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PURIFICATION, CHARACTERIZATION AND APPLICATIONS OF THERMOSTABLE ALKALINE PROTEASE FROM MARINE *STREPTOMYCES* SP. D1

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ABSTRACT

An extracellular alkaline protease from marine *Streptomyces* sp. D1 was isolated and characterized. The protease was purified using ammonium sulfate precipitation method, followed by dialysis and Sephadex G-100 gel chromatography, with an 8.82-fold increase in specific activity and a 12.8% recovery. The molecular weight was found to be 28 kDa, determined by SDS-PAGE. The purified enzyme was completely inhibited by phenylmethylsulphonyl fluoride, indicating presence of a serine protease. Protease enzyme was found to have maximum activity at 45^0C and pH 10, respectively. The enzyme was stable in pH range 8-10 and temperature 45-60^0C. The enzyme was found to be halotolerant, retains 92.5% of its initial activity after 6 h. The alkaline protease was found stable in presence of non-ionic surfactant (1% Triton X-100) retains 81.5% residual activity after 48 h and 92.57 % residual activity after 6 h with oxidizing agent (1% H_2O_2). The protease inhibited the growth of several pathogenic organisms such as *B. subtilis, E. coli, P. aeruginosa* and *S. aureus*. Enzyme also exhibited good hair removal activity from goat skin. These unique properties make this protease an ideal choice in food, pharmaceutical, leather and detergent industries.

KEYWORDS: ***treptomyces*, Alkaline serine protease, Purification, Enzyme stability, Antibacterial and Dehairing activity**

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INTRODUCTION

The enzymes are considered as "green chemicals" due to their eco-friendly in nature and possess wide range of applications from industrial sector to house-hold products[1]. There is increased demand of alkaline proteases for a wide range of applications in laundry detergent, textile, food processing, pharmaceuticals and leather industries[2, 3, 4]. Among them the thermostable proteases have been widely studied for industrial production such as detergent, weave processing, meat tendering and reducing the risk of contamination by other organisms at high temperature[5]. The use of enzymes for leather processing as alternatives of chemicals is expected to develop an environmental friendly leather processing process[6]. *Streptomyces* species are the most industrially important actinomycetes, due to their capacity to produce numerous secondary metabolites[7]. The ability of these bacteria to produce large amounts of enzymes, such as proteases with varied substrate specificities, presents its beneficial use[8]. While alkaline proteases from bacteria are extensively characterized, similar attention has not been paid to actinomycetes. To date antibiotics are the major bioactive compounds from the actinomycetes. Discovering such species, producing proteases with novel characteristics will be of great value to the enzyme industry for different applications[9, 10]. The present study describes purification, characterization of thermostable, salt-tolerant an alkaline serine protease from marine *Streptomyces* sp. D1.

MATERIALS AND METHODS

All bacteriological media components were purchased from Hi-Media, India. Sephadex G-100, bovine serum albumin (BSA), sodium dodicyl sulfate (SDS), cetyl trimethyl ammonium bromide (CTAB), polyacrylamide, Folin-Ciocalteau reagent and protease inhibitors were purchased from Sigma-Aldrich, India. All other chemicals were of analytical grade.

(i) Isolation and identification of strain D1

The extracellular alkaline protease producing actinomycetes was isolated from marine sediment obtained at the West coast of India[10]. Identification of strain was done by using scanning electron microscopy[11] and 16S r-DNA sequencing[12, 13].

(ii) Media and culture conditions

Fermentation was carried out in a 2 l bench scale fermenter (New Brunswick Scientific, USA) containing (g/l): xylose 30.0, alanine 20.0, NaCl 90.0, K_2HPO_4 8.0, and KCl 0.75. The fermentation media was then inoculated with 50 ml of seed culture aseptically and incubated for 12 days. The operating conditions during batch fermentation were temperature 37^0C, pH 10.0, agitation rate 150 rpm and the aeration rate 1.0 VVM.

(iii) Assay of protease activity

The protease assay was performed with the modification of method described by Nilegaonkar[14] *et al*. The reaction mixture containing 5 ml of 1% (w/v) casein was dissolved in 100 mM Glycine-NaOH buffer pH 10.0 and 1 ml of crude enzyme incubated at 45^0C for 20 min. The reaction was terminated by adding 5 ml of 5% (w/v) trichloroacetic acid. After 10 min, the reaction mixture was centrifuged at 4^0C and 6,000 rpm for 10 min. The supernatant (2 ml) was mixed with 5 ml of 5% (w/v) of sodium carbonate and 1 ml Folin and Ciocalteau reagent (1:3). After 10 min, the blue colour formed was measured with UV spectrophotometer at 660 nm. Calibration curve was plotted using tyrosine as a standard. One unit (U) of enzyme activity represents the amount of enzyme required to liberate 1 µg of tyrosine under standard assay conditions. Protein concentration was determined according to the Lowry method[15].

(iv) Isolation and purification of protease

Isolation of extracellular protease was done using method described by Banerjee and Negi[16]. The enzyme was precipitated by ammonium sulfate precipitation method. The precipitate was centrifuged at 10,000 rpm at 4^0C for 20 min and dissolved into minimum volume of 100 mM Glycine-NaOH buffer of pH 10.0. Partially purified enzyme was desalted by conventional dialysis for 48 h against the same buffer. Enzyme obtained from the above step was loaded onto a Sephadex G-100 column (2 × 20 cm), pre-equilibrated with same buffer at flow rate of 10 ml/h. The bound protease was eluted by applying a gradient increasing concentration of NaCl (0-0.5 M). The fractions were collected and analyzed for protease activity as well as protein content[17].

(v) SDS- PAGE electrophoresis[18]

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to method described by Laemmli using 12% polyacrylamide gel.

(vi) Effect of inhibitors and metal ions on protease activity[17]

The effect of different protease inhibitors such as, PMSF, IAA, Pestatin A and EDTA was investigated by incubating the enzyme for 30 min at 45^0C and residual activity was determined. Effect of different monovalent and divalent metal ions were determined by incubating protease sample for 30 min at 37^0C and the residual activity was measured.

(vii) Effect of pH, temperature, NaCl on the proteolytic activity and stability

Effect of pH on protease activity was determined in pH range 5-13 using casein as substrate at 45^0C for 30 min incubation time. The pH stability of the enzyme was investigated in the pH range of 5-13. Therefore, enzyme was mixed with same buffer solutions mentioned above and incubated at 45^0C for 1 h and residual protease activity was measured under standard assay conditions[17]. The optimum temperature for protease activity was determined by incubating the reaction mixture for 30 min at different temperatures ranging from 25 to 90^0C. The thermal stability was determined by pre-incubation of the enzyme at temperature ranges 25-90^0C for 15-90 min time interval and residual activity was measured by considering initial enzyme activity as 100%[19]. The effect of sodium chloride was determined by incubating the protease with different concentrations ranging 1-20%, w/v for 1 h and protease activity was measured. Salt tolerability of protease was determined by incubating enzyme with different percentage of NaCl for 6-48 h. Residual activity was determined as described above[20].

(viii) Effect of surfactant and oxidizing agent on stability of enzyme[17]

The effect of surfactant (1%) was determined by pre-incubating the protease enzyme with different surfactant and oxidizing agent solutions for 6-48 h at 45^0C before testing for protease activity. Residual activity was measured with respect to control, considered as 100%.

(ix) Assay for anti-microbial activity[21]

Antimicrobial activity of protease was carried out using well diffusion method and the zone of clearance was calculated. For evaluating the effect of protease on different pathogens, *Staphylococcus aureus* (NCIM-2079), *Escherichia coli* (NCIM-2345), *Bacillus subtilis* (NCIM-2063) and *Pseudomonas aeruginosa* (NCIM-2493) were taken as test organisms. The diameter of the zone of clearance was measured and expressed as arbitrary units per ml (AU/ml) as per the calculation below AU/ml = Diameter of the zone of clearance (mm) × 1000 / Volume taken in the well µl

(x) Dehairing activity[1]

In dehairing activity, fresh goat skin was cut to 4 cm^2 pieces and incubated with 10.0 ml of purified protease (50 U/ml in 100 mM Glycine-NaOH buffer, pH 10.0) for 6 h at 45°C. Goat skin treated with only buffer was taken as control. The skin pieces were virtually analyzed for dehairing activity.

RESULTS AND DISCUSSION

1. Identification of strain D1

The newly isolated marine strain D1 was exhibited good growth at the temperature range 37-45^{0}C on glycerol yeast extract agar medium. The colony characteristic of strain D1 was circular in shape, irregular outer surface, appeared chalky white in color. Spores were smooth and oval shaped about 1-2.5 µm in size. The strain was identified as member of *Streptomyces* genus based on the 16S r-DNA sequencing (Fig 1).

16S r-DNA sequencing of marine strain D1

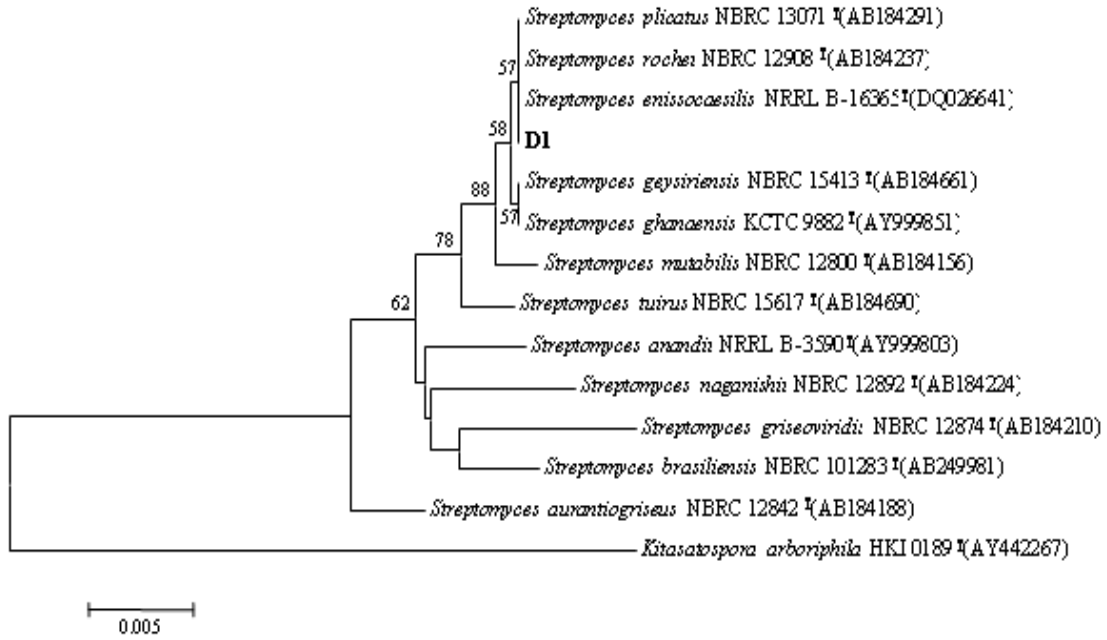

Figure 1
Neighbour-joining phylogenetic tree of strain D1 made by MEGA 4.0.

2. Purification of enzyme

Protease assay and total protein content was carried out at every step of purification and results are summarized in Table 1. It is observed that ammonium sulfate purified sample showed a specific activity 2.51 (IU/mg) being attained 1.49 fold purification and 79.2% recovery. Further dialysis of this enzyme exhibited a specific activity 5.83 (IU/mg) being attained 3.47 fold purification and 66.8% recovery. In the final step, Sephadex G-100 column purified enzyme was attained 8.82 fold purification, 14.8 (U/mg) specific activities and 12.8% recovery. Purified protease migrated as a single band in SDS-PAGE under reducing conditions, suggests that the purified protease was homogeneous. The apparent molecular weight of the purified protease as revealed by SDS-PAGE was about 28 kDa (Fig 2). Similar molecular weight a surfactant stable high alkaline protease was characterized from *Bacillus* sp. B001[22].

Table 1
Summary of protease purification steps from marine Streptomyces sp. D1

Purification step	Total protein (mg)	Enzyme recovery (%)	Enzyme activity (U/ml)	Specific activity (U/mg)	Purification (fold)
Cell-free supernatant	254	100	428	1.68	1
Ammonium sulfate	135	79.2	339	2.51	1.49
Dialyze	49	66.8	286	5.83	3.47
Sephadex-G100	3.1	12.8	46	14.8	8.82

Figure 2
SDS-PAGE gel electrophoresis of protease from Streptomyces sp. D1

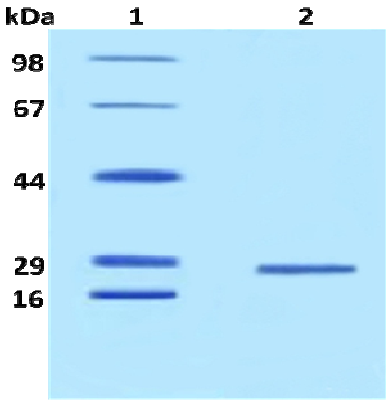

Figure 2 SDS-PAGE pattern of purified protease, Lane 1: Molecular mass marker protein, Lane 2: Sephadex G-100 purified protease.

3. Effect of inhibitors and metal ions on protease activity

The protease under investigation was completely inhibited by PMSF, indicating that enzyme belongs to a serine protease family[1] (Table 2). The addition of Ca^{2+}, Co^{2+} and Mg^{2+} increased protease activity by 135%, 118% and 110% of the control, respectively[21]. However, Cu^{2+}, Hg^{2+} and Zn^{2+} showed reduction of enzyme activity. Maximum inhibition of protease activity was observed in the presence of Fe^{2+} up to 8% of the control[22].

Table 2
Effect of inhibitors on protease activity

Inhibitor	Residual activity* (%)	
Concentration	**5 mM**	**10 mM**
Control	100	100
PMSF	2.23±0.14	1.89±0.10
IAA	96.88 ±1.01	96.78±0.68
EDTA	97.43±1.09	96.28±0.96
Pestatin A	97.09±0.63	96.33±1.65

**** The data given are mean values of three observations ± standard deviation (SD).***

4. Effect of pH, temperature and NaCl on enzyme activity and stability

The activity of protease was influenced with change in pH. The enzyme showed activity over pH range 5.0-13.0 with optimum activity at pH 10.0 (Fig 3A). The protease exhibited more than 54% activity at pH 8.0 and 13.0. The high activity of enzyme at high pH solutions is a very important factor for their utilization in laundry detergent additive[23]. The protease enzyme was found to be stable in buffer system with pH range 8.0 to 10.0 (100 mM glycine-NaOH buffer). There was 98.6% retention of enzyme activity observed with pH 10.0 buffer after 6 h however, activity suddenly decreased in acidic pH range approximately 51% activity retained at pH 5 after 6 h (Fig 3B). Further increase in incubation time to 48 h indicated only 30% and 47% of its initial activity at pH 5.0 and 13.0, respectively. The protease was found to be highly stable at pH 10.0 and retained 86.8% of its original activity after 48 h incubation. The pH stability of protease was higher than reported protease from *Bacillus* sp. stable at pH 9-10 up to 30 min[20]. The protease was most active at 45°C, though it showed considerable activity over the range of 45-60°C[17]. Further increase in temperature up to 50°C exhibited 96% of protease activity. The protease activity gradually declined above 50°C, found 83%, 79% at 70°C and 80°C respectively (Fig 3C). The thermal stability profile of protease (Fig 3D) indicate that enzyme was stable after 120 min retaining 96.1%, 92.1% and 83.6% of initial activity at 45°C, 50°C and 60°C, respectively. The enzyme was satisfactory stable at temperature range 60-80°C and retained 74.3% and 62.4% of its initial activities after 120 min at 70°C and 80°C, respectively. However, protease enzyme lost 50% of its initial activity after 90 min of incubation at 90°C and retained 23.7% activity after 120 min. Hence it is found that the alkaline protease form *Streptomyces* sp. D1 is thermostable and may be useful to various biotechnological processes. Very few reports on protease showed stability at higher temperature. Thermostability of protease from strain D1 is in agreement with the enzyme from *Bacillus mojavensis*[24]. The protease exhibited optimum activity at 12% NaCl concentration, this indicate its halotolerant nature (Fig 3E). Furthermore, increase in NaCl concentration activity of enzyme declined[20]. The stability of protease improved with increase in NaCl concentration and found maximum 92.5% of its original activity at 12% (w/v) NaCl after 6 h. It retained almost 73% and 67% of its activity in presence of 12% and 15% (w/v) of NaCl after 48 h incubation (Fig 3F). Protease retained 50% of activity after 48 h at 3% (w/v) of NaCl. Enzyme activity was drastically dropped to 39% without NaCl after 48 h. Thus, salt is probably necessary for the stability of enzyme.

Effect of pH, temperature and NaCl on activity and stability of protease from Strain D1

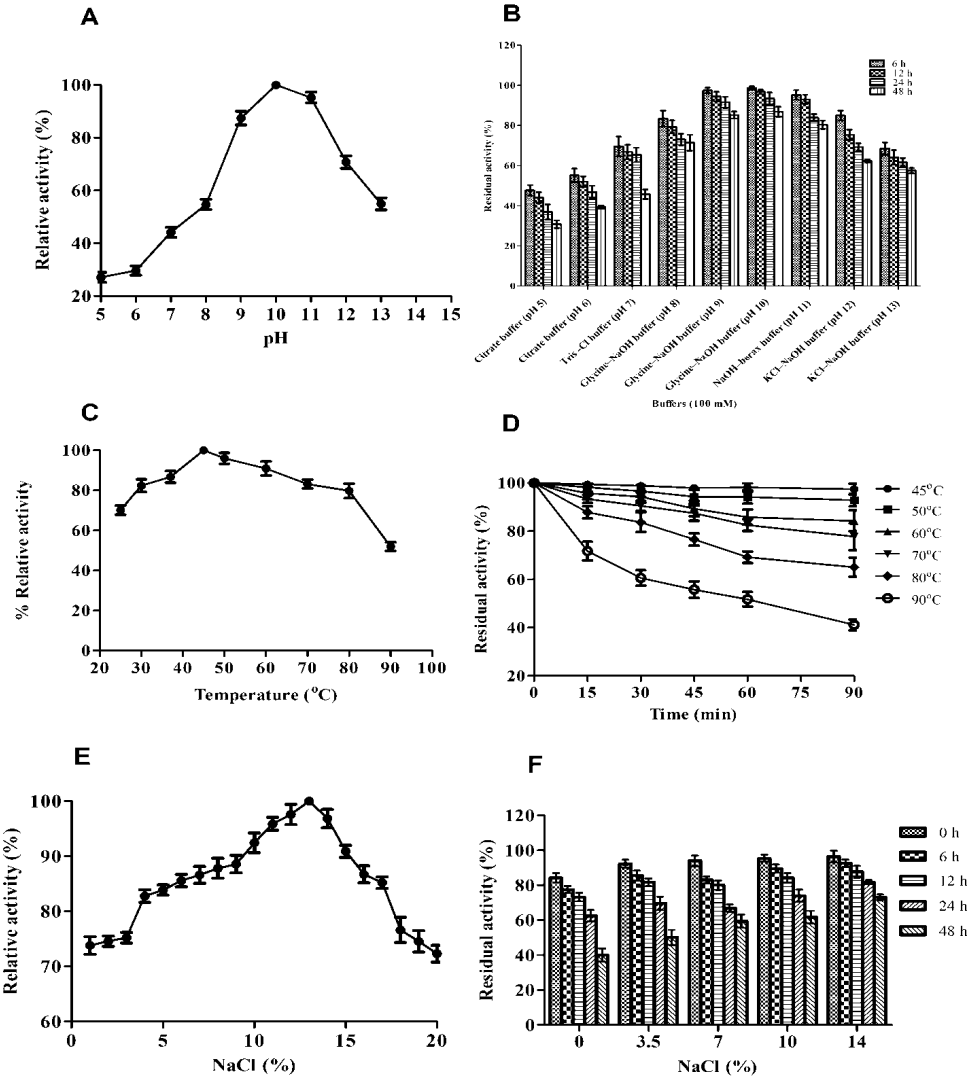

Figure 3

Effect of pH on [A] protease activity and [B] protease stability, Effect of temperature on [C] protease activity and [D] protease stability, Effect of sodium chloride on [E] protease activity and [F] protease stability.

5. Effect of surfactant and oxidizing agent on protease stability

The protease exhibited good stability in presence of non-ionic surfactants like Tween 20, Tween 80 and Triton X-100. Enzyme showed 10% increase in its residual activity with Triton X-100 while its activity retained to 96.2%, 95.1% with Tween 20 and Tween 80 after 6 h, respectively (Table 3). In the presence of strong anionic surfactant like SDS enzyme retained 65% of its original activity after 48 h. Protease retained 92% of its activity with strong oxidizing agent such as hydrogen peroxide (1%) after 6 h. Thus, protease was found to be stable in presence of non-ionic surfactants and oxidizing agents. This is a very important characteristic for potential use in detergent formulation[1, 24].

Table 3
Effect of surfactants on stability of protease enzyme

Surfactant	Residual activity* (%)			
	6 h	12 h	24 h	48 h
Tween-20	96.24 ± 3.20	87.48 ± 1.53	81.65 ± 2.90	65.64 ± 2.48
Tween-80	95.13 ± 2.56	84.85 ± 2.56	82.07 ± 3.98	64.25 ± 2.09
Triton X-100	110.72 ± 4.13	105.67 ± 3.17	100.88 ± 3.96	81.85 ± 4.13
SDS	97.57 ± 2.74	88.15 ± 2.39	81.03 ± 3.14	65.32 ± 4.00
H_2O_2	92.57 ± 4.06	84.49 ± 4.03	74.01 ± 3.77	53.28 ± 2.81

**** The data given are mean values of three observations ± standard deviation (SD).***

6. Antibacterial activity of protease enzyme

The isolated protease showed a wide activity against the pathogenic strains. The protease inhibited the growth of *E. coli*, *S. aureus*, *B. subtilis* and *P. aeruginosa.* The diameters of inhibition zone of various aliquots of protease are presented in Table 4. The protease showed maximum zone of clearance against gram positive, *B. subtilis.* The isolated protease exhibited strong antibacterial potential against pathogenic organisms. Similar type of result reported using partially purified protease from *Bacillus proteolyticus* CFR3001[21].

Table 4
Antibacterial activity of protease from Streptomyces sp. D1

Pathogenic organisms	Protease volume(µl)	Clearance zone (mm)	AU/ml
E. coli	25	13	520
	50	28	560
	75	44	586
B. subtilis	25	14	560
	50	30	600
	75	48	640
P. aeruginosa	25	11	440
	50	25	500
	75	39	520
S. aureus	25	12	480
	50	26	520
	75	41	546

7. Dehairing activity

In dehairing study, incubation of protease with goat skin for 12 h showed removal hairs very easily as compared to skin treated with buffer only (Fig 4). The dehairing activity of protease created interest of researchers because of significant reduction in toxicity, in addition to improvement of leather quality than chemical methodology[1, 25]. The present study confirmed the dehairing activity of this protease thus, its potential application in leather industry.

Dehairing activity of protease from Streptomyces sp. D1 on goat skin

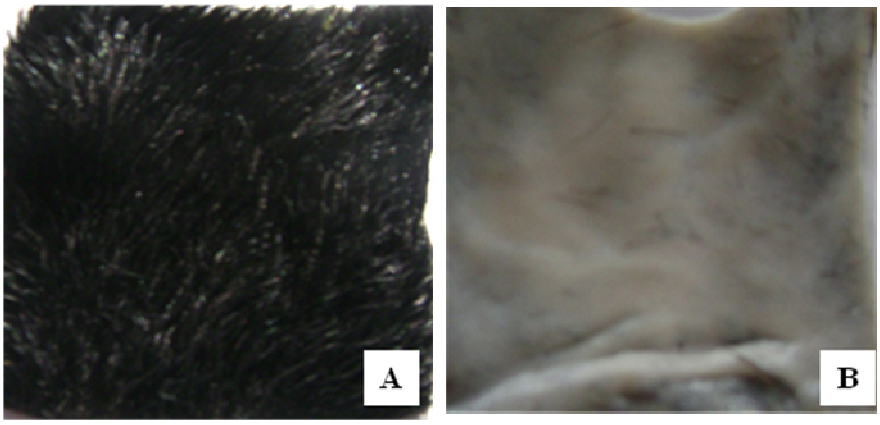

Figure 4
Dehairing activity of protease: [A] Goat skin (control) and [B] Dehairing Activity after protease treatment (12 h).

CONCLUSION

A thermo alkaline, surfactant and detergent tolerant protease produced by marine *Streptomyces* sp. D1 was purified and characterized. The protease was exhibited important properties such as, higher stability in the presence of a wide range of salt, pH and elevated temperature. The enzyme was demonstrated tremendous stability towards surfactant and oxidizing agent. Studies indicated its potential application for hair removal. The protease exhibited strong antibacterial activity against pathogenic organisms. Owing to its halotolerant thermo alkaline nature, its protease may have potential uses in industries such as detergent, food, pharmaceutical, and leather as well as molecular biology techniques.

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