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Isolation, Partial purification and Characterization of Keratinase from *Bacillus megaterium*

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Abstract

A positive keratinase-producing bacterium from soil sample was screened and identified as Bacillus megaterium. The selected growth medium contained feather meal as a sole source of carbon and nitrogen at pH 10.0. The partially purified keratinase has a molecular mass of 41±1 kDa, optimum pH in the alkaline region (9.0) and optimum temperature of 50ºC. K^+ and Co^{2+} slightly inhibited the enzyme activity whereas Hg^{2+} exhibited a complete inhibition. NEM, IAA, TLCK and NAI moderately inhibited the enzyme whereas PMSF strongly inhibited the keratinase suggesting serine at the active site; however EDTA did not inhibit the enzyme. The isolated strain was thermotolerant and alkalophilic bacterium which can therefore be used in leather processing and detergent industry.

Keywords: Keratin, alkalophilic, thermotolerant, keratinolytic activity.

Introduction

Keratin is an insoluble structural protein in epithelial cells of vertebrates and represents the major constituents of skin and its appendages such as nail, hair, feather, wool, hooves, scales, and stratum corneum[1]. One of the main characteristics of keratins is that they have high mechanical stability and resistance to proteolytic degradation, which depends on the disulphide and hydrogen bonds, salt linkages and other cross-linkings[2]. Williams et al.[3] reported that feathers are produced in large amounts as a waste by-product at poultry processing plants, reaching millions of tons per year worldwide. Feathers contain over 90% crude protein in the form of keratin[4].

Brutt and Ichida[5] reported different approaches to dispose feather waste, including land filling and burning; however these techniques can cause contamination of air, soil and water. Feathers can be hydrolysed by mechanical or chemical treatment to feedstuffs, fertilizers, glues and foils or used for the production of amino acids and peptides but these techniques lead to environmental pollution[6]. Cai et al.[7] suggested traditional ways to degrade feathers such as alkali hydrolysis and steam pressure cooking but these techniques may not only destroy the amino acids but also consume large amounts of energy. Xu et al.[8] reported that degradation of poultry feathers by keratinolytic proteases offers an alternative method for efficient bioconversion, nutritional enhancement and environmental friendliness.

Keratinases (3.4.21-/24/99) are the enzymes that can hydrolyze keratin substrates produced by a large number of bacteria, actinomycetes, and fungi. They are used as detergent additives to aid in the removal of proteinaceous stains[9], to enhance drug delivery[10], in the preparation of protein hydrolysates of high nutritional value[11], in the bioprocessing of used X-ray or photographic films for silver recovery[12], in leather processing[13], in agroindrustrial waste degradation[14] and in prion degradation[15]. Because of the numerous potential uses of keratinases, this study was undertaken to screen a bacterium that produces a highly active keratinase, to partially purify and characterize the enzyme.

Material and Methods

Preparation of feather meal: The feather meal was prepared from native chicken feathers as described by Tork et al.[16] with slight modifications. The feathers were cut with scissors in to small pieces of 3 to 4 cm long and washed several times with tap water. Defatting of feather pieces was done by soaking them in a mixture of chloroform: methanol (1:1) for 2 days followed by chloroform: acetone: methanol (4:1:3) for 2 days. The solvent was replaced every day. The feathers were finally washed several times with tap water to eliminate the solvent residual, dried for 24 h in sunlight, grinded using electrical mixer blender (Kenstar Senator PCMG 0120) and used as feather meal.

Isolation, screening and identification of keratinase-producing bacteria: Soil sample was collected from different poultry dump yards in Bangalore and was screened for keratinase-producing bacteria as follows: 1 g of the soil sample was suspended in distilled water. After soil sedimentation, 0.1 ml of the supernatant was spread on the surface of agar medium supplemented with feather meal as a sole carbon and nitrogen source. The modified feather meal agar plate[17] contained the following constituents ($g.L^{-1}$): NaCl (0.5), KH_2PO_4 (0.7), K_2HPO_4 (1.4), $MgSO_4$ (0.1) and feather meal (10). The pH of the medium was adjusted to 10.0 using sodium carbonate in

order to isolate alkalophilic microorganisms. The plates were incubated at 37°C till the colonies appeared. Development of keratinase-producing bacteria was confirmed by the presence of a clear zone around the bacterial colony. The colonies with highest keratin hydrolyzing ability were picked up and purified by repeated screening on the same medium. Pure cultures were maintained on casein agar plate slants at 4°C. The isolate was identified by microscopic examination and biochemical tests as described in the Bergey's manual of systematic bacteriology[18].

Crude keratinase production: Submerged fermentation was performed by inoculating pure culture of isolate into the production medium[19] containing feather meal (1%), yeast extract (0.01%), NaCl (0.05%), KH_2PO_4 (0.03%), K_2HPO_4 (0.04%) and $MgCl_2$ (0.01%), pH 10.0. The incubation was carried out at 37°C for 3 days. The broth was centrifuged (REMI C-30 BL centrifuge, India) at 10,000 rpm for 10 min and the supernatant was used as crude enzyme.

Preparation of soluble keratin and keratin solution: Soluble keratin was prepared from white chicken feathers by the method of Wawrzkiewicz et al.[20]. 10 g of native chicken feathers was mixed with 500 ml of dimethylsulfoxide (DMSO) and heated in a reflux condenser at 100°C for 2 h. Soluble keratin was then precipitated by addition of chilled acetone at −20°C for 2 h, followed by cooling centrifugation at 10000 rpm for 10 min. The resulting precipitate was washed twice with distilled water and dried at 40°C in a hot air oven (SERVEWELL, India). The keratin solution was prepared by dissolving 1 g of soluble keratin in 20 ml of 0.05 M NaOH, adjusting the pH to 7 with 0.1 M HCl and diluting the resulted solution to 200 ml with 0.05 M phosphate buffer (pH 7).

Determination of crude keratinase activity: The keratinolytic activity was assayed according to method of Vigneshwaran et al.[21]. 1.0 ml of crude enzyme was diluted in 0.05 M Tris-HCl buffer (pH 8.0) and was incubated with 1 ml of 1% soluble keratin solution at 50°C in a water bath for 10 min. The reaction was stopped by adding 2.0 ml of 0.4 M trichloroacetic acid (TCA). The resulted precipitate was removed by centrifugation at 10000 rpm for 10 min. 0.2 ml of the supernatant was taken and diluted with to 1.0 ml with distilled water. To this, 5.0 ml of alkaline cupper reagent was added, shaken well, and incubated for 10 min. After incubation period, 0.5 ml of Folin Ciocalteau (FC) reagent was added and incubated in the dark for 30 min to allow blue color development. The control was prepared by incubating enzyme solution with 2 ml of TCA without the addition of keratin solution. The absorbance was measured at 660 nm using a UV-spectrophotometer (SL 159, ELICO). One unit of alkaline keratinase was defined as the amount of enzyme required to liberate 1 µg of tyrosine per min per ml under the standard assay conditions.

Optimization of culture conditions for keratinase production: The effect of carbon source on the production of keratinase was tested by adding different concentrations of feather meal in the production medium. The effect of different nitrogen sources was tested by adding individual nitrogen source viz. tryptone, peptone and yeast extract in the production medium. The effect of pH and temperature on keratinase production was individually tested by taking the production media at different pHs and temperatures. The fermentation media were tested every day for keratinase production till a decline was observed in the enzyme activity. For time course analysis of keratinase production, the isolate was grown in the optimized growth medium and the activity was measured every day for a period of 7 days.

Protein estimation: The amount of protein present was assayed by the Lowry method[22] using bovine serum albumin (BSA) as standard. The colour developed was read at 660 nm.

Partial purification of keratinase: The cell free extract from fermentation broth was partially purified by acetone precipitation method[23]. Keratinase was precipitated by prechilled acetone (30-80%) fractionation. The acetone was added to the cell free extract in 3:1 ratio and incubated for 60 min at -20°C. The contents were subjected to centrifugation at 10000 rpm for 10 min. The supernatant was discarded carefully and pellet was dissolved in Tris-acetate buffer (pH 7).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE): In order to determine molecular weight, the partially purified enzyme and known molecular weight markers were subjected to electrophoresis. SDS-PAGE was performed with 12% polyacrylamide gels[24], and the gels were silver stained[25]. The molecular weight was determined by interpolation from a linear semi-logarithmic plot of relative molecular mass versus the relative mobility value.

Biochemical characterization of extracellular keratinase: The effect of pH on keratinase activity was investigated over a range of pH 3.0 to 11.0 using buffers of 0.2M strength each: citric acid buffer (pH 2.0-3.5), acetate buffer (pH 4.0-5.5), phosphate buffer (pH 6.0-7.5), Tris-HCl buffer (pH 8.0-10.0) and glycine-NaOH buffer (pH 10.5 to 12.0). The method of Tork et al.[16] was used with slight modifications. The assay was performed by preincubating 0.2 ml of enzyme with 0.8 ml of each of the pH buffers at room temperature. 1 ml of keratin solution was then added to each test tube and incubated for 20 min at 50°C. The reaction was arrested by adding 10% TCA and the activity was determined as described earlier. The effect of temperature on keratinase activity was studied by performing the standard assay procedure at optimum pH with a temperature range of 20°C to 90°C. Effect of different metal ions on keratinase activity was analysed using 10 mM of Fe^{2+}, Cu^{2+}, Zn^{2+}, Ca^{2+}, Mg^{2+}, Mn^{2+}, Hg^{2+}, Ba^{2+}, Na^{+} and K^{+}. The assay was performed by preincubating 0.2 ml of enzyme with 0.8 ml of buffer with respective metal ions at room temperature. The influence of group specific reagents on keratinase activity was investigated using phenylmethylsulphonylfluoride (PMSF), iodoacetic acid (IAA), N-acetylimidazole (NAI), N-ethylmalamide (NEM), tocylchloridemethylketone (TLCK) and

ethylenediaminetetraacetate (EDTA) of 10 mM concentration each. The activity was determined as described earlier.

Results and Discussion

Isolation, screening and identification of keratinase-producing bacteria: In the present investigation, bacteria were isolated from the soil collected from poultry dump yards. The colonies that showed a clear zone around them after addition of TCA solution on keratin agar plates (figure 1) were regarded as enzyme producers. Based on microscopic and biochemical tests, the isolate was identified as *B. megaterium*. Brandelli et al.[26] reported that keratinases are produced by a variety of bacteria mainly by *Bacillus* and *Streptomyces* species. Wood[27] isolated a feather degrading bacterium *Bacillus licheniformis* from soil.

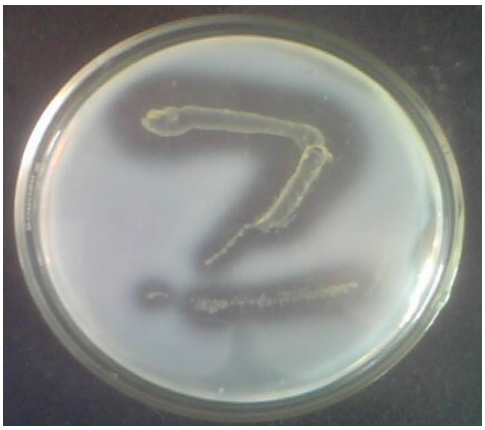

Figure-1
Plate with keratinase-positive colony grown on ground keratin agar medium showing clear zone around it after adding trichloroacetic acid solution

Optimization of culture conditions for keratinase production by the isolate: Effect of carbon and nitrogen source on enzyme production: *B. megaterium* was inoculated into media containing various concentrations of carbon source and different nitrogen sources. It was found that maximum keratinase production occurred at 1.5% concentration (figure 2) and in the presence of a yeast extract (0.1%) as nitrogen source (figure 3). Cheng et al.[28] reported that 1% feather powder gave the highest keratinase activity for *B. licheniformis* PWD-1. Studies undertaken by Lin and Yin[19] showed maximum keratinase production in *B. licheniformis* YJ4 with 0.5% feather meal after 72 h incubation. Kainoor and Naik[29] achieved maximum keratinase production in presence of 1% feather meal and 0.1 % yeast extract in the medium with *Bacillus* sp. JB 99. The increased concentration of feather decreased the enzyme production suggesting catabolic repression on keratinase production.

Effect of initial pH on enzyme production: The effect of pH on keratinase production by *B. megaterium* was studied and is shown on figure 4. Maximum enzyme production was achieved at pH 10.0. The production of protease increased as pH of the medium increases and reaches maximum at pH 10.0. After pH 10.0 there was a decrease in enzyme production. Likewise, maximum enzyme production occurred at pH 10.0 with alkalophilic bacterium *Bacillus* sp. JB99[29]. Srivastava et al.[30] reported pH 8.5 as optimum for keratinase production in *Bacillus* sp. SAA5. Cheng et al.[28] also reported the highest keratinase production by *B. licheniformis* PWD-1 at initial pH of 8.5. *B. megaterium* is thus a better alkalophilic keratinase-producing bacterium.

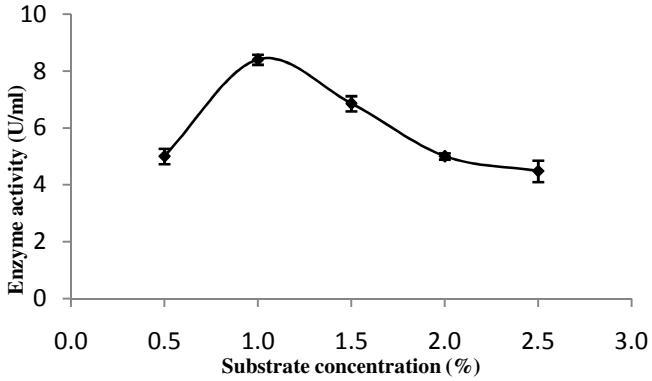

Figure-2
Effect of different concentrations of keratin on enzyme production. Data are mean ± SD (n = 3)

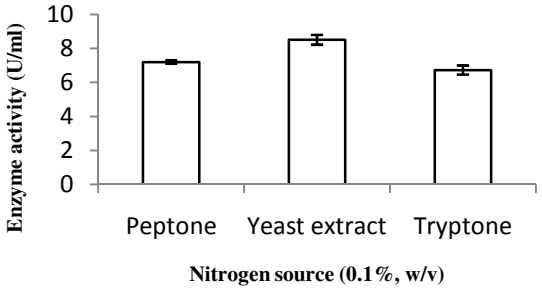

Figure-3
Effect of different nitrogen sources on keratinase production. Data are mean ± SD (n = 3)

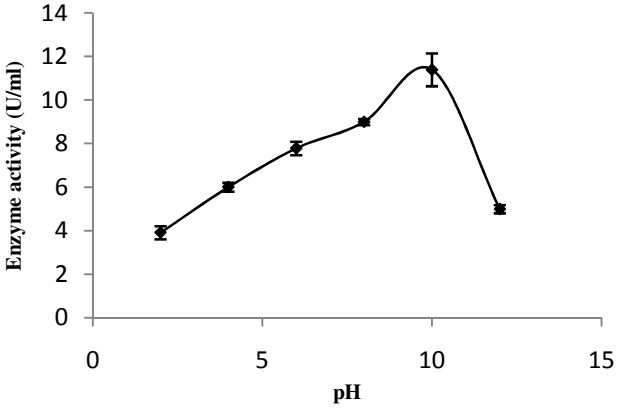

Figure-4
Effect of different pH on keratinase production, Data are mean ± SD (n = 3)

Effect of incubation temperature on enzyme production: The isolate was capable of producing keratinase in the range of temperatures tested with production maximum at 35ºC (figure 5). However, increase in temperature beyond 35ºC led to decline in enzyme production. Maximum keratinase production at 37ºC was found by Xu et al.[8] in *B. licheniformis* K-19. Brandelli and Riffel[31] reported the production of enzyme between 25 and 37ºC, with maximum activity and yield at 30ºC. Maximum temperature for keratinase production of 40ºC was recorded by Suh et al.[32] with *Bacillus subtilis* and *Bacillus pumilis*.

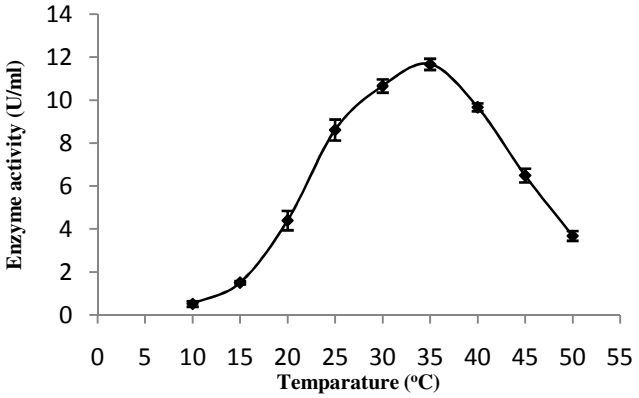

Figure-5
Effect of incubation temperature on keratinase production, Data are mean ± SD (n = 3)

Time course study of enzyme production: The maximum extracellular alkaline keratinase production was observed on the 3rd day when *B. megaterium* was grown in the optimized feather meal medium (figure 6). The cell free extract was harvested on this day and was used for further enzymatic studies. Likewise Lin and Yin[19] observed maximum keratinase production on the day3. Agrahari and Wadhwa[17] with *Bacillus thurengenesis* SN2 and Srivastava et al.[30] using *Bacillus* strain SAA5 reported maximum keratinase production on day 5 and 7, respectively.

Partial purification of keratinase: The enzyme was partially purified by acetone precipitation. The specific activity and fold purification of the enzyme increased with acetone purification (table 1) proving that acetone precipitation is suitable method for keratinase purification. Ali et al.[23] purified keratinase by acetone precipitation from *Aspergillus oryzae* NRRL-447 that showed 258.8 U/mg specific activity with purification fold of 2.31 and yield of 52.5 %. Suntornsuk et al.[33] purified keratinase from *B. licheniformis* FK 14 with 86 fold purification, specific activity of 218 U/mg and 25% recovery. Dozie et al.[34] reported that acetone precipitation increased the purity of keratinolytic protease from *Chrysosporium keratinophilum*.

SDS-PAGE: Partially purified enzyme showed one clear band having keratinolytic activity and a number of diffuse bands of different proteins. On comparison with the standard molecular weight markers, the apparent molecular weight of keratinase was found to be 41 ± 1kDa (figure 7). The molecular weight of keratinases varies between bacteria. Among the bacteria the lower molecular weight was observed in *Streptomyces albidoflavus* (18 kDa)[35] and the higher molecular weight was observed in *Kocuria rosea* (240 kDa)[36]. Molecular mass of keratinase purified from *Streptomyces thermoviolaceus* was estimated as 40 kDa by Chitte et al.[37]; 38 kDa by Brandelli[2] from the bacterium *Chryseobacterium* sp. kr6 and 65 kDa by Kumar et al.[38] from *Bacillus pumilis*.

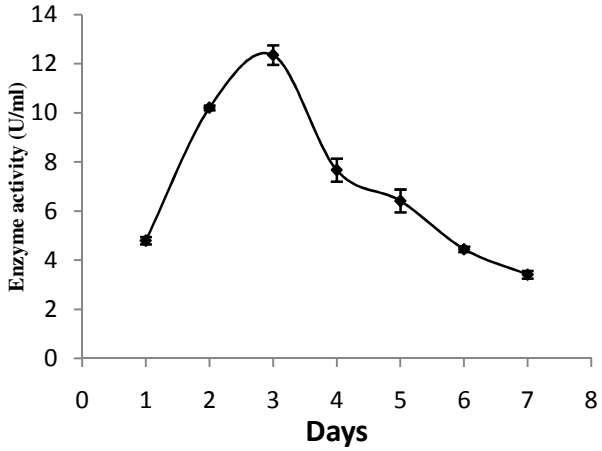

Figure-6
Time course analysis of keratinase production by *Bacillus megaterium* on different days, Data are mean ± SD (n = 3)

Biochemical characterization of extracellular keratinase: Effect of pH and temperature on partially purified enzyme activity: The maximum activity of the keratinase from *B. megaterium* was observed at pH 9.0 (figure 8) and 50ºC (figure 9) using keratin solution as substrate. Rozs et al.[39] reported maximum keratinase activity at pH 8.5 and 50ºC with *B. licheniformis* K-508; pH 10 and 40ºC was reported by Bernal et al.[36] with *K. rosea*; and pH 7 and 55ºC by Thys et al.[40] with *Microbacterium* sp. The purified keratinase is thus a better thermotolerant and alkalophilic enzyme.

Effect of metal cations on enzyme activity: The effect of various metal ions was tested on the activity of partially purified keratinase in the presence of keratin solution as substrate. It was found that K^+ and Co^{2+} slightly inhibited the enzyme activity whereas Hg^{2+} exhibited a complete inhibitory effect. Zn^{2+} inhibited the enzyme whereas Fe^{2+}, Ca^{2+}, Mg^{2+}, Ba^{2+}, Cu^{2+} and Na^+ moderately inhibited the enzyme (figure 10). Keratinase activity was also decreased in the presence of Hg^{2+} and Zn^{2+} in *Bacillus* sp. JB 99[29], and in *B. licheniformis* YJ4[19]. Keratinase inhibition by Hg^{2+} may suggest that a free cysteine is present at or near the active site. Lusterio et al.[41] suggested that inhibition by Hg^{2+} is not just related to binding of the thiol groups but may be a result of an interaction with tryptophan residues or with the carbonyl group of amino acids in the enzyme.

Effect of group specific reagents on keratinase activity: Effect of group specific reagents on partially purified enzyme was studied in the presence of keratin solution as substrate. NEM, IAA, TLCK and NAI moderately inhibited the enzyme whereas PMSF strongly inhibited the keratinase; however EDTA did not inhibit the enzyme (figure 11). The inhibition of enzyme by PMSF suggested that the isolated enzyme could be a serine protease. PMSF also inhibited keratinase extracted from *B. licheniformis*[33], *B. licheniformis* YJ4[19] and *Bacillus* sp. JB 99[29]. Enzyme activity was also inhibited by group specific reagents like TLCK, IAA, NEM and NAI indicating that serine, cysteine, tyrosine and lysine may play an important role in the catalytic activity. This inference is based on the already available literature on the known group specific reagents and their target amino acid residues which get modified leading to inhibition. EDTA displayed no effect on activity of the enzyme, thus the enzyme is not a metalloprotease.

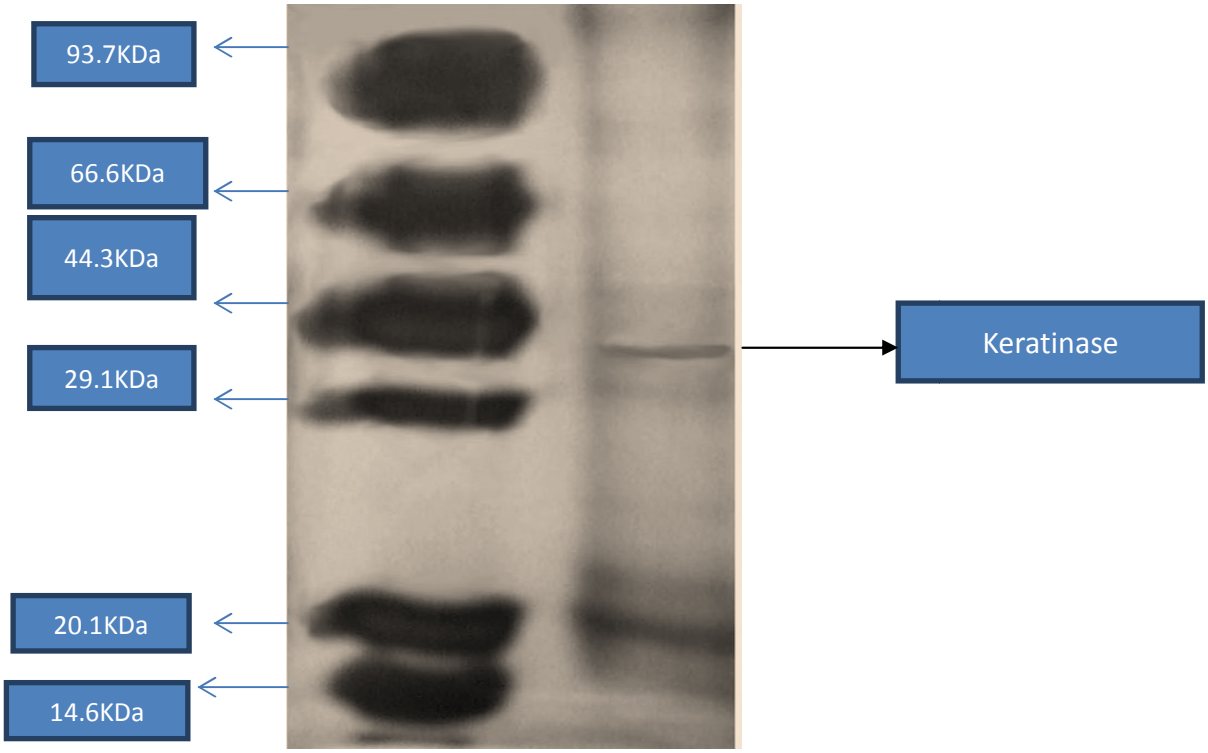

Figure-7
SDS-PAGE pattern of partially purified keratinase isolated from *Bacillus megaterium*. Lane 1: Molecular weight markers, Lane 2: partially purified keratinase

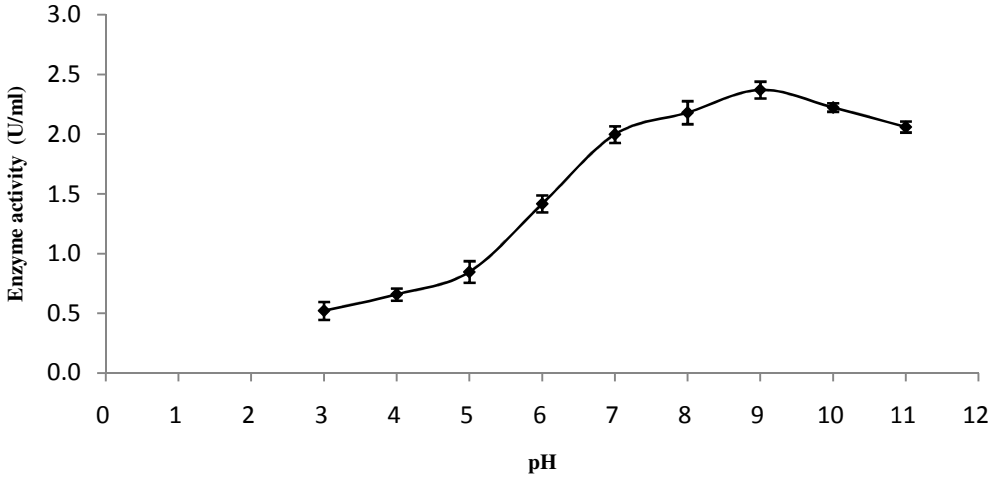

Figure-8
Effect of pH on the activity of partially purified alkaline keratinase from *Bacillus megaterium*, Data are mean ± SD (n = 3)

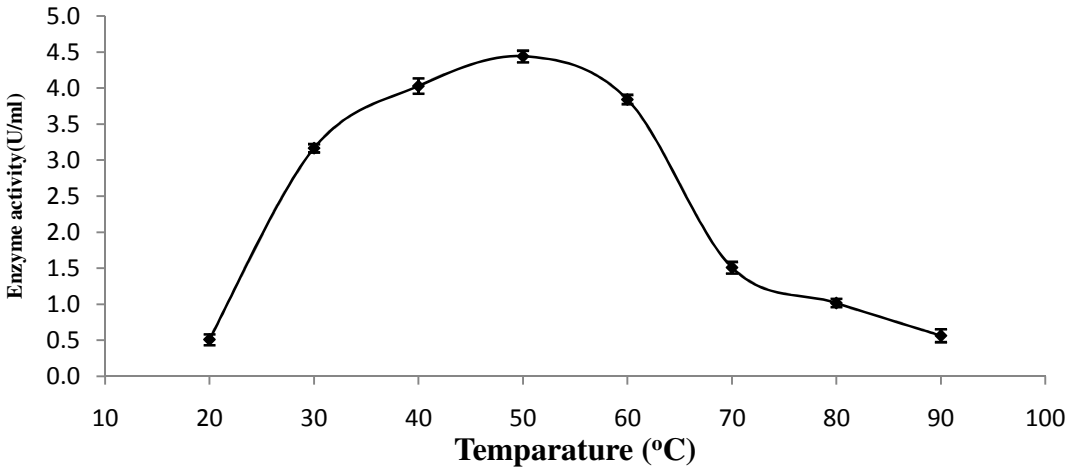

Figure-9

The influence of temperature on the activity of partially purified alkaline keratinase from *Bacillus megaterium*. Data are mean ± SD (n = 3)

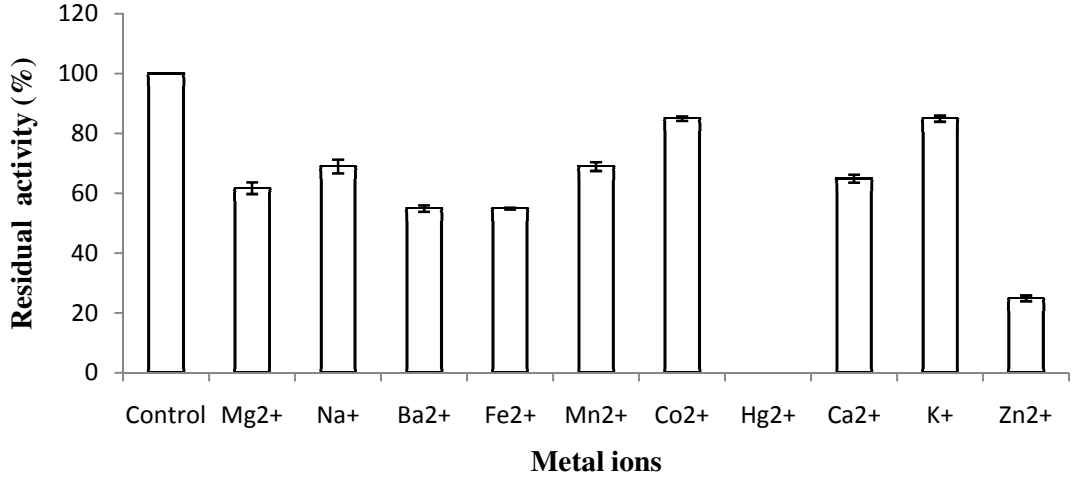

Figure-10

Effect of metal ions on the activity of alkaline keratinase of *Bacillus megaterium*, Values represents mean ± (n=3)

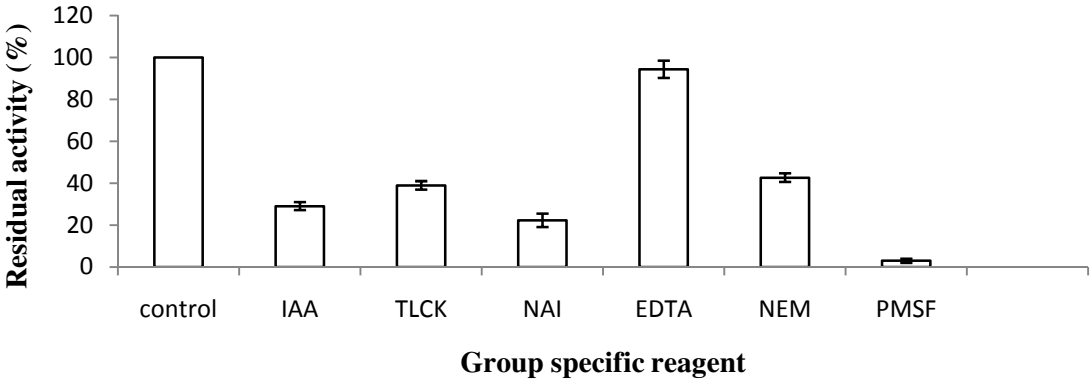

Figure-11

Effect of group specific reagents on the activity of alkaline keratinase of *Bacillus megaterium*, Data are mean ± SD (n = 3)

Conclusion

A positive keratinase producing bacterium from soil was identified as *B. megaterium*. The inhibition of enzyme by PMSF suggested that the isolated enzyme could be a serine protease. *B. megaterium* was found to be better thermotolerant and alkalophilic bacterial strain which can therefore be utilized in leather processing and detergent industry. It can be suggested that *B. megaterium* is a potential source of alkaline keratinase for biotechnological applications and can be effectively used in the large scale production of enzyme for commercial purposes.

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