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Production and Purification of Novel Alkaline Protease Producing *Bacillus firmus* BAAP-43 isolated from Tannery and Leather Industries Soils

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ABSTRACT: Proteolytic enzymes are the enzymes that bring about degradation of the proteins into peptides and amino acids. Proteases enzymes are one of the most important groups of industrial enzymes accounting for more than 65% of the total market for industrial enzymes used in laundry detergent, brewing, the leather, and dairy, pharmaceutical and food industries, and in the production of protein. Protease can hydrolyze the hardly soluble protein on fabric into soluble peptide chain and amino acid in detergent solution. Smoothness. A protease producing microorganism was isolated from tannery and leather industries soil collected from tannery effluent contaminated sites. Based on the 16S rRNA sequencing morphological, biochemical, and molecular characterization the isolate was identified as *Bacillus firmus* BAAP-43. Maximum protease production was achieved at 45□C and pH of 9.5 and multiple sources of carbon for its alkaline proteases production; glucose was the best source of carbon, peptone was found to be the best nitrogen source for the maximum enzyme production. Mg^{2+} enhances the growth and enzyme production and among the different substrates used, casein showed maximum enzyme production. The enzyme was purified by ammonium sulphate precipitation, DEAE- Cellulose and Sephadex G-100 gel chromatography. The Purified protein was with specific activity of 18360.6 U/mg protein was obtained with 43.1 purification fold and 18.7% recovery percentage. Molecular weight of purified enzyme was as 30.2 kDa by SDS-PAGE.

Keywords: Alkaline Protease, *Bacillus firmus,* Purification, Characterization.

INTRODUCTION

Proteases are extracellular hydrolytic enzymes capable of hydrolysing large protein molecules to smaller peptides or amino acids that can be utilized by microorganisms. Proteases or peptidases are classified as proteolytic enzymes Because of their specificity, stability, and activity under a wide variety of physical environments, bacterial proteases are synthesized in huge amounts. Proteolytic enzymes make up more than 65% of commercial enzyme production worldwide, while alkaline proteases account 35% of the proteolytic enzymes. Enzymes are valuable products because to their huge applications in a wide variety of sectors, including food, detergents, leather and paper, meat processing, cheesemaking, bioremediation, and silver recovery from photographic film. Moreover, these enzymes can be used in therapeutically to treat harmful lesions and inflammation (Fazilat, 2016; Parameswaran *et al*., 2018). The increased usage of these alkaline proteases used as the detergent additive is mostly due to the cleaning capabilities of these enzymes in most of the detergents industries. Bacteria is considered as the most excellent source to produce three major types of proteases i.e., acid, neutral and alkaline. Amongst them, neutral and alkaline proteases have a great application potential in the enzyme detergent and leather industry because of the increasing trend of development of ecofriendly technologies (Ullah *et al*., 2022).

Proteases are the most significant and useful microbial enzymes. They have a broad applications and are widely employed in the biotech, pharmaceutical, chemical, and agricultural sectors. Proteases are also used in biofuels and other agricultural and medicinal products. Because of their various potential applications, physiological traits, and capacity to create multifunctional enzymes and other metabolites, the majority of companies exploit proteases from *Bacillus* sp (Voigt *et al*., 2004). Proteases are a group of enzymes that remain active in very basic pH range of 7-13 with an optimal pH of 10 are the alkaline proteases (Furhan and Sharma 2014). The use of alkaline proteases as industrial catalysts has greatly increased in recent years. The alkaline protease producing *Bacillus* sp. found its natural habitat in open-air laundromat (Dhobi Ghat) soil, which possesses attractive extracellular enzyme producing abilities with extreme pH and 6,7 temperature tolerance. The enzymes from these strains belong to metallic type or serine centre

proteases that sustain in harsh process parameters (Shaikh and Dixit 2017) There is a need to find new microorganisms to produce new enzymes that are multifunctional properties to meet the current industrial demand. Microbial proteases with proteolytic activity over a wide range of pH and temperature compared to other proteases isolated from animal and plant sources (Walsh, 2014). Most microbial enzymes are extracellular in nature and produced in the culture medium; therefore, the isolation of enzymes from the medium is very simple and reliable. Proteases are used as a one of the ingredient in different brands of commercial detergents to improve the cleaning efficiency by eliminating proteins such as milk, blood and food stains. The action of alkaline proteases in a detergent depends on many factors such as pH, temperature, detergent composition, and the type of washing machine used. The key to utilizing alkaline protease as a balance in detergent products is the strength of the enzyme in the detergent. Therefore, we attempt to identify a new variety of alkaline protease with good quality, multi functional and stable properties that make it suitable as an additive in detergents (Mahakhan *et al.*, 2023). The current study's objectives were to attempt to enhance the purification, characterization, and application of alkaline protease from BAAP-43

MATERIALS AND METHODS

Isolation and screening of microorganisms. Sediment and water samples collected from tannery and leather industries situated in and around Erode District, Tamil Nadu, India collected in sterile plastic containers were brought to the laboratory under low temperature. Protease producing microorganisms were isolated from serially diluted samples by plating with Nutrient agar. All the isolated bacterial strains were screened for extracellular protease production in skim milk agar plates for which the bacterial strains were streaked at the centre and the plates were incubated for 48 hours at 37°C. A positive reaction is indicated by a clear zone formed around the colony. The strain that showed very good growth and protease activity was selected for further studies. The bacterial strain was adapted to selective biochemical tests (Bergey's manual of Determinative Bacteriology (1994) and further characterised by 16S rRNA gene sequencing.

Screening of bacterial alkalophilic protease.

Using skim milk agar medium, which contains skim milk 1.0%, peptone 0.5%, sodium chloride 5%, and agar 2.5%, individual bacterial colonies were tested for the synthesis of proteolytic enzymes. Before sterilization, the pH of the medium was adjusted to 9.0 using 1 N HCL and 1 N NaOH. After 48 hours of incubation at 37°C, the inoculated plates were examined for zones of clear, which suggest proteolytic activity.

Assay for proteolytic activity. A modified Kunitz technique was used to measure the activity of the protease (caseinolytic) (1947) The reaction mixture (0.6 mL) contains of 300 μL of 1% casein in 200mM glycine-NaOH buffer (pH 9.0) and 300 μL of enzyme solution or supernatant. The reaction was initiated by adding an enzyme solution at 40◦C. The reaction was stopped by adding 0.4M trichloroacetic acid (TCA, 300 μL) after the reaction was kept for 15 min. The reaction mixture was further kept on ice for another 10 min. Then it was centrifuged at 4◦C for 10 min at 10000 rpm. Further 0.3mL of supernatant solution, mixed with 1.5mL of a 0.4M Na_2CO_3 solution in 2.1mL distilled water, and 0.3mL of Folin-Ciocalteu reagent were further incubated for 20 min at 40◦C. The amount of L-tyrosine liberated from casein was measured by the absorbance at 680 nm by using a UV-VIS spectrophotometer. L-tyrosine was used as a reference for the standard curve. One unit of protease enzyme activity was defined that protease that liberates 1 μg/mL of tyrosine per min.

Optimization studies. The effects of culture conditions on growth and alkaline protease production were measured by culturing *Bacillus firmus* BAAP-43 in nutrient broth and incubated at 37°C. The organism was grown at different temperature ranging from 10 to 60ºC, and different concentration of NaCl at 1, 2, 3, 4, 5 and 10%. pH effect on growth and enzyme was assayed at different pH ranges from 5.0 to 12.0. Carbon sources such as organic and inorganic nitrogen sources was tested for the growth and enzyme production, the test organism was inoculated with minimal salt medium with glucose, lactose, sucrose, maltose, fructose, and mannitol and various organic and inorganic nitrogen sources at 1.0% (w/v). Peptone, yeast extract, beef extract, tryptone and inorganic nitrogen sources such as ammonium sulphate, sodium nitrate and potassium nitrate were added separately. For the influence of metal ions on growth and enzyme production, the test organism was inoculated in medium supplemented with Mn^{2+}, Cu^{2+}, Zn^{2+}, Fe^{2+}, K^{+}, Co^{2+}, Ba^{2+}, Ca^{2+}, EDTA and Mg^{2+} at a final concentration of 2.0 mM. Growth was measured at 600 nm and the enzyme activity was assayed after 40 hrs of incubation. All the tests are in triplicates.

Purification of extracellular alkaline protease from test strain BAAP-43

1000 mL of BAAP-43 was used to inoculating 2000 mL of medium in 4 L Hoffkin's flask. After 40 hrs of incubation, the cells were harvested by centrifugation at 8000 × g for 30 min at 4ºC and the supernatant is used for the purification process. All the purification steps were carried out at 4ºC.

Protein determination. Protein was estimated by the method of Bradford (1976) by using Bovine Serum Albumin as a standard. During the each step of purification, protein was estimated by absorbance at 280 nm.

Extraction and purification of extracellular alkaline protease from test strain BAAP-43. The Culture supernatant was precipitated by using 80% ammonium sulphate and the precipitate protein was centrifuged and the pellet was resolved in 30 mM Tris–HCl (pH 9.0) and dialyzed. The dialyzed protein was used for the next step of purification process.

Phenyl sepharose chromatography. A column of phenyl sepharose (1.0 × 7.0 cm, pre-equilibrated with

Tris-HCl (30 mM; pH 9.0) and filled with dialyzed protein at a flow rate of mL/min. A linear gradient of NaCl (0-1.25 M) in Tris-HCl to enhance the release of the bound enzyme (30 mM; pH 9.0). The fractions that had specific activity more than 300 U/mg were combined, lyophilized, and employed for the next step of purification

Gel filtration chromatography. After pre-equilibrating with 30 mM of Tris-HCl (pH 9.0), this sample was applied to a column packed with Sephadex G-100. After that, the column was eluted using an equilibration buffer. F ractions of (3 mL) were collected and tested for protein concentration and protease activity.

Reverse phase high performance liquid chromatography (RP-HPLC). Reverse phase high performance liquid chromatography were used to further evaluate the purity of the fractions.

Homogeneity and determination of purified alkaline protease. The silver staining technique was used to determine the molecular weight of the purified protein by Blum *et al.* (1987).

RESULTS AND DISCUSSION

Isolation and screening of alkaline protease producers. In the current investigation, 68 bacterial strains were isolated from sediment samples from tanneries and leather industries that were collected in and around the Erode District of Tamil Nadu, India. Among the 68 isolates, one isolate exhibited strong growth and a high hydrolytic zone while using skim milk agar as the only carbon/energy source during the extracellular protease production. Isolates were designated as BAAP- 1 to BAAP- 68. Our results were supported by Similar kind of research was carried by Siddharthan *et al.* (2016). The isolate BAAP-43 was chosen for future investigations because, during the first initial screening, it showed highest hydrolytic ability against protein sources. Narendra *et al.* (2012) reported that about 25 organisms were recovered from different fields near to Ravulapalem village, East Godavari district, Andhra Pradesh and Jani *et al.* (2016) reported out of 50 protease-producing isolates only 10 isolates were identified as potent producers of alkaline protease based on the size of clear zone around the colonies on the screening media plate having skimmed milk agar.

Phenotypic and biochemical characterization of BAAP-43. The colony of the test isolate BAAP-43 was granular glossy, round, slightly elevated, opaque, and had a rhizoidal edge. The cells were motile Gram-positive rods that ranged in size from 0.6 to 1.0 µm in width and 1.2 to 3.8 µm in length. Table 1 shows the morphological and physiological characters of the isolate BAAP-43. Based on characters provided in Bergey's manual of Determinative Bacteriology (1994), the test organism was identified as *Bacillus* sp (1994). It was reported that *Bacillus* species are well known and effective for the production of the protease when compare to the other microbial flora (Kuebutornye *et al*., 2019).

Molecular characterization of the test strain based on 16S rRNA gene sequence homology. Partial sequence of 1240 bases of the amplified 16S rRNA gene sequence of the test organism BAAP-43 showed 100% homology with a maximum score of 2290 out of the total score of 2290 to the 16S rRNA gene sequence of *Bacillus firmus* strain CAS 7 in the GenBank (Accession No. HQ116811) and it was designated as *Bacillus firmus* BAAP-43. Results presented here agreed with the previous literature, as several *Bacillus* species are known to be good alkaline protease producers and have been widely used in various industries (Al-Dhabi *et al*., 2020). Similar to this, several researchers were able to describe and identify potential strains by 16s rDNA sequencing analysis (Waghmare *et al*., 2015).

Growth and extracellular alkaline protease production by *Bacillus firmus* BAAP-43. Extracellular alkaline protease production from the test isolate improved after 24 hours and reached a maximum (398 U/mL) at 44 hours of incubation. (Fig. 1). There was a slight drop in protease production (293 U/mL) after 44 hours, which also happened to coincide with a decline in growth, but protease production was maintained at close to maximum between 36 and 44 hours. The present study was highly supported by *Bacillus cereus* strain isolated from soil (Ahamed *et al*., 2016; Shaikh and Dixit 2017)

Effect of pH on growth and extracellular alkaline protease production by *Bacillus firmus* BAAP-43. At pH 9.5, growth and alkaline protease production was recorded maximum (412 U/mL) whereas at pH values below 8.5 and above 10.0, both growth and enzyme production slightly decreased (Fig. 2). Consequently, the findings unmistakably show that pH has a crucial impact on the test isolate's capacity to grow and produce proteolytic enzymes. Similarly Kumar and Vats (2010) are also reported same pattern but the enzyme production was extended to 72 hours. Present study results were supported by the enzyme synthesis was increased with increase of medium pH towards alkaline range from neutrality (Mahakhan *et al.*, 2023).

Effect of temperature on growth and alkaline protease production by *Bacillus firmus* BAAP-43. Among the different temperatures, the 45ºC showed highest growth and enzyme production (391 U/mL) when compared to all other temperatures. Whereas 40ºC and above 45ºC recorded 50% of enzyme production and growth (Fig. 3). Kumari and Premila (2023) also reported that the optimum temperature for protease production was 45ºC for *Bacillus* sp.

Effect of salinity on growth and alkaline protease production by *Bacillus firmus* BAAP-43. In the presence of NaCl up to 5% in the medium, the organism produced enzymes well and grew well. However cells grown with 5% NaCl had the maximum alkaline protease production, at 401 U/mL. Maximum inhibition of both growth and alkaline protease production was recorded with 10% sodium chloride, even though considerable levels of growth and alkaline protease production were observed at 1-4% NaCl (Fig. 4). Our results was supported by Marathe *et al.* (2018) showed a salt tolerance of up to 5% NaCl beyond which protease activity declined and ceased.

Effect of various carbon and nitrogen sources on the growth and alkaline protease production by *Bacillus firmus* BAAP-43. Peptone supported the highest production of protease out of all the carbon and nitrogen sources studied, and it produced 399 U/mL. and tryptone, beef extract, and yeast extract recorded 301, 234, and 189 U/mL respectively. Only glucose and fructose were found to promote the growth and enzyme production when the various carbon sources were investigated for their capacity to do so. In the presence of glucose the organism produced 402 U/mL of enzyme. (Fig. 5). The same results were recorded by Elgammal *et al*. (2020) who applied 0.5% peptone using *Bacillus megaterium*. Whereas carbon sources optimization in a similar study of protease production supports our finding that glucose acts as a promoter for better protease production by Patil and Kurhekar (2020).

Effect of metal ions on growth and alkaline protease production by *Bacillus firmus* BAAP-43. Effects of various metal ions on growth and enzyme production were investigated; Mg2+ to a final concentration of 2 mM supported the highest enzyme production of 385 U/mL and followed by ferric ions where as Cu, Hg, and Co metal ions are completely inhibited growth and enzyme production. (Fig. 6). Although the test organism was able to grow and produce the enzyme in the absence of added metal ions where as growth was supported by Mn, Zn, Fe and Ba and enzyme production was drastically inhibited. Metal cations have a great effect on improving the activity and stability of proteases. Mg+ is an activator of various enzymes that are widely involved in material metabolism and energy metabolism. Simillarly Mg2+ ions showed increasing production of enzyme by Mahakhan *et al*. (2023). Where the magnesium sulphate and manganese sulphate enriched medium enhanced the protease production in *Bacillus gibsonii* 6BS15-4.

Purification of extracellular alkaline protease from *Bacillus firmus* BAAP-43. Concentrating of the enzyme was achieved by precipitation of the enzyme by ammonium sulphate and by dialysed. Total protein concentration was estimated by Bardford method and the total amount of protein in crude was found to be 102 mg (Table -2). The maximum activity of protease was obtained with 60, 70, 80 and 90% ammonium sulfate saturation. Similarly kind of high activities of enzyme was observed by (Ullah *et al*., 2022) at 70% of ammonium sulphate concentration level.

Purification of alkaline protease by DEAE Cellulose Chromatography

DEAE Cellulose column was loaded with 102 mg of protein that had 312854 U of Protease activity recovered from ammonium sulphate fractions. DEAE Cellulose Column had five protein peaks, the fractions 15–35 showed protease activity (0.475 – 0.850 M NaCl concentration). Yield 18 mg of protein was obtained which contained 196300.8 U specific activity (Table 2).

Purification of alkaline Protease by Sephadex G-100 Gel Filtration Chromatography. Active fractions from DEAE Cellulose were pooled, concentrated, and lyophilized before being dialyzed against Tris-HCl buffer (30 mM; pH 9.0). In SDS-PAGE, the enzyme's purity was tested. The purity of the enzyme was checked on SDS-PAGE. At final the enzyme was purified to 43.1 fold , with a final recovery of 18.7%. (Table 2). Purified alkaline protease has a specific activity of 18360.6 U/mg protein.

Homogeneity and Molecular mass determination of purified alkaline protease from *Bacillus firmus* BAAP-43

SDS-PAGE analysis of the purified enzyme showed single band indicating the homogeneity of the enzyme (Fig. 7a). Purified enzyme was subjected to RPHPLC analysis; it showed a single distinct peak with a retention time of 11.5 minutes, clearly indicating that the purified enzyme was homogeneity (Fig. 7b). Based on the relative mobility, the molecular mass of the purified alkaline protease was calculated to be 30.2 kDa. Similar results were reported by Lakshmi *et al.* (2018) reported the use of ammonium sulfate precipitation, ion exchange (DEAE-Cellulose) and gel filtration (Sephadex G-100) chromatographic techniques for the purification of protein with increase in the purity of the protein by 17.04-fold and also reported the similar molecular weight from *Bacillus cereus* strain S8. The molecular mass is comparable to other purified alkaline proteases of *Bacillus* sp (Zaman *et al*., 2023).

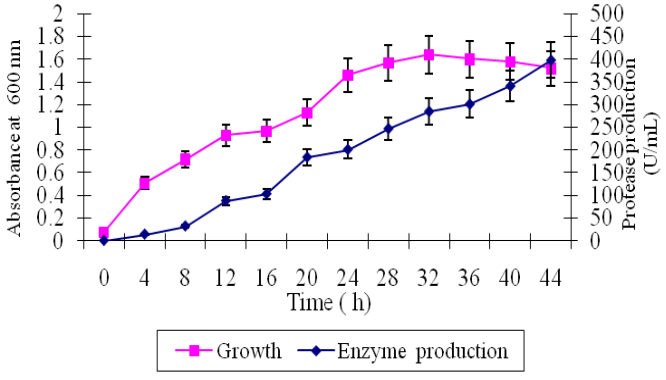


Fig. 1. Effect of different incubation time on growth and Alkaline protease production by *Bacillus firmus* BAAP-43.

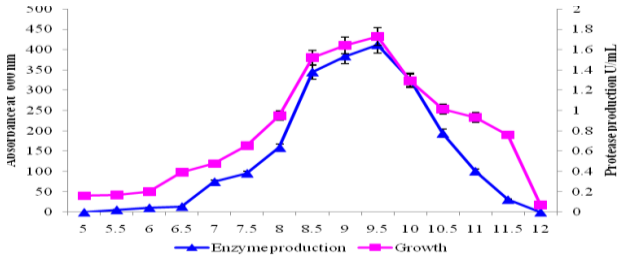


Fig. 2. Effect of pH on growth and Alkaline protease production by *Bacillus firmus* BAAP-43.

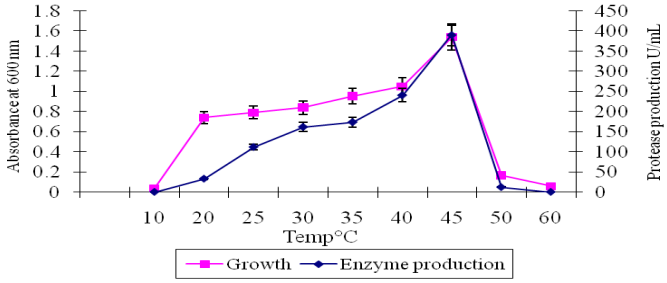


Fig. 3. Effect of temperature on growth and Alkaline protease production by *Bacillus firmus* BAAP-43.

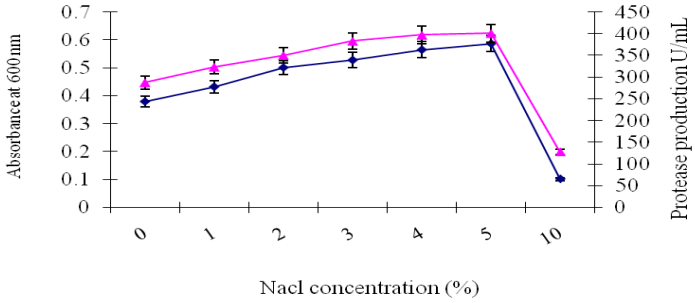


Fig. 4. Effect of NaCl concentration on growth and Alkaline protease production by *Bacillus firmus* BAAP-43

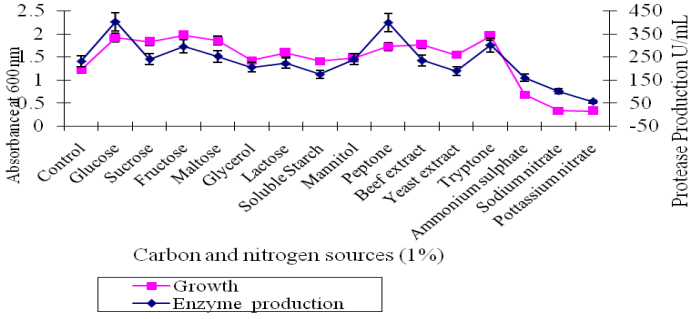


Fig. 5. Effect of carbon and nitrogen on growth and Alkaline protease production by *Bacillus firmus* BAAP-43.

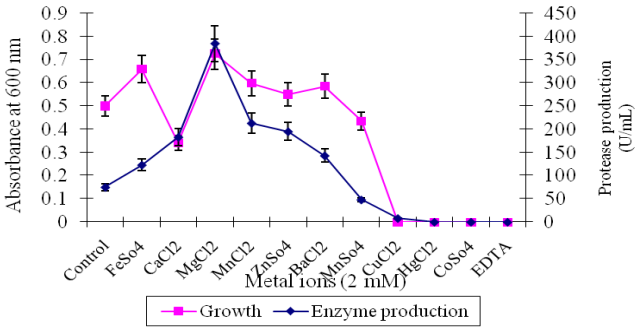
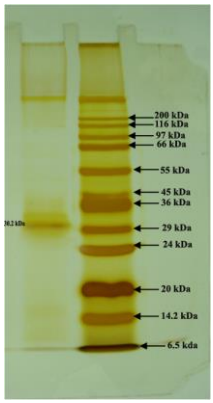
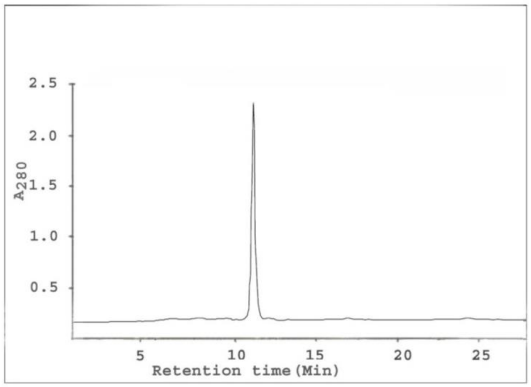


Fig. 6. Effect of metal ions on growth and Alkaline protease production by *Bacillus firmus* BAAP-43.



(A)



(B)

Lane -1 – Marker, Aprotinin, bovine lung (6,500), α-Lactalbumin, bovine milk (14,200) Trypsin inhibitor, soybean (20,000), Trypsinogen, bovine pancreas (24,000), Carbonic anhydrase, bovine erythrocytes (29,000), Glyceraldehyde-3-phosphate dehydrogenase, rabbit muscle (36,000), Ovalbumin, chicken egg (45,000), Glutamic dehydrogenase, bovine liver (55,000) , Albumin, bovine serum (66,000), Phosphorylase B, rabbit muscle (97,000) β-Galactosidase, *E. coli* (116,000), Myosin, porcine heart (200,000)
Lane -2 - Purified protein from after Gel filtration (5 µg)

Fig. 7 (A) Homogeneity of purified alkaline Protease on SDS – PAGE (B) Purified fraction of RP-HPLC chromatography.

Table 1: Physiological and biochemical characteristics of Isolate BAAP-43.

Test	Results
Colony morphology	
Configuration	Circular
Margin	Rhizoid
Elevation	Slightly raised
Surface	Granular Shiny
Pigment	---
Opacity	Opaque
Physiological and Biochemical tests	
Growth at 25 - 42°C	+
Growth at pH (4 – 10)	+
Growth in NaCl (2 – 7%)	+
Growth in anaerobic agar	–
Gram's Reaction	Positive
Cell Shape	Rods
Size (µm)	Length: 1.2 – 3.5 µm; Width: approx.0.6 - 1.0 µm
Arrangement	Short chains
Motility	+
Growth on MacConkey Agar	-
Indole production	-
Methyl Red test	+
Voges Proskauer test	-
Citrate utilization	-
Acid from glucose	+
Gas from glucose	-
H_2S production	-
Casein hydrolysis	+
Gelatin hydrolysis	+
Urea hydrolysis	-
Starch hydrolysis	+
Nitrate reduction	+
Catalase Test	+
Oxidase Test	+
Tween 20 hydrolysis	-
Tween 40 hydrolysis	+
Tween 80 hydrolysis	-
Glucose	+
Sucrose	+
Mannitol	+
Galactose	+
Fructose	+

Table 2: Purification chart.

Source	Protein (mg)	Total activity (U)	Specific activity (U/mg protein)	Recovery (%)	Purification fold
Culture filtrate	652	277752	426	100	1
Ammonium sulphate precipitation	102	312854.4	3067.2	57.9	7.2
DEAE - Cellulose	18	196300.8	10905.6	29.2	25.6
Sephadex G-100	9.5	174425.7	18360.6	18.7	43.1

CONCLUSIONS

In the present study, native alkaline protease producing alkalophilic bacteria was isolated from local tannery and leather industries sediment soil samples as a potent producer of alkaline protease enzyme. The new isolate was identified as *Bacillus firmus* BAAP-43 based on biochemical tests and molecular identification using 16S rRNA sequence analysis. Culture conditions such as optimum incubation time, temperature, pH, Carbon, Nitrogen sources, Metal ions and substrates for the maximum growth and enzyme production were determined. The molecular weight of the purified alkaline protease was estimated to be 30.2 kDa. Further, a complete large-scale process optimization, purification, and characterization work is needed to achieve inexpensive production of alkaline protease.

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