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Characterization of the keratinolytic activity of indigenous *Bacillus subtilis* keratinase

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ABSTRACT

Keratins are water insoluble proteins of our environment. Being extremely resistant to degradation by proteolytic enzymes, keratins are digested mainly by alkali and keratinase enzymes. The aim of present study was to characterize the indigenous keratinase for potentials of keratin-degrading activities. We report the purification and characterization of keratinase from Bacillus subtilis isolated from soil of District Khairpur. The keratinase possessed good stability with >90% activity for one month and 50% activity after two months at 4°C. The enzyme was active for 48 h at room temperature. The temperature range for production and activity was between 37-50 ° C with optimum at 37° C from purified enzyme. Enzymatic activity was observed over a wide range of pH (8.0-11.0) however, optimum pH was found to be 10.0 indicating alkaliphic nature of this keratinase. The molecular mass of the keratinase was 30 kDa. The keratinase activity was not inactivated in presence of PMSF and EDTA but affected by HgCl2. This property of the enzyme indicated that it was a cysteine protease. Gelatin appeared as preferred substrate followed by keratin. Psoriasis scale hydrolysis and dehairing activities showed by this enzyme qualify it as a novel keratinase. Alkaliphilic, thermotolerant keratinase (a cysteine protease) from an indigenous strain of Bacillus subtilis exhibited substantial keratinolytic activities such as dehairng of goat skin and psoriases scale hydrolysis and appeared as a novel keratinase.

Key words: Keratinase, keratin, dehairing, enzyme

INTRODUCTION

Keratins, the heterogenic proteins of our environment present in hair, 57 feather, hooves, wool, horns, nail, stratum and cornium [1] and characterized by high content of cystine, arginine and serine. Keratins, due to the presence of the disulfide linkages and their coil coiled structure are highly resistant to acids and some protease enzymes, therefore, keratinous material is water insoluble and extremely resistant to degradation by proteolytic enzymes such as trypsin, pepsin, and papain, however, it is easily hydrolyzed by alkalis and keratinase enzymes [2] A group of proteolytic enzymes which are able to hydrolyze insoluble keratins more efficiently than other proteases are called keratinases [3.4.99] [3]. They are produced by some insects and mostly by microorganisms such as bacteria, actinomycetes and fungi. The best studied keratinases from bacteria belong to genera *Bacillus* [4].The prospective use of keratinases involve diverse applications where keratins need to be hydrolyzed, such as in the leather and detergent industries, textiles, waste bioconversion, and cosmetics medicine such as drug delivery through nails and degradation of keratinized skin [5]. Proteases represent an essential fraction of the global enzyme sales, and a relevant part of this market is accounted by bacterial proteases [6]. Keeping in view the important role of keratinase,

the aim of present study was to characterize and evaluate the keratianse produced from indigenous bacterial strains from soils of Khairpur District Sindh Pakistan.

EXPERIMENTAL SECTION

Screening of Proteolytic Bacteria from Soil Samples

Sampling and isolation of keratinolytic bacteria from soil was performed as described previously [7] with few modifications. Soil sample (0.1 gram) was directly plated on melted and cooled Casein agar medium (10g/L w/v caseine, Uni Chem; agar agar BioChem Malaysia, 15 mL /plate). Before the agar solidified, the plates ware rotated to distribute the soil in the medium. After solidification, plates were incubated at 37^o C for 1-2 days. This procedure was performed with each sample in duplicates. After incubation the plates were observed for the clearing zones around the colonies to signify protease production. A single colony with a clearing zone was picked up and inoculated on nutrient agar (BioChem Malaysis) plates and incubated ate 37^o C for 24 h. The isolated keratinolytic bacteria were identified by cell morphology, motility, Gram staining reaction and biochemical profiling using standard microbiological and biochemical techniques.

Screening of Proteolytic Activity of Enzyme on Gelatin Agar

Culture of *B. subtilis* isolated from the soil was inoculated and incubated on gelatin agar (gelatin 1.5% w/v, Sigma Aldrich St. Louis, MO USA; 2%, w/v agar agar BioChem Malaysia) and incubated at 37 ^{o} C for 24 h for production of the zone of hydrolysis (the zone of clearing surrounding *Bacillus subtilis*) indicated that the strain was able to hydrolyze gelatin. *Screening of Proteolytic Activity of Enzyme on Skimmed Milk Agar*

Crude keratinase enzyme was prepared as described previously [7]. Briefly, the *B. subtilis* was grown on basal salt medium (K_2 $HPO_4$1.5g; Mg SO_4. $7H_2O$ 0.025g; Ca Cl_2 0.025 g; Fe SO_4. $7H_2O$ 0.015g; Zn SO_4. $7H_2O$ 0.005g; Bovine serum albumin 05 mg/mL dissolved in 500 mL distilled water, 5g/L ground chicken feather powder. pH was adjusted with 1N HCl to 7.5 and volume made up to 1000 mL with distilled water). The chicken feathers were washed, dried and ground in grinder (Anex, Germany) prior to adding to the medium. The medium was autoclaved, inoculated with pure culture and incubated at 37 ^{o} C for 72 h on orbital shaker (150 rpm). The cultures were centrifuged at 8000 rpm, at room temperature for 10 minutes. The extra-cellular keratinase in supernatant was collected and passed through 0.45µm pore size membrane filter (Acrodisc) and was used as crude enzyme. For observation of proteolytic activity of the enzyme, skimmed milk agar [7] was used. Two wells were made in skimmed milk agar plates in duplicate with sterilized borer. In one well 100µl of Basal salt medium [7] was added as a control. In the other well, 100µl of crude keratinase was added as a test. Then plates were incubated at 37 ^{o} C for 24 h. After incubation period, plates were observed for the zone of hydrolysis.

Assay for Keratinase Activity

The assay was performed according to the method of Cheng et al., [8] with some modifications. Keratin azure (Azure dye-impregnated sheep's wool keratin, Sigma- Aldrich St. Louis, MO USA) was used as a substrate. It was first frozen at 0^o C and then ground into a fine powder. The keratin azure suspension used as substrate comprised 5mg keratin azure powder suspended in 1mL 50mM Tris- HCl buffer (pH 8.0). Keratin azure, the Azo dye-impregnated keratin (Sigma- Aldrich St. Louis, MO USA) was used as a chromogenic non-specific substrate for keratinase activity assay. Upon proteolysis, soluble peptide fragments, purple in color due to Azo dye-impregnation, were released and detected by absorbance at 595 nm. The reaction mixture contained 1mL keratin azure suspension and 200µl crude enzyme and purified enzyme (this study). The control comprised a 1 mL keratin azure suspension and 200µl basal salt feather medium. The reactions (reaction mixture and the control) were carried out in shaking incubator at 37°C with constant agitation of 200 rpm for one hour. After incubation, the reactions were stopped by adding 1mL trichloroacetic acid (5% v/v, Sigma- Aldrich St. Louis, MO USA) in both the reaction and control tubes. The unreacted soluble substrate was removed by centrifugation at 3000 rpm for 10 min at room temperature and the liberation of the azo-dye in the supernatant was measured at 595 nm. One unit (U) keratinase activity was defined as the amount of enzyme causing 0.01 absorbance increase between the sample and control at 595 nm under given conditions. *Ammonium Sulphate Precipitation/Salting out* The protocol used was that described by Cheng et al., [8]. briefly, salting out was performed by the addition of solid ammonium sulphate (Sigma- Aldrich St. Louis, MO USA) in a 100 mL crude enzyme during continuous stirring to achieve 30% saturation and then centrifuged to remove the pellet. The crude enzyme was precipitated from the supernatant by the addition of solid ammonium sulphate with gentle stirring until 70% saturation, allowed to stand for 12 h at 4^{o}C and centrifuged at 6000 rpm for 10 minutes at room temperature. The pellet was collected and resuspended in 50mM Tris- HCl buffer (pH 8.0);

dialyzed against 2 liters 50mM phosphate buffer (pH 8.0) overnight 127 at 4 ^{o}C and continued for further 3 h after changing the buffer.

DEAE Sephadex A-50 Chromatograph

The method used was essentially of Zhang et al. [1]. The column (1.0 × 35 cm) was packed with DEAE Sephadex A-50 (Sigma-Aldrich St. Louis, MO USA) matrix and equilibrated with 50 mM Tris HCl buffer (pH 8.0). The dialyzed enzyme (10mL) was loaded, washed with two bed volumes (50 mL) of 50mM Tris- HCl Buffer (pH 8.0) to remove the free proteins and eluted with a gradient of 0.1-1 M NaCl in 50mM Tris HCl buffer pH (8.0). Fractions were collected at a flow rate of 1mL/min. All the fractions were analyzed for keratinase activity by performing the keratinase assay as described earlier.

Estimation of Protein

Protein concentration was measured by the method of Bradford [9], using bovine serum albumin (BSA, (Sigma-Aldrich St. Louis, MO USA) as standard. The specific activity was expressed as the enzymatic activity (U) per mg of protein

Assessment of Purity and Estimation of Molecular weight of Purified Keratinase

SDS -PAGE (Sodium Dodecyl Sulfate Poly Acrylamide Gel Electrophoresis) was performed according to Laemmli [10] using BioRad mini gel electrophoresis apparatus. The molecular mass of the keratinase was calculated by comparing the electrophoretic mobilities of the protein marker with the electrophoretic mobility of keratinase.

Effect of Temperature on Keratinolytic Activity of Purified Enzyme

The purified keratinase (200 µL) was added to 1mL keratin azure suspension and incubated in shaking 150 incubator at 37, 40, 45 and 50^{o} C at 200rpm for one hour. For the control 1mL keratin azure suspension in the same buffer was rotated for one hour at 37, 40, 45 and 50 ^{o} C at 200rpm and activity measured by keratinase assay.

Effect of pH on Keratinolytic Activity of Purified Enzyme

The effect of pH on the keratinolytic activity was assayed at 37^{o} C using buffers of various pH ranging from pH 5.0 to 12.0. The effect of pH was studied by carrying out the keratinase assay in the pH range of 3.0–12.0 using 50mM buffers: pH 3.0–6.0 (citrate phosphate buffer) pH 7.0-8.0 (sodium phosphate buffer), pH 9.0-10.0 (glycine- NaOH buffer), pH 11.0 (phosphate hydroxide buffer), and pH 12.0 (hydroxide-chloride buffer). All chemicals were from Sigma-Aldrich St. Louis, MO USA. The activity was measured using keratinase assay.

Hydrolysis of Protein substrates by Purified Keratinase

To determine the hydrolysis of protein substrate by purified keratinase, the substrates (2% final concentration) include bovine albumin (Research Organics, Cleaveland OH. USA), Casein (Uni Chem, Greenville California USA), Collagen-I and Collagen-III ((Bio Trend, Destin USA), Gelatin (Sigma- Aldrich St. Louis, MO USA) and Keratin (MP Biomedicals, California USA) were incubated 37^{o} C for 30 minutes with 200 µl purified keratinase and activity was measured by keratinase assay.

Effect of Inhibitors on Keratinase Activity

The effect of different inhibitors on keratinolytic activity of purified enzyme was assessed at 37 ^{o}C using 5mM phenylmethylsulfonyl fluoride (PMSF), 10mM ethylenediamine tetraacetic acid (EDTA) and 10mM mercuric chloride (HgCl2). All chemicals were purchased from Sigma-Aldrich St. Louis, MO USA. The 200µl purified enzyme was incubated with 1mL of each inhibitor at 37 ^{o}C for 10 minutes. After incubation, 1mL of keratin azure solution was added into each of the tubes and further incubated at 37 ^{o} C for one hour. For the control, 200µl enzyme was incubated 172 with 1mL keratin azure solution at 37^{o} C for one hour. After incubation, the reactions were stopped by adding 1mL of trichloroacetic acid (5%) in the test and control tubes; centrifuged at 3000rpm for 10 minutes. The supernatant was spectrophotometrically measured for the release of azo dye at 595nm wave length.

Psoriasis Scale Hydrolysis

Human psoriasis scales (5.0 g, obtained from a local dermatology clinic) were autoclaved and incubated at 37^{o} C for 24-72 h with 5mL (59 U/mL activity) crude enzyme and as control in 5mL basal salt medium as described previously. After incubation, the tubes were observed for the hydrolysis of psoriasis scales and keratinase -treated and untreated scales were observed under microscope (x40).

Dehairing of Goat Skin

Skin of goat was obtained from local slaughter house. The skin was cut into 2x1 inch pieces and washed with distilled water and autoclaved. One piece of skin was placed into a flask containing 500mL of crude keratinase enzyme (59 U/mL activity) and another piece of skin was placed into another flask as a control containing 500mL of basal salt medium [7]. Then both flasks were incubated in shaking incubator at 37 ^{o}C at 50rpm/min for 24 h. After incubation the skin was observed for dehairing.

RESULTS

Screening of Proteolytic Bacteria from Soil Samples

B. subtilis produced zone of hydrolysis of 8mm on skimmed milk agar as shown in Fig. 1. This indicated that the *B. subtilis* possessed proteolytic activity. The activity in the crude enzyme was also investigated using well diffusion method in gelatin agar. The crude enzyme showed zone of hydrolysis of gelatin hydrolysis as shown in Fig. 2, indicating that proteolytic activity was indeed present.

Fig. 1 Screening of Proteolytic Bacteria from Soil Samples

The figure shows zone of hydrolysis on skimmed milk agar by *B. subtilis* after 24 h of incubation at 37^{o} C.

Fig 2 Screening of Proteolytic Activity of Crude Keratinase

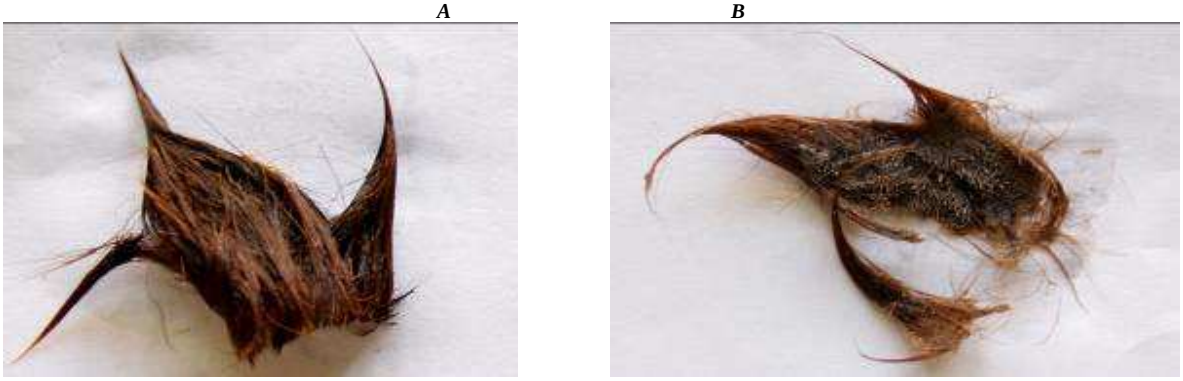
The figure shows zone of hydrolysis on gelatin agar after 24 h of incubation with crude keratinase at 37^{o} C.
S= sample (crude keratinase), C= control (basal salt medium)

Dehairing of Skin by the Enzyme

Dehairing of goat skin was observed as a result of keratinolytic activity shown by the crude keratinase and hair was removed from the skin. It was noted that the dehairing started with in 12 h and complete removal was observed after 24 h incubation. (Fig. 3 A, B and Fig. 4 A, B). However, the skin remained intact when visually inspected

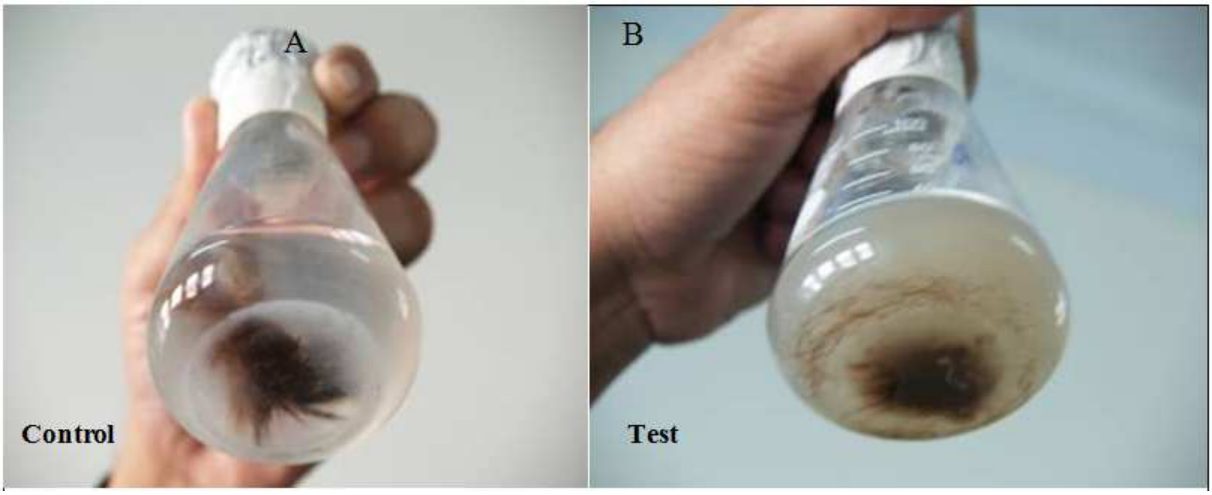
Fig 3 Hair removal from goat skin by keratinase from *B. subtilis*

A ***B***



A= Goat skin used as a control **B= Hair removal from Goat skin (in 24 h)**

Fig. 4 Dehairing activity on goat skin by keratinase from *B. subtilis*



A= Goat skin in basal salt medium used as a control **B= Initiation of de hairing on goat skin (in 12h)**

Psoriasis Scale Hydrolysis

Keratinase enzyme of *B. subtilis* hydrolyzed the scales as revealed by microscopy that bigger scales were converted into small ones. Microscopic observation further showed hydrolyzed cells that were separated from the scales and appeared singly indicating the potential keratinase activity (Table 1)

Table 1 Effect of Crude Keratinase from *B. subtilis* on Human psoriases scales

Time in hours	Cells/HPF Test (+/- SEM)	Cells/HPF Control (+/-SEM)	*p*-value (unpaired *t*-test) Two-tailed
24	44 (6)	39 (4.1)	0.0741
48	86 (9.2)	41(6.3)	0.0002
72	93 (5.g)	51(4.7)	0.0053

The human psoriases scales (5.0 g/50 mL) were incubated with crude keratinase as described in materials and methods. The table shows values in cells/HPF (high power field) under microscope. The values are from two separate experiments.

Purification of Enzyme through DEAE- Sephadex-A50 Chromatography

Keratinase activity was detected from fraction 5 to 10 with peak activity in 8th fraction as determined by keratinase assay. The results of the purification steps are summarized in Table 2. The overall purification factor was about 38 fold**,** and the final yield was 45%. The final product had a specific activity of about 460 U/mg.

Table 2 Purification profile of Keratinase from *B. subtilis*

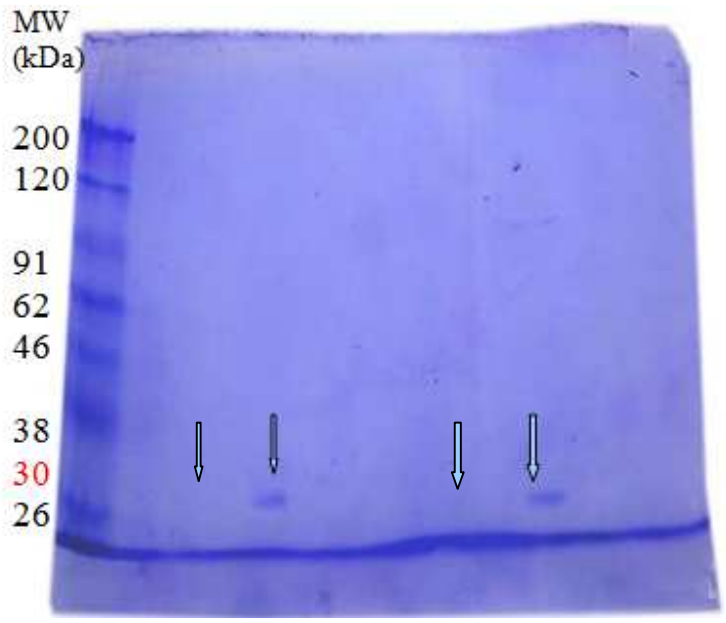
Steps	Volume (ml)	Total activity (U)	Total protein (mg)	Specific activity U/mg	Purification factor	Yield (%)
Crude (1)	250	2783	230 (0.92mg/ml)	12.1	1.0-fold	100
ASP (2)	50	1896	48 (0.96mg/ml)	39.5	3.2-fold	68
DEAE (3) Sephadex-A 50	18	9200	20 (1.1mg/ml)	460	38fold	485

Steps: (1) Crude keratinase; (2) ammonium sulfate precipitation product; (3) chromatography product.

Determination of Molecular Mass of the Purified Keratinase

SDS-PAGE of the pooled fractions revealed a single band at position 30 kDa (Fig. 5) indicated that the enzyme was purified to homogeneity

Fig. 5 SDS- PAGE of the Purified Keratinase Enzyme



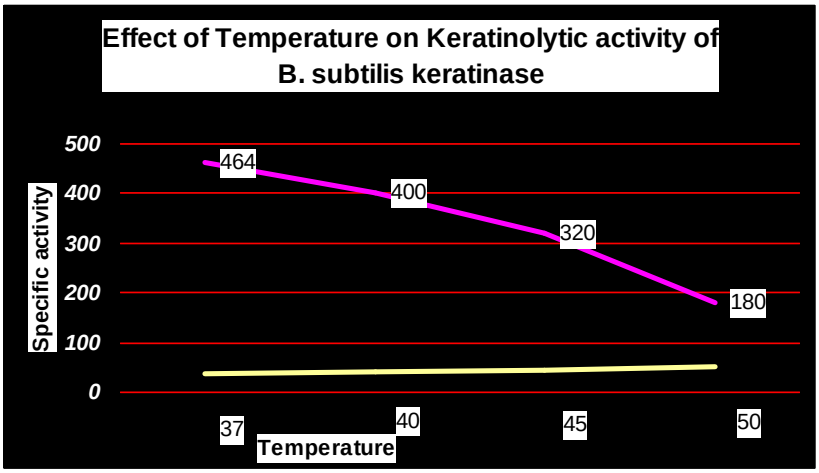
Purified keratinase enzyme (pooled fractions from 5^{th} -10^{th}) was resolved by SDS-PAGE (10%) as described in material and methods. The gel shows molecular weight marker (lane 1 from left side) and purified keratinase at 30 kDa position (lane 4 and 8).

Characterization of the Purified Keratinase Enzyme

Effect of Temperature

Fig. 6 shows the effect of temperature on the hydrolysis of keratin azure by purified keratinase enzyme from *B. subtilis*. Enzyme showed the keratinolytic activity at different temperatures viz 25, 37, 40 and 45^{o} C but at 50^{o} C, declining enzymatic activity was found. The maximum keratinolytic activity (460 U/mg) was observed at 37^{o} C.

Fig. 6 Effect of temperature on keratinolytic activity of *B. subtilis* keratinase

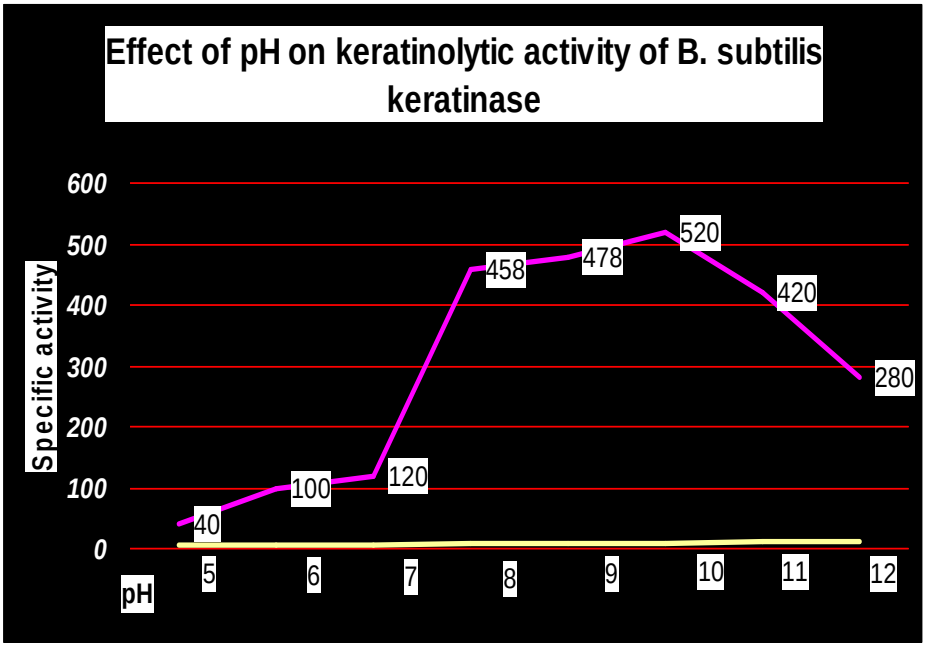


The purified keratinase from of *B. subtilis* was added tol keratin azure suspension and incubated at 37, 40 , 45 and 50^{o} C as described in materials and methods. Pink line represents keratinolytic activity of keratinase at different temperatures. Yellow line represents the activity in the control at temperature tested.

Effect of pH

A range of pH from 5.0 to 12.0 was tested on the purified keratinase enzyme. The enzyme exhibited maximum keratinolytic activity (520 U/mg) at pH 10.0 followed by the 420 U/220 mg at pH 11 and activity was found from pH 8.0 to 11.0 (Fig.7).

Fig. 7 Effect of pH on keratinolytic activity of *B. subtilis* keratinase



The figure shows the effect of pH on keratinase enzyme. Keratinase was assayed at 37^{o} C using buffers of various pH ranging from 5.0 to 12.0 as described in materials and methods. Pink line represents keratinolytic activity of keratinase at different pH. Yellow line represents the activity in the control at different pH tested

Hydrolysis of Protein Substrates by Purified Keratinase

Table 3 shows the hydrolyzing activity of enzyme on different protein substrates (bovine albumin, casein, collagen-I, collagen-III, gelatin and keratin) by purified keratinase enzyme of *B. subtilis*. It was observed that gelatin and keratin were preferred substrates where greater enzyme activity was observed (540U/mL and 260 U/mL respectively) followed by casein and bovine albumin. No activity was found against collagen I and III.

Table 3 Hydrolysis of Protein Substrates by Purified Enzyme of *B. subtilis*

Protein substrate	Keratinase Activity Unit/ml	Specific Activity Unit/mg	SD	SEM
Bovine Albumin	17.5	140	8.49	6
Collagen-I	0	0	0	0
Collagen-III	0	0	0	0
Gelatin	67.5	540	4.24	3
Casein	20	160	2.83	2
Keratin	32.5	260	9.90	7

Values are mean of two separate experiments

Effect of Inhibitors on Keratinolytic Activity

We used PMSF (Phenyl Methyl Sulfonyl Fluoride; a serine protease inhibitor) and EDTA (ethylene diamine tetra acetic acid; a metallo- protease inhibitor) for determining the nature of purified keratinase. It was observed that the activity of purified keratinase produced from *B. subtilis* was completely inhibited by HgCl2 (mercuric chloride) as compared to PMSF and EDTA (Table 4) where partial inhibition was observed, indicating that the enzyme did not belong to serine or metalloprotease but a cysteine protease.

Table 4 Effect of Inhibitors on keratinase enzyme from *B. subtilis*

Inhibitor	Residual Enzyme Activity (%)	SD	SEM
None	100	00	00
$HgCl_2$	00	00	00
PMSF	50	7.07	5
EDTA	75	2.83	2

Values are mean of two separate experiments

DISCUSSION

This study presents the keratinase production from an indigenous *B. subtilis* strain isolated from Khairpur district Sindh Pakistan. The screening study revealed that this strain possessed keratinase activity. The isolation of keratinase enzyme has previously been reported from *B. licheniformis.* [11] as well as from other *Bacillus* species [12-15]. Keratinase from *B. subtilis* has been reported by Cheng et al. [5] however, in present study the keratiolytic activity was accompanied with dehairing property without collagenolytic activity. Similar effects have also been reported previously [16-18]. Scales of skin from a patient of psoriasis were partially converted into single cells when incubated with crude keratinase. It is assumed that alkaline conditions aid in breaking disulfide linkages and assist rapid degradation of keratin. This indicated that this activity of the enzyme could further be employed for future pharmaceutical applications Single band of resolved purified protein on SDS-PAGE showed that the keratinase was purified to homogeneity. The overall purification factor was about 38 fold**,** and the final yield was 45%. The final product had a specific activity of about 464 U/mg. The estimated molecular mass of keratinase in present study appeared to be 30kDa. Molecular masses of keratinases from 18 to 200 kDa have been reported in literature [5]. For *Bacillus* species, they are of medium size, such as 33 kDa (*B. licheniformis*) [11], and 24 kDa [19].

The complete mechanism of temperature control in enzyme production is although not well understood [20]; the enzyme synthesis and energy metabolism in *Bacillus* is controlled by temperature and oxygen uptake [2]**.** The keratinase enzyme activity was retained up to 50^o C and thereafter declined. This indicated that the keratinase from this strain was thermotolerant. The scope of this study was to evaluate thermostablity of the keratinase therefore activity assays at lower temperatures were not performed. In published literature, temperature optima for protease production from *Bacillus* varied from 34 to 60° C depending on the bacterial strain used. It has been reported that optimum temperature for maximum protease production in *B. alcalophilus* [21], *B. pumilus* [18], and *Bacillus* sp.

JB-99 [22] was in mesophilic to thermophilic ranges. Our study support previous reports as *B. subtilis* growth at 25° C in present study was very slow that indicated the mesophilic nature of this strain (data not shown).

The pH is an important factor which affects the cellular function. The pH significantly determines abiotic factors, such as carbon availability [6] nutrient availability and the solubility of metals [23, 24]**.** Higher activity of keratinase in present study was obtained at pH 10 where for the growth, pH 7.0 was optimum. This showed that the *B. subtilis* was neutrophilic strain but the keratinase was alkaliphilic. A similar observation has been reported by Han et al**.** [13] for different strains of *Bacillus*. The high pH requirements for maximum protease production by *Bacillus* sp. are reported in literature [22 -24, and 25] which is consistent with our findings.

In present study, three enzyme inhibitors viz. HgCl2, PMSF and EDTA were used to observe their effect on the activity of keratinase from the *B. subtilis.* No complete inhibition was observed with EDTA and PMSF suggesting that the protease was not a metallo-protease or senine protease respectively. However, complete inhibition with HgCl2 occurred may be due to surfactant effect on the enzyme that categorized it as a cysteine protease. Substrate specificity by keratinase from *B. subtilis* was high for gelatin followed by keratin, casein, and bovine albumin and enzymatic activity was absent on collagen- I and collagen- III that is a desirable feature of a protease for leather industry. The keratinase dehaired the goat skin within 24 h at pH 8.0 and ambient temperature 37^{o} C. Earlier studies with the dehairing protease from *B. subtilis* S14 have reported elastase, keratinase and collagenase activities [14] while the present enzyme had no collagenolytic properties. The enzyme used in dehairing process should not be collagenolytic in nature, so that hide matrix remains intact. pH plays an important role in dehairing process [16]. Similar to our study, Annapurna et al. [17] has reported pH 10 for optimum dehairing activity of an enzyme isolated from *Bacillus* sp. In general, the dehairing process requires activity of enzyme under alkaline condition; this criterion was satisfied by keratinase from *B. subtilis* in our study and this novel enzyme could be used in pharmaceutical applications and leather industry and as additives in animal feed to improve feather meal digestibility.

CONCLUSION

Indigenous strain *Bacillus subtilus* was isolated identified and characterized for the production of keratinase enzyme. This novel strain produced secreted, extracellular keratinase at 37 ^{o} C, pH 7.0. Purified enzyme was highly active at 37 ^{o} C and retained its activity up to 50 ^{o} C and at alkaline pH 10- 12 (pH optima 11.0). PMSF and EDTA partially inhibited the enzyme while HgCl2 substantially inhibited the activity indicates that it is a cysteine protease. The enzyme possessed good dehairing property and ability to hydrolyze psoriasis scales. For the first time, we report this new keratinase that is able to de-hair without sodium sulfide. The processing time, as well as the pH activity range and the avoidance of collagen damage, are properties that make this new enzyme an excellent candidate for industrial applications.

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