a thiol-activated, oxidation stable keratinase from *Bacillus pumilus* KS12. SAGE-Hindawi Access to Research. 2010;7.
18. Lonata E, Canganella F, Bianconi G, Benno Y, Sakamoto M, Capasso A, Rossi M, La Cara F. A novel keratinase from *Clostridium sporogenes* bv. Pennavorans Nov., a thermotolerant organism isolated from solfataric muds. Microbiology Research. 2008;163:105-112.
19. Armin EK, Zahra HF, Hatice KG. Keratinolytic protease production and characterization from *Bacillus* sp. isolated from poultry wastes. International Journal of Applied Biology and Pharmaceutical Technology. 2015;6(63).
20. Dastager GS, Chan JL, Wen-Jun L, Chang-Jin K, Dayanand A. Production, characterization and application of keratinase from *Streptomyces gulbargensis*. Bioresour. Technol. 2009;100:1868-1871.
21. Bo X, Qiaofang Z, Xianghua T, Yunjuan, Yand ZH. Isolation and characterization of a new keratinolytic bacterium that exhibits significant feather-degrading capability. Afr. J. Biotechnol. 2009;8(18): 4590-4596.
22. Venkata S, Francois NN, Sunil SM. Isolation, partial purification and characterization of keratinase from *Bacillus megaterium.* International Research Journal of Biological Sciences. 2013;2(2): 13-20.
23. Kainoor PS, Naik GR. Production and characterization of feather degrading keratinase from *Bacillus* sp. JB 99. India Journal. Biotechnol. 20109:384-390.
24. Lin HH, Yin LJ. Feather meal and rice husk enhanced keratinase production by *Bacillus licheniformis* YJ4 and characterization of produced keratinase. Journal of Microbiology, Science and Technology. 2010;18(3):458-465.
25. Cai C, Chen J, Qi J, Yin Y, Zheng X. Purification and characterization of keratinase from a new *Bacillus subtilis* strain. J Zhejiang Univ Sci B. 2008;9(9):713-720.
26. Han M, Luo W, Gu Q, Yu X. Isolation and characterization of a keratinolytic protease from a feather-degrading bacterium *Pseudomonas aeruginosa* C11. African Journal of Microbiology Research. 2012;6(9):2211-2221.

Peer-review history:
The peer review history for this paper can be accessed here:
http://sciencedomain.org/review-history/18943