Appl Biochem Biotechnol (2016) 178:1666–1686
DOI 10.1007/s12010-015-1974-7

Production, Purification, and Biochemical Characterization of Thermostable Metallo-Protease from Novel *Bacillus alkalitelluris* TWI3 Isolated from Tannery Waste

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Received: 28 October 2015 / Accepted: 28 December 2015 /
Published online: 9 January 2016

Abstract Protease enzymes in tannery industries have enormous applications. Seeking a potential candidate for efficient protease production has emerged in recent years. In our study, we sought to isolate proteolytic bacteria from tannery waste dumping site in Tamilnadu, India. Novel proteolytic *Bacillus alkalitelluris* TWI3 was isolated and tested for protease production. Maximum protease production was achieved using lactose and skim milk as a carbon and nitrogen source, respectively, and optimum growth temperature was found to be 40 °C at pH 8. Protease enzyme was purified using ammonium sulfate precipitation method and anion exchange chromatography. Diethylaminoethanol (DEAE) column chromatography and Sephadex G-100 chromatography yielded an overall 4.92-fold and 7.19-fold purification, respectively. The 42.6-kDa TWI3 protease was characterized as alkaline metallo-protease and stable up to 60 °C and pH 10. Ca^{2+}, Mn^{2+}, and Mg^{2+} ions activated the protease, while Hg^{2+}, Cu^{2+}, Zn^{2+}, and Fe^{2+} greatly inhibited it. Ethylenediaminetetraacetic acid (EDTA) inhibited TWI3 protease and was activated by Ca^{2+}, which confirmed that TWI3 protease is a metallo-protease. Moreover, this protease is capable of dehairing goat skin and also removed several cloth stains, which makes it more suitable for various biotechnological applications.

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Keywords Protease · *Bacillus alkalitelluris* · Tannery · Dehairing · Destaining

Introduction

Hydrolytic enzymes play a crucial role in protein hydrolysis via disrupting the peptide bonds between amino acids in polypeptide chains. Proteases are among most vital hydrolytic enzymes which are predominantly synthesized by a diverse group of microorganisms (i.e., bacteria, yeast, and molds) and also found in plant and animal tissues [1]. Proteases account for 60 % of the total enzyme sales in world market, which is used in various industries including food, leather, dairy, pharmaceutical, diagnostic, and waste management [2, 3].

Based on the enzymatic activity at various pH conditions, microbial proteases are classified as acidic, neutral, and alkaline proteases. Among all the protease classes, alkaline proteases are most commonly used in many industries due to its versatile activity. Alkaline proteases are used in detergent industries to remove the proteinaceous dirt from cloths and also used in the tannery industries to remove hairs from animal skin [4, 5].

In recent years, tannery industries have been significantly contributing in the economic development of developing countries. The waste produced from these industries has also increased excessively. The pollution control board's screening of waste disposal from these industries and awareness among the consumers create a plenty of pressure on the leather industries to develop a new method of leather processing [6]. A major bottleneck in the leather processing is to dethrone the hair from the skin without inflicting damage. Traditionally hair is removed by using highly concentrated saturated lime and sodium sulphide [7]. The boundless utility of toxic chemicals (i.e., sulphide) pollutes the environment and also intensely affects the health of leather industry workers. Hence, the leather industries tend to implement enzymatic dehairing process, which is more economical and environmentally secure [8].

Although many proteases have been screened and isolated from diverse group of microorganisms, the search for a novel enzyme with added potent dehairing properties without supplementation of chemicals is increasing up to the minute. The chief objective of this study was to isolate the protease-producing bacterium from the dumping sites of tannery industries and optimize the production parameters for maximal protease production. Apart from this, we intend to purify the protease enzyme and analyze its stability under distinct conditions. Finally, we intend to assess the enzyme's dehairing properties on goat skin and its stain-removing ability for blood and dirty motor oils.

Materials and Methods

Collection of Soil and Water Samples

The protein-rich tannery effluent samples were obtained from two discrete places of combined effluent of tannery industries at Thomaiyarpuram (10° 19′ 59″ N latitude and 77° 57′ 31″ E longitude), Dindigul district, Tamilnadu, India. Soil and water samples were aseptically collected in presterile containers and transported to the microbiological laboratory for further screening at 4 °C.

Bacterial Isolation and Screening for Protease Production

The protease-producing bacterial strains were isolated from tannery effluent samples by serial dilution method. Appropriate dilutions were spread on skim milk agar media containing peptone (0.1 %, *w*/*v*), NaCl (0.5 %, *w*/*v*), agar (2.0 %, *w*/*v*), and skim milk (10 %, *w*/*v*) and incubated at 37 °C for 48 h. After incubation, individual colonies were maintained as a pure culture in agar stabs and glycerol stocks. To screen the potential protease-producing bacteria, all pure cultures were spread on same medium. Formation of halo zone around colonies resulting from casein hydrolysis was considered as confirmation of proteolytic activity. Totally, 12 strains (6 from soil and 6 from water) produced clear zone around the colonies, and TWI3 was selected for further study. Morphological and physiological characteristics of TWI3 were observed according to standard protocols [9].

Identification by 16S rDNA Sequencing and Phylogenetic Analysis

The proteolytic strain TWI3 was further identified by 16S rRNA gene sequencing employing standard protocol [9]. The overnight bacterial cultures were used for genomic DNA isolation using DNeasy Blood and Tissue kit (Qiagen, USA). Universal bacterial primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) were used to amplify the 16S rDNA from genomic DNA. PCR reaction comprising 35 cycles, including initial denaturation (94 °C for 5 min), denaturation (94 °C for 1 min), annealing (56 °C for 30 s), and elongation (72 °C for 1.5 min). The final PCR products were purified by QIAquick PCR purification kit (QIAGEN, India). Purified PCR products were sequenced by using automated gene sequencer (ABI prism 3730 DNA analyzer, USA). Resulting sequence was searched for sequence similarity in GenBank database. The alignment of partial 16S rRNA sequence was performed by using basic local alignment search tool (BLASTn). Phylogenetic tree was constructed by using Mega 6 software. Approximately 1400 bp of the 16S rRNA gene sequences of the strains and related species were used for reconstructing phylogenetic trees with the neighbor-joining method. The statistical reliability of the tree was evaluated by bootstrap analysis of 1000 replicates. *Escherichia coli* were used as outgroup.

Protease Enzyme Production

Pure culture of each isolates was inoculated (1.0 O.D at A_{600}) into the 50-ml production medium containing 0.1 % (*w*/*v*) peptone, 0.5 % (*w*/*v*) glucose, 0.05 % (*w*/*v*) NaCl, and 0.01 % (*w*/*v*) $MgSO_4 \cdot 7H_2O$ and incubated at 37 °C for 48–72 h in shaking incubator at 150 rpm. The fermented broth was centrifuged at 6000×*g* for 15 min at 4 °C. The cell-free culture supernatant (CFCS) was filter sterilized through 0.22-μm membrane filter (Millipore, USA) and used as enzyme source for protease assay.

Purification of Protease Enzyme

The CFCS was collected after 48 h of incubation by centrifugation at 8000×*g* for 20 min at 4 °C. The saturated ammonium sulfate was added to the CFCS and incubated at 4 °C for 3 h. The concentration of ammonium sulfate was increased in 20 % increments from 20 to 80 % saturation in order to precipitate enzyme. The precipitate was separated by centrifugation at 10,000×*g* for 10 min at 4 °C and dissolved in 20 mM Tris hydrochloride (pH 8) and dialyzed

against the same buffer at 4 °C for 24 h using 12- and 14-kDa cutoff dialysis membrane (HiMedia, India) by continuous stirring [10]. The dialyzed enzymes were pooled together and eluted by DEAE-cellulose column (5 cm × 25 cm) with linear gradient of 0–0.75 mol/l NaCl in buffer. All the protein fractions were stored at −20 °C, further purified using Sephadex G-100 column equilibrated with 0.05 M of phosphate buffer. The protein fractions were collected and protein concentration was measured at 280 nm (Spectronic 20D, India). To avoid the denaturing of protein, all the steps were performed at 4 °C. Purified protein fractions were determined by the method described Bradford [11] using bovine serum albumin (BSA) as standard. To determine the molecular weight of the purified protease 12 % Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli [12].

Protease Activity Assay

Protease assay was conducted by using casein as a substrate as described by Kunitz [13] with some modifications. Summarily, 0.5 ml of enzyme was mixed with 0.5 ml of 2 % casein (dissolved in 0.1 M phosphate buffer of pH 10.0) solution incubated at 40 °C for 10 min. After incubation, the reaction was terminated by addition of 1 ml chilled 10 % (*w*/*v*) trichloroacetic acid (TCA). For control assay, 0.5 ml of heat killed enzyme was used as control; similarly, a test blank was also prepared by adding TCA prior to addition of enzyme. The reaction mixture was centrifuged at 6000×*g* for 15 min at 4 °C, and 1 ml of supernatant from test and control tubes was transferred to fresh tubes. In each tube, 5 ml of 0.44 M Na_2CO_3 and 1 ml of 0.5 N Folin Ciocalteu reagents (FCR) were added and incubated at 30 °C in dark. The absorbance was measured at 660 nm (Spectronic 20D, India). One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol tyrosine per minute.

Optimization of Cultural Conditions

The optimal conditions required for the bacterial growth such as temperature and pH were optimized. To optimize the temperature, production medium (pH 7) was inoculated with test strain and incubated at different temperatures (20 to 80 °C) for 48 h and assayed for enzyme activity. Similarly, the initial pH of the growth medium was adjusted ranging from 4 to 10 with 1 N HCl or 1 N NaOH solution. During pH optimization, the temperature (30 °C) and incubation period (48 h) were maintained constant.

Moreover, the optimal incubation period for higher protease production was also determined by subjecting the inoculum to varying incubation periods such as 0, 24, 48, and 72 h while keeping other parameters such as pH and temperature constant and assayed for enzyme activity. Enzyme production was directly correlated with the concentration of inoculum provided in the production medium; therefore, the effect of inoculum size on protease production was performed. Varied inoculum concentrations (i.e., 0.5, 1, 1.5, 2, and 2.5 ml) of isolate were inoculated to production medium and protease assay was executed.

Optimization of Carbon and Nitrogen Sources

In order to determine the appropriate carbon and nitrogen source for maximal protease production, different carbon sources such as fructose, sucrose, lactose, starch, glucose, and maltose were substituted at 1 % (*w*/*v*) concentrations as a sole carbon source. The enzyme assay was performed after 48-h incubation.

Similarly, the effect of various organic nitrogen sources on the growth and enzyme production of TWI3 were determined by substituting peptone with other nitrogen sources such as beef extract, yeast extract, casein, soybean, and skim milk. During this process, the other growth conditions such as temperature, pH, and incubation period were kept constant.

Characterization of Protease Enzyme

Effect of pH on the Stability of Enzyme

The pH stability of the protease enzyme was investigated at various pH ranging from 4 to 12. Casein (2 %) was used as a substrate for enzyme assay, and various buffer systems, i.e., sodium acetate (pH 4–6), phosphate buffer (pH 7), glycine–NaOH (pH 8 and 9), and carbonate buffer (pH 10–12), were used to adjust the pH in enzyme reaction. Two millimeters of enzyme was mixed with 2 ml of 0.1 M above mentioned buffer solutions and incubated at 40 °C for 2 h. The activity of enzymes was examined by previously mentioned assay method.

Effect of Temperature on the Stability of Enzyme

Thermal stability of protease enzyme was determined by pre-incubating the enzyme (pH 8) at different temperatures ranging from 30 to 90 °C for 30 min. The enzyme without heat treatment was considered as control (100 % activity). The activity of enzymes was assayed by previously mentioned assay procedure.

Effect of NaCl Concentration on the Stability of Enzyme

Halo stability is a vital characteristic of protease enzyme for tannery saline waste water treatment; hence, the effect of various NaCl concentrations on protease activity was investigated by incubating the reaction mixture with equal volumes at varying NaCl concentrations ranging from 0.5 to 2.5 M and incubated for 20 min at 40 °C. The residual activity of protease was measured as per the standard assay procedure.

Effects of Metal Ions on Protease Activity

The effect of various metal ions on the activity of purified protease enzyme was estimated by using reaction mixtures containing $HgCl_2$, $FeSO_4$, $CuSO_4$, $CaCl_2$, $MnSO_4$, $MgSO_4$, $ZnSO_4$, KCl, and $NiCl_2$ at the concentration of 1 and 5 mM and was incubated for 1 h at 40 °C. Reaction mixture without metal ions was considered as control (100 % enzyme activity). The enzyme activity was measured by previously mentioned procedure.

Effect of Inhibitors on Protease Activity

The effect of different protease inhibitors on the activity of protease enzyme was tested using ethylenediaminetetraacetic acid (EDTA), phenylmethylsulfonyl fluoride (PMSF), β-mercaptoethanol, and indoacetamide at the concentration of 1 and 5 mM; reaction mixture without inhibitor was used as control.

Effect of Detergent on Protease Activity

The effect of detergents and oxidants on protease enzyme was analyzed using sodium dodecyl sulphate (SDS), Tween 80, Triton X-100, and H_2O_2 at the concentration of 1 and 5 % (*w*/*v*). To examine the effect of inhibitors/detergents, purified enzyme was pre-incubated with and without each inhibitor/detergents (control) for 1 h at 40 °C. The protease assay was performed using standard protocols.

Substrate Specificity and Enzyme Kinetics

Substrate specificity of purified protease enzyme was estimated using various natural substrates such as casein, gelatin, bovine serum albumin, chicken feather keratin, human-hair keratin, and collagen. The enzyme activity was measured by incubating 200 μl of purified enzyme with 200 μl of substrate (5 mg/ml) in 100 mM phosphate buffer (pH 7) at 40 °C. To obtain kinetic constants (K_m and V_{max}) of purified protease, initial velocity of casein hydrolysis was determined and casein was used as a substrate at various concentrations (0.1–2 %, *w*/*v*).

Dehairing of Goat Skin

The goat skin was procured from slaughter house and cut into smaller pieces (5 cm^2) for dehairing experiment. The goat skin was soaked in water for several hours to remove unwanted dirt, blood, and other contaminants. Each piece of goat skin was incubated with 2 % enzyme (50 U ml^{-1} in 100 mM glycine–NaOH buffer, pH 10.0) and 10 % water. Control experiment was performed by incubating goat skin with a paste of lime (10 %) and 3 % sodium sulfide (60 % purity) and 10 % water for 18 h at 37 °C. After incubation, the skin was dehaired manually and analyzed visually [4, 6].

Destaining of Dirty Motor Oil and Blood Stain

To perform the destaining experiment, protease enzyme was tested for its compatibility with commercial detergent (Surf Excel™). The detergent was diluted with distilled water (7 mg/ml) and incubated with crude enzyme for 30 min at 60 °C, and residual protease activity was determined. Application of protease as a detergent was studied on white cloth pieces (10 cm × 10 cm) stained with blood and grease/motor oil. The stained cloths were treated with 1 ml commercial detergent (7 mg/ml) and/or 2 ml enzyme (1000 IU/ml) with 100 ml of distilled water in separate flask and incubated at 60 °C for 30 min. After the incubation, cloth pieces were taken out, rinsed with water, and dried. Visual examination of various pieces exhibited the effect of enzyme on removal of blood and grease stains. Untreated white cloth with blood stain and grease stain were used as control [14].

Scanning Electron Microscopy

To analyze the surface and cross section of enzyme-treated goat skin, samples were dehydrated and freeze dried. Freeze dried samples were chopped into 5 × 2 mm size and mounted on aluminum stubs and sputter-coated with gold. The electron micrographs of both surface and cross-sectional view were recorded at various resolutions using Vega3 TE Scan (USA) electron microscope with an accelerating voltage of 10 kV [8].

Statistical Analysis

All experiments were done in triplicates, and the results were expressed as mean ± standard deviations. SPSS 13.0 statistical software (SPSS Inc., Chicago, IL, USA) was used for data analysis. To study the significant differences between samples, one-way ANOVA was calculated with a significance level of $P < 0.05$.

Results and Discussion

Isolation and Identification of Proteolytic Bacteria

In this study, proteolytic bacterial strains were isolated from tannery soil and effluent. After the incubation period, we selected 12 colonies based on zone around the colonies which is the indicator for protease production. Among them, six colonies were isolated from tannery soil (TSI3, TSI4, TSI5, TSI6, TSI7, and TSI10), and six colonies were isolated from tannery effluent (TWI1, TWI3, TWI6, TWI8, TWI9, TWI10). Individual colonies were purified through repeated streaking on fresh agar plates. All positive protease producing colonies were cultivated in casein liquid medium for 2 days, and protease activity was measured. The strain TWI3 exhibited higher proteolytic activity (275 ± 2.8 U/ml) compared with others (Fig. 1) and selected for further studies.

Identification and Molecular Characteristics of TWI3

The bacterial strain TWI3 is a Gram-positive, rod-shaped, facultatively anaerobic bacterium producing endospores and motile by means of peritrichous flagella. The strain is oxidase-positive and able to grow well at pH 7.0–11 (optimal pH 8) and at 20–50 °C (optimal 40 °C). Based on Bergey's Manual of Determinative Bacteriology, these physiochemical experiment results suggested that this TWI3 strain belongs to the *Bacillus*

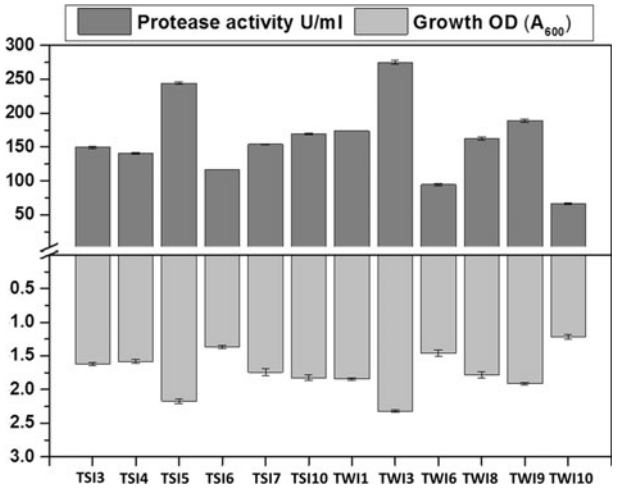

Fig. 1 Protease activity of bacterial strains isolated from tannery soil and tannery effluent

family. The 16s rRNA sequencing and pylogenetic analysis revealed that the TWI3 is affiliated with genus *Bacillus* and closely related to *Bacillus alkalitelluris* (99 % identity), and the closet neighbors were found to be *Bacillus salsus* A24 (96 % identity) and *Bacillus litoralis* KUDC1714 (95 %) [15]. The genomic G + C content of TWI3 strain was found to be 35.85 mol%. The 1236-bp 16s rRNA sequence was deposited in NCBI database under the accession number of KT751273 (Fig. 2).

Protease Production by TWI3

To determine the optimal cultivation time required for the maximal growth and production of alkaline protease, we performed growth kinetic study for 96 h using production medium. After a 4-h lag phase, exponential growth was observed up to 36 h, reaching stationary phase after 42 h (Fig. 3). The strain TWI3 started secreting the alkaline protease enzyme at early exponential phase (6 h), but the maximal protease production was achieved at the beginning of stationary phase (325.86 ± 24.42 U/ml). A similar kind of secretion pattern was observed by Joo et al. [16]. We found sharp decline in protease production after 42 h, which may be due to the autolysis of protease enzyme, triggered by accumulation of enzyme in production media. Apart from this, we found that the protease production was directly proportional to the bacterial growth (Fig. 3).

Purification of Protease Enzyme

Extracellular alkaline protease from culture supernatant of *B. alkalitelluris* TWI3 strain was purified by 20 to 80 % of ammonium sulphate precipitation followed by DEAE-cellulose and Sephadex G-100 column chromatography with the recovery of 71.68, 21.62, and 6.81 %, respectively (Table 1). The final purification fold is 7.19, which has specific activity around 421.16 U/mg. Molecular weight of purified alkaline protease was determined by SDS-PAGE

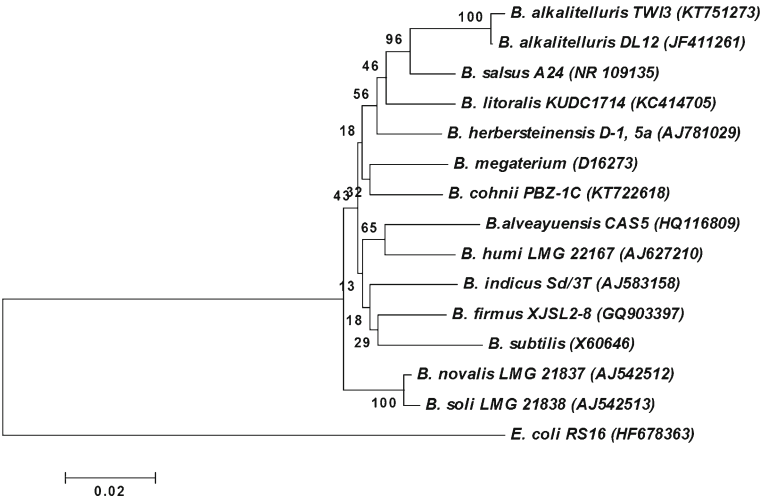

Fig. 2 Neighbor-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the relationships between strain TWI3 and related taxa. Numbers at branching nodes are bootstrap percentages (based on 1000 replications)

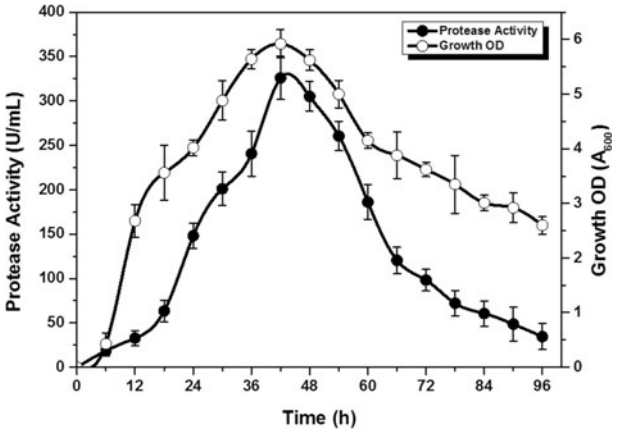

Fig. 3 Growth kinetics (*filled circles*) and protease production (empty circle) of TWI3 strain. Cells were grown in production media at pH 7 at 37 °C with 150 rpm agitation. Samples were collected in every 6 h. Results were expressed as mean ± standard deviations of three replicates

analysis, and a single band was observed around 42.6 kDa and was also confirmed by zymogram analysis using casein as a substrate (Fig. 4).

Optimization of Temperature and pH

The major difficulty in production of protease enzyme is to optimize the production parameters such as temperature, pH, and suitable carbon and nitrogen sources for higher production in industries. The protease production is severely affected by media components such as C/N ratio, availability of metabolizable sugars, salts, and various ions [17, 18]. The TWI3 strain exhibited cell growth and protease production at various temperatures ranging from 20 to 80 °C. The maximum cell growth and protease production were observed at 40 °C (Fig. 5a), and protease production decreased to 45.36 and 31.12 U/ml at 70 and 80 °C, respectively, which suggests that the enzyme production sharply decreased at elevated temperature ranges. On the other hand, TWI3 showed ability to grow at high temperatures up to 70 °C, which indicates that this *B. alkalitelluris* TWI3 is a thermotolerant bacterium.

Similarly, the bacterial growth and enzyme production were analyzed at various acidic and alkaline pH conditions (4 to 10). The TWI3 strain is able to grow well at all tested pH ranges, but maximal cell growth and protease production (279.4 U/ml) were recorded at pH 8, and the protease production slightly decreased to 256.4 and 251.2 U/ml at pH 9 and 10, respectively

Table 1 Purification profile of alkaline protease from *B. alkalitelluris*TWI3

Purification steps	Total activity (U min^{-1})[a]	Total protein (mg)	Specific activity (U mg^{-1})	Recovery (%)	Purification fold
CFCS	918.46	69.34	167.26	100	1.0
Ammonium sulphate precipitation (80 %)	643.14	18.35	238.42	71.68	2.36
DEAE-cellulose	218.49	6.35	318.49	21.62	4.92
Sephadex G-100	103.15	1.63	421.16	6.81	7.19

CFCS cell-free culture supernatant

[a] Total activity was measured spectrophotometrically using casein as substrate

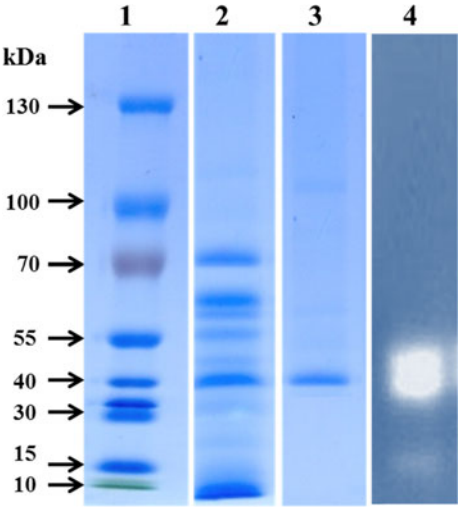

Fig. 4 SDS-PAGE analysis of crude and purified protease enzyme from TWI3 strain. *Lane 1*: molecular marker (PageRuler™ Prestained Protein Ladder, 10 to 180 kDa). *Lane 2*: crude protease. *Lane 3* purified protease. *Lane 4*: zymogram of purified protease with casein as a substrate

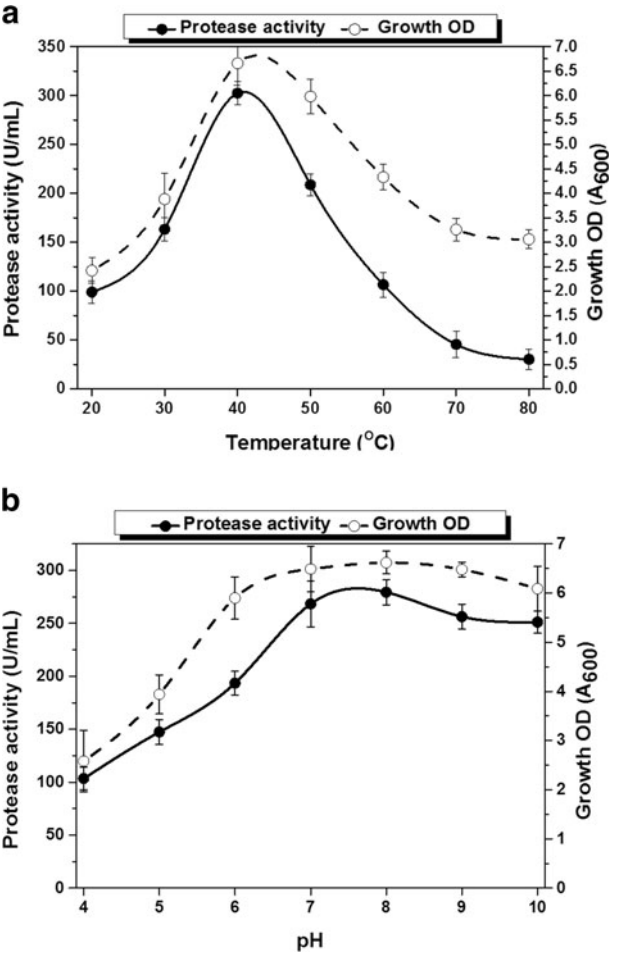

Fig. 5 Effect of temperature (**a**) and pH (**b**) on the growth and production of protease enzyme from TWI3

(Fig. 5b). Optimum pH for the protease production was 8 to 10, which suggests that alkaliphilic nature of the organism and enzyme.

Optimization of Carbon and Nitrogen Sources

The microorganism required specific nutrient supplement for their growth and production of several metabolites. Carbon and nitrogen are very essential elements for normal cell growth and functions. To optimize the effective carbon and nitrogen sources, different carbon and nitrogen sources were supplemented (1 % *w*/*v*) to the production medium. The *B. alkalitelluris* TWI3 demonstrated diverse growth rate and protease production in various carbon sources. The maximum protease production was observed in media supplemented with lactose as a carbon source (316.2 U/ml). However, addition of glucose, maltose, and starch as a carbon source drastically decreased the protease production to78.5, 73.69, and 66 %, respectively, compared to maximum yield (Fig. 6a).Vijay Anand et al. [19] and Ibrahim and Al-Salamah [20] reported earlier that lactose can increase the protease production. Previous studies have postulated that enzyme production is regulated by physiological mechanism, and addition of

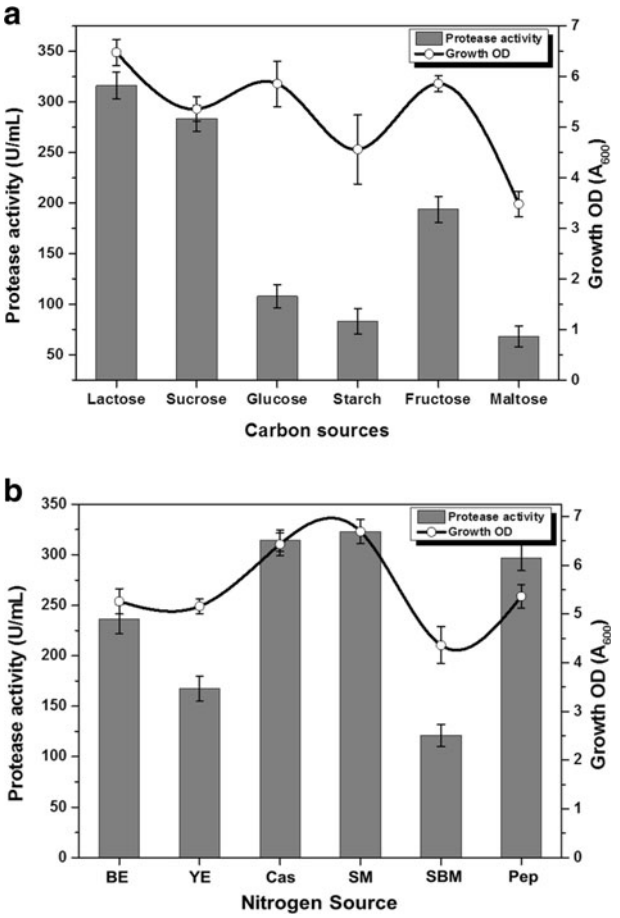

Fig. 6 Effect of various carbon (**a**) and nitrogen (**b**) sources on the growth and production of protease enzyme from TWI3

pure carbon sources (i.e., glucose and maltose) represses the enzyme production via catabolic repression in liquid culture media [21, 22].

To optimize the suitable nitrogen source for higher protease production, distinct organic nitrogen sources were tested since inorganic sources are expensive and hence not suitable for large scale production. The maximum protease production was observed with the addition of skim milk (322.8 U/ml) followed by casein (314.05 U/ml), peptone (297.0 U/ml), and beef extract (236.4 U/ml; Fig. 6b). In previous reports, several other authors have proved that the organic carbon sources such as skim milk [23], casein [24], peptone [25], and beef extracts [26] increased the protease production when supplemented at 1 % concentrations by using other proteolytic bacteria. Supplementation of soybean meal and yeast extract did not increase the protease production.

Characterization of Purified Protease Enzyme

Effect of pH on Protease Enzyme

The purified protease enzyme from *B. alkalitelluris* TWI3 showed catalytic activity in wide ranges of alkaline pH (8 to 11) indicating that the enzyme is an alkaline protease. The maximum relative protease activity (100 %) was observed at pH 10. Furthermore, it also exhibited 42.17 and 63.18 % relative activity at pH 6 and 12, respectively (Fig. 7a). These findings suggest that protease from TWI3 is active at wide ranges of pH. In addition, TWI3 protease showed maximum stability at pH 10 (100 %) and also retained 98 % stability at pH 8, 9, and 11 (Fig. 7a). The wide pH stability of TWI3 protease suggests that this could be a potential candidate to be used in the tannery and detergent industries.

Effect of Temperature on Protease Enzyme

To determine thermostability of TWI3 protease, the enzyme was pre-incubated at wide ranges of temperature (30 to 90 °C). Maximum relative protease activity was observed at 60 °C (98.46 %) and enzyme activity slightly decreased at 70 °C (85.18 %) and 80 °C (64.32 %; Fig. 7b). TWI3 also showed little activity at 90 °C (34.32 %). Similarly, the TWI3 protease enzyme showed 100 % stability up to 60 °C and decreased slightly at increased temperature. These results indicate that the purified protease form TWI3 is a thermostable protease enzyme. Thermostability is a key characteristic of protease enzymes required in various industries (i.e., detergent and leather processing). Since TWI3 possess thermostability over wide ranges of temperatures, it can potentially be used in many industries.

Effect of NaCl Concentration on Stability of Enzyme

Halo stability of the purified protease enzyme was determined by addition of varying concentrations of NaCl (0–2.5 M). The TIW3 protease displayed significant activity with presence of up to 1.5 M NaCl concentrations; however, the maximum enzyme activity was observed without addition of NaCl. The enzyme activity slightly decreased with increasing NaCl concentration. The stability experiments revealed that TWI3 protease was 100 % stable up to 1.5 M NaCl concentration and slightly decreased at 2 M (84.10 %) and 2.5 M (71.19 %; Fig.7c). Similar results were observed by Shivanand and Jayaraman [27]. They demonstrated that *Bacillus aquimaris* strain VITP4 was stable up to 2 M NaCl. Principally, TWI3 protease

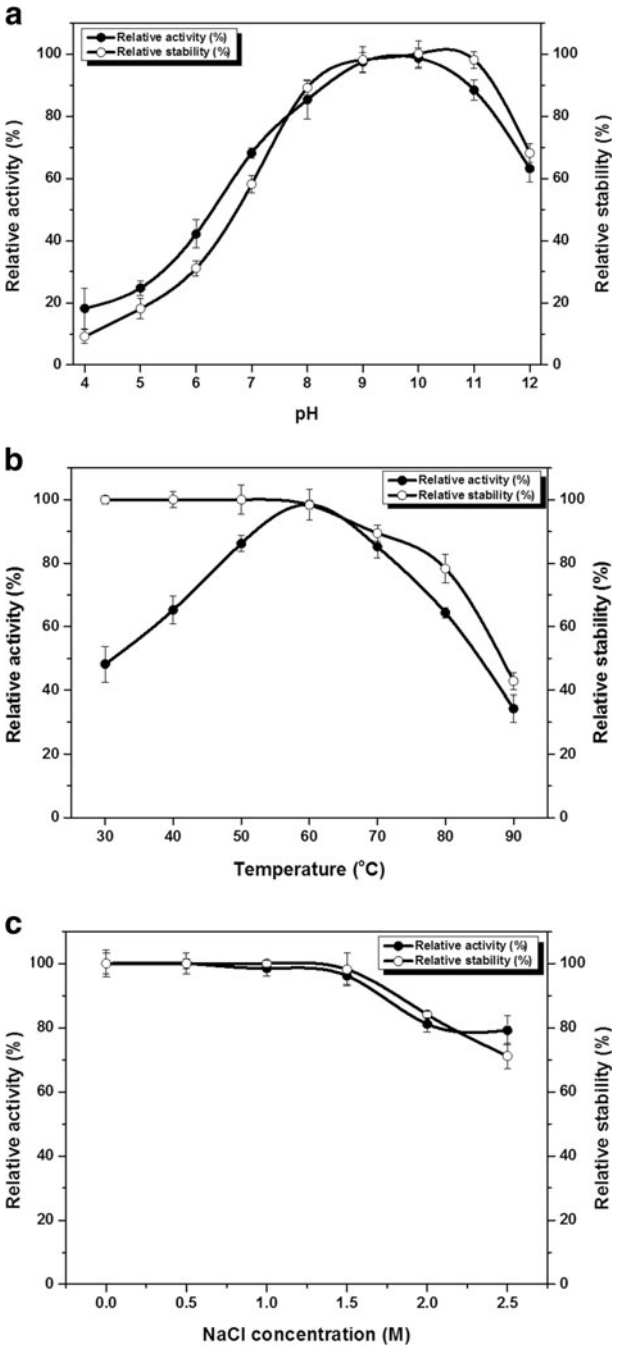

Fig. 7 Effect of temperature (**a**), pH (**b**), and NaCl concentrations (**c**) on relative activity and relative stability of purified protease of TWI3

demonstrated activity and stability at higher concentration of NaCl. This halotolerant nature of this enzyme suggests that this could be a useful candidate for several biotechnological processes involving usage of high salt concentrations such as leather processing.

Effect of Metal Ions on Protease Activity

Metal ions have determined role in the function of an enzyme; some of them stimulate the catalytic process and some of them inhibit it. The effects of various metal ions on protease activity of TWI3 are summarized in Table 2. The protease activity increased remarkably when the enzyme was treated with Ca^{2+} (146.46 %), Mn^{2+} (138.72 %), and Mg^{2+} (123.15 %) at 5 mM concentrations (Table 2). Besides this, significant inhibitory effect on the protease activity was observed with Hg^{2+} (0 % at 5 mM), Ni^{2+} (7.15 % at 5 mM), and Zn^{2+} (18.93 % at 5 mM). Similarly, the enzyme was partially inhibited by Fe^{2+} (76.48 %) and K^{+} (62.18 %) ions (Table 2). Several researchers have postulated that Ca^{2+} ions may stabilize the protease enzyme and prevent it from autolysis, thereby increasing the catalytic activity of the enzyme [14, 24, 28]. Stimulatory effect of Mg^{2+} ion on the proteases of *Bacillus* sp. B001 and *Bacillus alveayuensis* CAS 5 has been reported earlier by several researchers [11, 18]. The stability of *B. alkalitelluris*TWI3 protease in the presence of Ca^{2+} ion makes it a more suitable candidate for usage in the detergent formulations.

Effect of Inhibitors on Protease Activity

The influence of various protease inhibitors on enzyme activity generally provides insights into the nature of enzyme, active site center, and its cofactor requirements [29, 30]. Protease inhibitors such as serine protease inhibitor (PMSF), indoacetamide (cysteine protease inhibitor), and β-mercaptoethanol displayed no significant effect on enzyme activity. While EDTA (metallo-protease inhibitor) caused 67.65 and 97.95 % inactivation of enzyme at 1- and 5-mM concentrations, respectively (Table 3). Protease from TWI3 is stimulated by Ca^{2+} ions and inhibited by EDTA, which clearly indicates that the purified enzyme from *B. alkalitelluris* TWI3 belongs to metallo-protease family. Our findings are concordant with previous research studies which reported alkaline metallo-protease secreted by various halotolerant strains *B. aquimaris* VITP4 [27], *Jeotgalicoccus* sp. [24], *B. alveayuensis* CAS5 [14], *Bacillus* sp. B001 [31], and *Bacillus cereus* [29].

Table 2 Effect of various metal ions on the activity of purified alkaline protease from *B. alkalitelluris*TWI3

Metal ions	Relative activity (%)[a]	
	1 mM	5 mM
Control	100 ± 0.16	100 ± 1.52
$HgCl_2$	2.76 ± 0.56	0.00 ± 0.00
$FeSO_4$	83.46 ± 1.56	76.48 ± 2.65
$CuSO_4$	38.13 ± 6.25	24.15 ± 0.09
$CaCl_2$	138 ± 2.46	146.46 ± 1.92
$MnSO_4$	121.63 ± 1.57	138.72 ± 1.68
$MgSO_4$	118.94 ± 4.18	123.15 ± 1.82
$ZnSO_4$	37.45 ± 2.54	18.93 ± 2.19
KCl	82.14 ± 0.93	62.18 ± 3.56
$NiCl_2$	18.43 ± 1.06	7.15 ± 1.34

[a] Control without addition of any metal ion was considered as 100 % activity (210.2 U/ml)

Table 3 Effect of various inhibitors, detergents, and oxidants on the purified alkaline protease from *B. alkalitelluris* TWI3

	Concentration	Relative activity (%)[a]
Control	0	100 ± 0.15
Inhibitors		
EDTA (mM)	1	32.35 ± 2.45
	5	2.05 ± 0.35
PMSF (mM)	1	96.48 ± 0.92
	5	83.81 ± 1.25
β-Mercaptoethanol (mM)	1	93.45 ± 1.26
	5	91.65 ± 3.16
Indoacetamide (mM)	1	98.64 ± 0.96
	5	92.15 ± 1.86
Detergents		
SDS (%)	1	32.47 ± 1.57
	5	2.47 ± 0.36
Tween 80 (%)	1	93.56 ± 1.56
	5	18.00 ± 0.08
Triton X-100 (%)	1	81.64 ± 1.59
	5	1.36 ± 0.06
Oxidants		
H_2O_2	1	135.62 ± 2.64
	5	108.19 ± 6.56

EDTA ethylenediaminotetraacetic acid, *PMSF* phenymethylsulphonyl fluoride, *SDS* sodium dodecyl sulphate

[a] Control without addition of any inhibitor/detergents/oxidants was considered as 100 % activity (210.2 U/ml)

Effect of Detergent/Oxidants on Protease Activity

The protease enzymes which successfully retain their catalytic efficiency during exposure to various harsh environments are considered for industrial applications. The influence of various detergents and oxidizing agents on TWI3 protease was tested. Significant protease activity was observed when the enzyme was treated with 1 % (*w*/*v*) surfactants such as Tween 80 (93.56 %) and Triton X-100 (81.64 %), but the activity dramatically decreased to 18 and 1.36 %, respectively, with higher concentration of same surfactants (5 % each; Table 3). Similarly, SDS (detergent) strongly inhibits the enzyme activity up to 32.47 and 2.47 % at 1 and 5 %, respectively (Table 3). Uyar et al. [32] and Ahmetoglu et al. [33] reported that most of the available proteases exhibited lower activity and stability at the presence of anionic surfactants including SDS. Additionally, the alkaline protease activity was not affected by the addition of potential oxidizing agent hydrogen peroxide at 1 % concentration but slightly decreased (108.19 %) at 5 % concentration.

Substrate Specificity and Enzyme Kinetics

To identify the appropriate substrate for the maximum protease activity, various natural substrates were employed. The results demonstrated that higher proteolytic activity with

casein followed by gelatin and bovine serum albumin indicates the broad substrate specificity of the enzyme. The TWI3 protease shows little activity on chicken feather keratin as a substrate, but no activity was detected on fibrous proteins such as human hair keratin and collagen (Table 4). Similarly Waghmare et al. [34], Nilegaonkar et al. [31], and Annamalai et al. [14] also demonstrated the higher proteolytic activity with casein, gelatin, and BSA as substrate. To determine the enzyme kinetics of TWI3 protease, Michaelis–Menten's constant (K_m) and maximum velocity (V_{max}) were calculated. The TWI3 had K_m and V_{max} values of 38.0 mg/ml and 253.0 U/ml, respectively.

Dehairing of Goat Skin

Several protease enzymes possess keratinolytic activity along with their protease activity, which is not preferred in leather processing industries for the removal of hair from skin or hide [14]. Similarly, collagenase activity of several proteases is also considered as a major bottleneck in leather processing industries. However, the results from substrate specificity assay revealed that the *B. alkalitelluris* TWI3 protease does not exhibit any keratinase activity. To validate this statement, we also performed the in vivo dehairing experiments using goat skin. We compared the dehairing efficiency of purified TWI3 protease with chemical method and goat skin treated with distilled water and glycine–NaOH buffer was used as control (Fig. 8). The goat skin treated with TWI3 enzyme displayed complete removal of hair by selective degradation of the soft keratin tissue in the follicle, thereby pulling out intact hairs without affecting tensile strength of the leather. On the other hand, chemically treated skin has short hairs on the skin surface. Likewise, enzymatically treated skin appeared to be cleaner, whiter, smooth, and silky (Fig. 8d) compared to the skin treated with chemicals (Fig. 8c) and control (Fig. 8a, b). These results suggests that the enzymatic dehairing of skin/hide can be a good alternative to chemical dehairing process as pointed out by many researchers [35, 36].

Scanning Electron Microscopy

Additionally, supporting our dehairing experimental data, we also used scanning electron microscopy (SEM) to evaluate the effect of enzyme treatment on goat skin. Electron micrographs of enzymatically or chemically treated and untreated goat skin are shown in Fig. 9. The surface view of untreated skin using SEM shows some hair (Fig. 9a), but the chemically treated skin shows some residual hair, which confirms the incomplete removal of hair from the hair follicles (Fig. 9b). However, enzymatically dehaired goat skin was cleaner and the surface

Table 4 Substrate specificity of purified alkaline protease enzyme from *B. alkalitelluris* TWI3

Natural substrates	Enzyme activity (U/ml)
Casein	432.13 ± 13.56
Gelatin	284.16 ± 9.43
Bovine serum albumin	197.52 ± 17.16
Chicken feather keratin	43.15 ± 1.54
Human hair keratin	0
Collagen	0

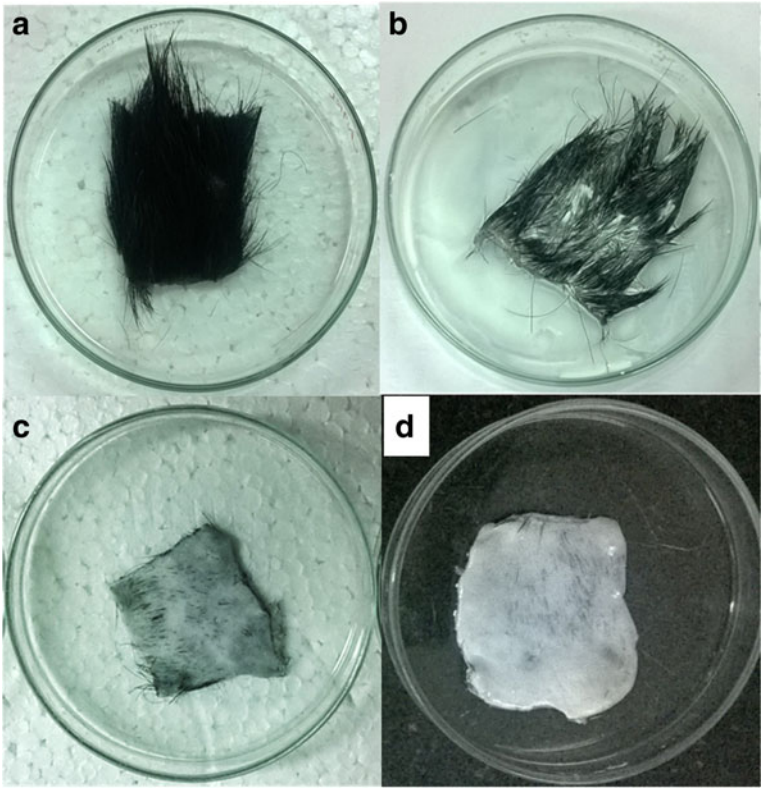

Fig. 8 Dehairing of goat skin (**a**) goat skin treated with distilled water, (**b**) goat skin treated with 100 mM glycine–NaOH buffer (pH 10.0), (**c**) chemically treated goat skin after 24 h at 37 °C, (**d**) enzymatically treated with purified TWI3 protease(50 U l^{-1}) after 24 h at 37 °C

appeared to be smoother compared to chemically treated skin, and there is no residual hair observed on the skin surfaces. The arrow mark in figure represents the complete removal of hair form hair follicles without any damage to the skin (Fig. 9c).

Similarly, the cross-section view of goat skin elucidated well-separated fiber bundles in enzymatically treated skin (Fig. 10b); however, the splitting of fiber bundle is more visible in

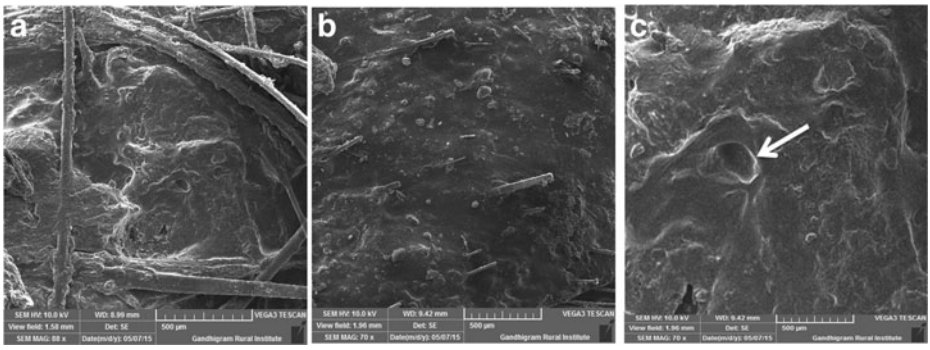

Fig. 9 Scanning electron microscopic view of goat skin surface: **a** untreated goat skin, **b** goat skin treated with lime-sulphide, and **c** goat skin treated with protease enzyme

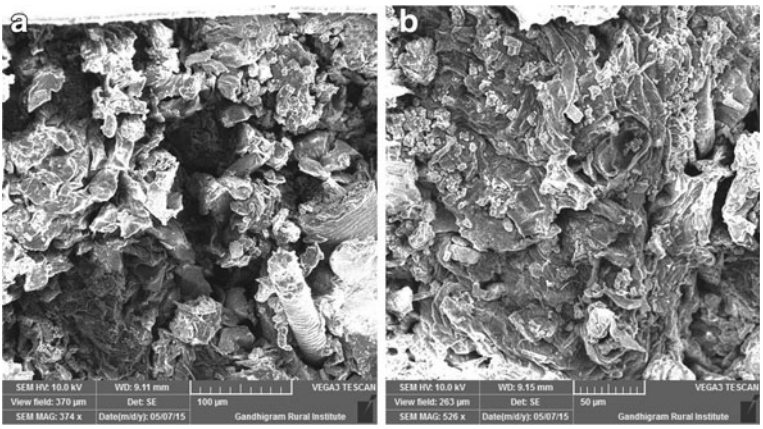

Fig. 10 Scanning electron microscopic view of goat skin cross section: **a** goat skin treated with lime–sulphide and **b** goat skin treated with crude protease enzyme

chemically treated skin (Fig. 10a). The plumping and swelling of the goat skin play a major role in the fiber opening during lime sulphide treatment. During enzymatic dehairing process, the enzyme initially opens up the fiber bundles, but due to insufficiency of exposure time, the fiber bundles open partially. The partially opened fiber bundle could further accelerate the penetration of protease through collagen matrix to act upon anchoring protein around the hair follicle eventually facilitating the removal of hair [37]. Due to these characteristics, enzymatically treated leather appears to be more compact than the chemically processed leather [6]. All of these microscopic observations confirmed the visual assessments, that the enzymatic dehairing process did not damage the intact skin.

Destaining of Dirty Motor Oil and Blood Stain

To demonstrate the destaining ability of TWI3 protease, cloths were stained with blood or dirty motor oil (Fig. 11a, f) and washed with protease with or without commercial detergent. The stained cloth washed with distilled water acted as control (Fig. 11b, g). The blood stains were partially removed from the cloth when treated with enzyme alone or detergent alone (Fig. 11c, d), but complete removal of blood stain was observed when treated with the combination of enzyme and detergent (Fig. 11e). However, the TWI3 protease can easily remove the proteinaceous stain like blood, but dirty motor oil stains were slightly retained in the cloths after treatment with enzyme or detergent or both (Fig. 11h–j). These results confirm the compatibility of TWI3 alkaline protease with other commercial detergents and thus increase the possibility of this enzyme to be used in detergent industries. These results are in agreement with the results of other researchers who already demonstrated the destaining of blood stain by proteases of *Bacillus brevis* [38] and *B. alveayuensis* CAS 5 [14].

Conclusion

In recent years, various microorganisms have been isolated from natural environment and successfully utilized for production of many commercially valuable products like

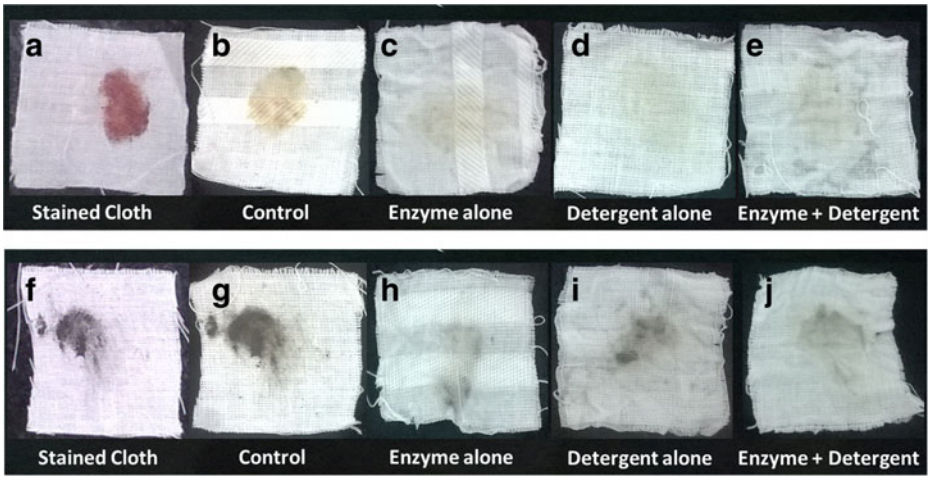

Fig. 11 Destaining properties of protease enzyme with or without commercial detergent (Surf Excel™). **a** Cloth stained with blood, **b** stained cloth washed with water (control), **c** stained cloth washed with crude enzyme, **d** stained cloth washed with detergent, **e** stained cloth washed with crude enzyme along with detergent, **f** Cloth stained with dirty motor oil, **g** stained cloth washed with water (control), **h** stained cloth washed with crude enzyme, **i** stained cloth washed with detergent, and **j** stained cloth washed with crude enzyme along with detergent

pigments, enzymes, and secondary metabolites. In this study, we isolated several potential protease-producing bacterial strains from tannery dumping sites. The bacterial strain was identified as *B. alkalitelluris* TWI3, and optimum cultural conditions like pH, temperature, and nutrient sources were optimized for higher production. We found that lactose and skim milk are good carbon and nitrogen sources, respectively. The purification of protease from TWI3 revealed the presence of a 42.6-kDa alkaline metallo-protease, which showed optimal activity at 60 °C and pH 9.5 and showed remarkable stability at the presence of various metal ions, inhibitors, detergents, oxidant, and NaCl (Table 5).The halotolerant, non-collagenase, and non-keratinolytic *B. alkalitelluris* TWI3 protease was also capable of dehairing goat skin and removed various stains, which makes it more suitable for the application of this enzyme in various industries like detergent and tannery. To the best of our knowledge, this is the first study to demonstrate the protease production by *B. alkalitelluris* isolated from tannery waste.

Table 5 Biochemical properties of purified alkaline protease enzyme from *B. alkalitelluris* TWI3

Biochemical property	Value
Molecular weight	42.6 kDa
Type of protease	Alkaline metallo-protease
Optimum pH	9.5
Optimum temperature	60 °C
*K*m value for casein (mg/ml)	38.0
*V*max value for casein (U/ml)	253.0

Acknowledgments The authors are thankful to the Department of Biology, Gandhigram Rural Institute-Deemed University, Gandhigram, Tamilnadu, India, for providing laboratory facility to carry out the entire research. We also thankful to Mr. Narendrakumar for English correction.

Compliance with Ethical Standards

Conflict of Interest We declare that we have no conflict of interest.

References

1. Asker, M. M. S., Mahmoud, M. G., Shebwy, K. E. L., & Aziz, M. S. A. (2013). Purification and character ization of two thermostab le protease fractions from Bacillus megaterium. *Journal of Genetic Engineering and Biotechnology, 11*(2), 103–109.
2. Rao, M. B., Tanksale, A. M., Ghatge, M. S., & Deshpande, V. V. (1998). Molecular and biotechnological aspects of microbial proteases. *Microbiology and Molecular Biology Reviews, 62*, 597–635.
3. Rai, S. K., & Mukherjee, A. K. (2010). Statistical optimization of production, purification and industrial application of a laundry detergent and organic solvent-stable subtilisin-like serine protease (Alzwiprase) from Bacillus subtilis DM-04. *Biochemical Engineering Journal, 48*(2), 173–180.
4. Belhoul, M., Jaouadi, N. Z., Rekik, H., Bejar, W., Touioui, S. B., Hmidi, M., Badis, A., Bejar, S., & Jaouadi, B. (2015). A novel detergent-stable solvent-tolerant serine thiol alkaline protease from *Streptomyces koyangensis* TN650. *Int. J. Biological Macromol., 79*, 871–882.
5. Joshi, S., & Satyanarayana, T. (2013). Characteristics and applications of a recombinant alkaline serine protease from a novel bacterium Bacillus lehensis. *Bioresource Technology, 131*, 76–85.
6. Sundararanjan, S., Kannan, C. N., & Chittibabu, S. (2011). Alkaline protease from *Bacillus cereus* VITSNO4: potential application as a dehairing agent. *Journal of Bioscience and Bioengineering, 111*, 128–133.
7. Thanikaivelan, P., Rao, R. J., Nair, B. U., & Ramasami, T. (2004). Progress and recent trends in biotechnological methods for leather processing. *Trends in Biotechnology, 22*(4), 181–188.
8. George, N., Chauhan, P. S., Kumar, V., Puri, N., & Gupta, N. (2014). Approach to eco-friendly leather: characteristics and application of an alkaline protease for chemical free dehairing of skins and hides at pilot scale. *Journal of Cleaner Production, 79*, 249–257.
9. Smibert, R.M., & Krieg, N.R. (1994). Phenotypic characterization, in Methods for general and molecular bacteriology (Gerhardt P, Murray RGE, Wood WA, Krieg NR, eds), Washington, pp. 607–54.
10. Gomori, G. (1995). Preparation of buffers for use in enzyme studies. In S. P. Colowick & N. O. Kaplan (Eds.), *Methods in Enzymology* (pp. 138–146). New York: Academic Press.
11. Bradford, M. M. (1976). A rapid and sensitive for the quantitation of microgram quantities of protein utilizing the principle of proteinedye binding. *Analytical Biochemistry, 72*, 248–254.
12. Laemmli, U. K. (1970). Cleavage of structural proteins during assembly of head of bacteriophage T4. *Nature, 227*, 680–685.
13. Kunitz, M. (1947). Crystalline soybean trypsin inhibitor: II. General properties. *Gen. Physiol., 30*, 291–310.
14. Annamalai, N., Rajeswari, M. V., & Balasubramanian, T. (2014). Extraction, purification and application of thermostable and halostable alkaline protease from *Bacillus alveayuensis* CAS 5 using marine wastes. *Food and Bioproducts Processing, 92*, 335–342.
15. Lee, J. C., Lee, G. S., Park, D. J., & Kim, C. J. (2008). *Bacillus alkalitelluris* sp. nov., an alkaliphilic bacterium isolated from sandy soil. *International Journal of Systematic and Evolutionary Microbiology, 58*, 2629–2634.
16. Joo, H. S., Kumar, C. G., Park, G. C., Paik, S. R., & Chang, C. S. (2003). Oxidant and SDS-stable alkaline protease from *Bacillus clausii* I-52: production and some properties. *J.Appl. Microbiol, 95*, 267–272.
17. Patel, R. K., Dodia, M. S., Joshi, R. H., & Singh, S. P. (2006). Purification and characterization of alkaline protease from a newly isolated haloalkaliphilic Bacillus sp. *Process Biochemistry, 41*, 2002–2009.
18. Deng, A., Wu, J., Zhang, Y., Zhang, G., & Wen, T. (2010). Purification and characterization of a surfactant-stable high-alkaline protease from Bacillus sp. B001. *Bioresource Technology, 101*, 7100–7106.
19. VijayAnand, S., Hemapriya, J., Selvinb, J., & Kiran, S. (2009). Production and optimization of haloalkaliphilic protease by an extremophile Halobacterium sp. Js1, isolated from Thalassohaline environment. *African Journal of Basic & Applied Sciences, 1*(3–4), 49–54.
20. Ibrahim, A. S. S., & Al-Salamah, A. A. (2009). Optimization of media and cultivation conditions for alkaline pro tease production by alkaliphilic Bacillus halodurans. *Research Journal of Microbiology, 4*, 251–259.

21. Zambare, V., Nilegaonkar, S., & Kanekar, P. (2011). A Novel extracellular protease from Pseudomonas aeruginosa MCM B-327: enzyme production and its partial characterization. *New Biotechnology, 28*, 173–181.
22. Kanekar, P. P., Nilegaonkar, S. S., Sarnaik, S. S., & Kelkar, A. S. (2002). Optimization of protease activity of alkaliphilic bacteria isolated from an alkaline lake in India. *Bioresource Technol, 85*, 87–93.
23. Gouda, M. K. (2006). Optimization and purification of alkaline proteases produced by marine Bacillus sp. MIG newly isolated from Eastern Harbour of Alexandria. *Polish Journal of Microbiology, 55*, 119–126.
24. Mokashe, N., Chaudhari, A., & Patil, U. (2015). Optimal production and characterization of alkaline protease from newly isolated halotolerant Jeotgalicoccus sp. *Biocatalysis and Agricultural Biotechnology., 4*(2), 235–243.
25. Olajuyigbe, F. M. (2013). Optimized production and properties of thermostable alkaline protease from Bacillus subtilis SHS-04 grown on groundnut (Arachis hypogaea) meal. *Advances in Enzyme Research, 1*(04), 112.
26. Kumar, R. S., Ananthan, G., & Prabhu, A. S. (2014). Optimization of medium composition for alkaline protease production by Marinobacter sp. GA CAS9 using response surface methodology — A statistical approach. *Biocatalysis and Agricultural Biotechnology, 3*, 191–197.
27. Shivanand, P., & Jayaraman, G. (2009). Production of extracellular protease from halotolerant bacterium, *Bacillus aquimaris* strain VITP4 isolated from Kumta coast. *Process Biochemistry, 44*, 1088–1094.
28. Manikandan, M., Kanna, V., & Pasic, L. (2011). Extraction, purification and characterization of a protease from Micrococcus sp. VKMM 037. *Environmental Technology, 32*, 1487–1495.
29. Doddapaneni, K. K., Tatineni, R., Vellanki, R. N., Rachcha, S., Anabrolu, N., Narakuti, V., & Mangaomoori, L. N. (2009). Purification and characterization of a solvent and detergent- stable novel protease from *Bacillus cereus. Microbiological Research, 164*, 383–390.
30. Arulmani, M., Aparanjini, K., Vasanthi, K., Arumugam, P., Arivuchelvi, M., & Kalaichelvan, P. T. (2007). Purification and partial characterization of serine protease from thermostable alkaliphilic *Bacillus laterosporus*-AK1. *World Journal of Microbiology and Biotechnology, 23*, 475–481.
31. Nilegaonkar, S. S., Zambare, V. P., Kanekar, P. P., Dhakephalkar, P. K., & Sarnaik, S. S. (2007). Production and partial characterization of dehairing protease from *Bacillus cereus* MCM B-326. *Bioresource Technology, 98*, 1238–1245.
32. Uyar, F., Porsuk, I., Kizil, G., & Yilmaz, E. I. (2011). Optimal conditions for production of extracellular protease from newly isolated *Bacillus cereus* strain CA15. *Eurasian Journal of Biosciences, 5*, 1–9.
33. Ahmetoglu, N., Bekler, F. M., Acer, O., & Guven, R. G. (2015). Production, purification and characterisation of thermostable metallo-protease from newly isolated. *EurAsian Journal of BioSciences, 9*, 1–11.
34. Waghmare, S. R., Gurav, A., Mali, S., Nadaf, N. H., Jadhav, D. B., & Sonawane, K. D. (2015). Purification and characterization of novel organic solvent tolerant 98kDa alkaline protease from isolated *Stenotrophomonas maltophilia* strain SK. *Protein Expression and Purification, 107*, 1–6.
35. Tiwary, E., & Gupta, R. (2010). Medium optimization for a novel 58 kDa dimeric ker- atinase from *Bacillus licheniformis* ER-15: biochemical characterization and application in feather degradation and dehairing of hides. *Bioresource Technology, 101*, 6103–6110.
36. Abraham, J., Gea, T., & Sánchez, A. (2014). Substitution of chemical dehairing by proteases from solid-state fermentation of hair wastes. *Journal of Cleaner Production, 74*, 191–198.
37. Thanikaivelan, P., Rao, J. R., Nair, B. U., & Ramasami, T. (2002). Towards zero discharge tanning: a shift from chemical to biocatalytic leather processing. *Environmental Science & Technology, 36*, 4187–4194.
38. Banerjee, U. C., Sani, R. K., Azmi, W., & Soni, R. (1999). Thermostable alkaline protease from *Bacillus brevis* and its characterization as a laundry detergent additive. *Process Biochemistry, 35*, 213–219.