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Characterization of a Novel Surfactant and Organic Solvent Stable High-alkaline Protease from New *Bacillus pseudofirmus* SVB1

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ABSTRACT

Recently, alkaline proteases are of great importance due to their several industrial (detergent, tanning, textile and dairy industries, organic synthesis, peptide synthesis) and environmental applications. The objective of this study was to purify and characterize a novel surfactant and organic solvent stable high-alkaline protease from a new *Bacillus pseudofirmus* SVB1 (Shampa Venkata *Bacillus* isolate 1). The potential of the culture broth of a newly isolated *Bacillus pseudofirmus* SVB1 have evaluated in hydrolyzing natural proteins *viz.*, egg albumin, blood, poultry feather and goat skin hair. An unusually large (85 kDa) serine alkaline protease was purified from the cell supernatant. High stability of the alkaline protease was found in the presence of surfactants, oxidizing agents, heavy metal ions and particularly organic solvents. The maximum activity was observed at an optimal pH of 10.0 and temperature of 40°C. The results obtained in this research study inferred that the purified alkaline protease was a novel surfactant and organic solvent tolerant enzyme with potential applications for various industrial processes.

Key words: High-alkaline protease, surfactant-stable, organic solvent stable, heavy metal tolerant, purification

INTRODUCTION

Alkaline proteases (EC.3.4.21-24, 99) are of immense interest due to their wide applications in detergent, tanning, textile and dairy industries, organic synthesis, peptide synthesis, instant recovery of silver from photographic plates and waste water treatment (Gupta *et al.*, 2002). Alkaline proteases contribute greatly in the global market of industrial enzymes (Banerjee *et al.*, 1999). In general, microbial proteases are extracellular and would simplify the downstream processing of the enzyme as compared to proteases obtained from plant and animal sources (Ozturk *et al.*, 2009). Despite the long list of protease producing microorganisms, only a few are considered as appropriate producers for commercial exploitation, being 'Generally Regarded as Safe' (GRAS), non-toxic and non-pathogenic. In view of this, search for new microorganisms that are able to produce novel alkaline protease, is being carried out till date on the globe (Jayasree *et al.*, 2009).

Efficient purification in downstream processing remains the essential prerequisite for subsequent commercial exploitation of any industrial enzyme. Majority of the purification procedures for extracellular enzymes involve concentration of the culture filtrate either with salt/solvent precipitation or by ultrafiltration followed by purification in a combination of chromatographic procedures (Huang *et al.*, 2003; Al-Omair, 2010; Vijayaraghavan *et al.*, 2011; Nkechi and Kayode, 2007; Salama *et al.*, 2008; Padmakar *et al.*, 2005). Previous reports on purification of alkaline protease describe various column chromatographies, *viz.*, ion exchange as well as hydrophobic interaction chromatography and affinity chromatography or gel filtration chromatography (Patel *et al.*, 2006). Apart from these conventional procedures, more sensitive and advanced procedures such as FPLC (Abbas *et al.*, 1989), converging-diverging foam fractionation (Banerjee *et al.*, 1993), crystallization (Park *et al.*, 1997) and preparative PAGE (Phadatare *et al.*, 1992) have been used for the purification of alkaline proteases. After the purification process, the homogeneity of the enzyme preparation is checked by either polyacrylamide gel electrophoresis or gel-filtration or Iso-electric Focusing (IEF) before further characterization of the enzyme.

Commercialization of alkaline proteases depends on their stability during isolation, purification and storage as well as on their robustness against solvents, surfactants and oxidants. These properties are known to vary with the nature of the organism from which the enzyme is produced. Thus, study of kinetics and catalytic behavior of enzyme isolated from any new strain is indispensable (Huang *et al.*, 2003; Patel *et al.*, 2006).

In the present study, a new *Bacillus pseudofirmus* SVB1 that produced alkaline protease was isolated and identified. The alkaline protease was purified to obtain a homogeneous preparation and detail biochemical characterization of the purified enzyme has been reported.

MATERIALS AND METHODS

Chemicals and reagents: All salts or media constituents *viz.*, NaCl, NaOH, MgSO₄.7H₂O, CaCl₂, NH₄NO₃, KH₂PO₄, K₂HPO₄ and FeCl₃ were purchased from Central Drug House (Pvt) Ltd. (CDH) India. Trichloroacetic Acid (TCA), acetic acid, Tris, HCl and borax were procured from Merck, India. Biological grade casein, gelatin, nutrient broth, Luria-Bertani broth and agar were obtained from Himedia, India. Protein molecular weight markers and Ezeebblue gel stainer[®] were procured from Bangalore Genei, India. Bovine Serum Albumin (BSA) and egg albumin were purchased from Sigma chemicals, India.

Microorganisms and culture conditions: The soil samples were collected in the premises of a tannery industry located at Park Circus, Kolkata, India and diluted in sterile saline solution (0.9% w/v). The diluted samples were plated onto Nutrient Agar (NA) plates (pH 10.0) containing (g L⁻¹) beef extract 1.0, yeast extract 2.0, peptone 5.0, NaCl 5.0, agar 20.0 and were incubated at 30°C. After 36 h, the isolates grown on the plates were streaked on Gelatin Agar (GA) Plate (pH 10.0) containing (g L⁻¹) gelatin 5.0, NaCl 5.0 and agar 20.0 to screen potential alkaline protease producers. Most efficient strain was selected based on its ability to form colony in very less time and large zone of clearance on gelatin agar plate. Cultures were regenerated every 2-3 weeks on a fresh nutrient agar plates from the frozen stock culture.

Inoculum preparation and alkaline protease production: The inoculum was prepared by adding a loop full of pure culture into 50 mL of medium as described previously (Sen *et al.*, 2009a). 1% v/v of inoculum from the culture (A₆₀₀ ~1.0) was added into 500 mL Erlenmeyer flasks

containing 100 mL of the sterile medium containing (in g L⁻¹) casein (7.64), MgSO₄·7H₂O (0.81), CaCl₂ (0.09), NaCl (4.5), FeCl₃ (0.36), NH₄NO₃ (0.58), K₂HPO₄ (2.75) and KH₂PO₄ (2.75) for alkaline protease production from SVB1, as described previously (Sen *et al.*, 2009a, b). The culture flasks were incubated in an orbital shaking incubator at 28°C and 191 rpm. Samples were withdrawn at regular interval of time for measurement of growth and alkaline protease production. The cell free extract was used as crude alkaline protease preparation for further purification and natural protein digestion studies.

Digestion of natural proteins: The culture broth (supernatant) from log phase culture of SVB1 (2 mL) was incubated with slaughter house wastes *viz.*, blood clot, coagulated egg or poultry feather in borax-NaOH buffer (pH 10.0) at 30°C for 12 h. Borax-NaOH buffer (pH 10.0) was used to replace enzyme in the negative control experiment. Dehairing was carried out with 2 mL of the culture broth (supernatant) of SVB1 in 25 mM borax-NaOH buffer at pH 10.0. Hide of a freshly butchered goat was cut into pieces of approximately 5×5 cm and washed with distilled water repeatedly to remove all extraneous matter. After brief air drying, the hide was weighed (between 2 to 3 g) and transferred to 90 mm Petri plate. The control used was 25 mM borax-NaOH buffer of pH 10.0 instead of culture broth (supernatant). Incubations were carried out for 6 h after which hair was scraped off gently from the hides with fingers.

Purification

Acetone precipitation: The culture supernatant containing the extracellular enzyme was first subjected to acetone precipitation where acetone fractions of 0-20, 20-40, 40-60 and 60-80% (v/v) were collected by centrifugation at 8000 rpm and the pellet obtained were air-dried at 4°C and suspended in a minimal volume of 25 mM borax-NaOH buffer (pH 10.0).

Ion exchange chromatography: The 40-60% acetone fraction was applied to a 2×25 cm CM-650 TOYOPEARL® column (Tosho Corporation, Tokyo, Japan). The column was pre-equilibrated with 25 mM Tris-HCl, pH 8.5. Proteins were eluted with a linear gradient of pH 8.0 (with 25 mM Tris-HCl) to pH 10.0 (with 25 mM borax-NaOH buffer). All the purification steps were conducted at 4°C.

Polyacrylamide gel electrophoresis: Sodium Dodecyl Sulphate-polyacrylamide Gel Electrophoresis (SDS-PAGE) of the previously purified enzyme fractions were carried out to check the homogeneity of the enzyme preparation and to determine its molecular weight. SDS-polyacrylamide gel electrophoresis was performed in Mini PROTEAN® Tetra Cell system (BIO-RAD, USA) using 1.5 mm thick gels, following the method of Laemmli (1970).

Zymography: Zymography on SDS-PAGE was performed according to the method of Garciacarreno *et al.* (1993). Finally, the gel was stained with Ezeblue® where clear zone on the blue background indicated the presence of protease.

Characterization

Determination of the optimum pH for activity and stability: The experiments were carried out to evaluate effect of pH (5.0-12.0) on the purified alkaline protease activity. Buffer systems (0.05 M) for different pH values are phosphate buffer (for pH 5.0-7.5), Tris-HCl (for pH 8.0-9.0),

borax-NaOH (for pH 9.5-11.0) and sodium phosphate (for pH 11.5-12.0). Enzyme was incubated in buffer at 30°C for 0-60 min and samples were withdrawn at regular time interval.

Determination of the optimum temperature and thermal stability: Optimum temperature of the enzyme activity was measured by incubating the enzyme reaction mixture at different temperatures (15-50°C) during assay in 25 mM borax-NaOH buffer (pH 10.0). To determine thermal stability, the enzyme preparation was pre-incubated for 1 h at different temperatures (30-50°C) and the residual activity was measured.

Effect of various metal ions: Effect of various metal ions (Na⁺, Mg²⁺, Ca²⁺, Mn²⁺, Zn²⁺, Cu²⁺, Cd²⁺, Hg²⁺ and Fe³⁺) on enzyme activity was studied by incubating it at room temperature in the presence of above mentioned metal ions (10 mM) as described by Rao *et al.* (2009) for 1 h. The residual activity of the enzyme was measured under standard assay conditions.

Effect of protease inhibitors: The influence of different protease inhibitors, *viz.*, Phenyl Methyl Sulphonyl Fluoride (PMSF), (serine protease inhibitor), Ethylene Diamine Tetra Acetic Acid (EDTA) (metalloprotease inhibitor), iodoacetamide (cysteine protease inhibitor) and Pepstatin A (aspartic protease inhibitor) was investigated by incubating the enzyme for 30 min at 30°C in the selected protease inhibitor contained in the assay mixture at a final concentration of either 1.0 or 5.0 mM. The residual proteolytic activity of the enzyme was determined under standard assay conditions.

Effect of surfactants/detergents and oxidizing agents: The effect of 1% final concentration of different surfactants (SDS in w/v, Tween-80, Triton X-100 in v/v) and oxidants (H₂O₂ in v/v) on proteolytic activity of the purified alkaline protease was studied by pre-incubating it for 4 h in the above solutions at 30°C before testing for protease activity. A parallel control was kept with enzyme and buffer with substrate in the absence of any surfactant or oxidizing agents.

Effect of various organic solvents: In the organic solvent stability test, 1.0 mL of organic solvent was added to 3.0 mL of the purified enzyme solution and incubated at 30°C in shaking condition at 150 rpm for 30 min. The remaining proteolytic activity using casein as substrate was measured. Stability was expressed as the remaining proteolytic activity relative to the solvent-free controls.

Substrate specificity: Proteolytic activity with various protein substrates including BSA, casein, egg albumin and gelatin was assayed by mixing 0.5 U of the enzyme with 840 µL of assay buffer containing the protein substrates (0.6% w/v). After incubation at 30°C for 30 min, the reaction was stopped by addition of 896 µL of TCA mixture (0.11 M TCA, 0.22 M sodium acetate and 0.33 M acetic acid) followed by 30 min incubation at room temperature. Undigested protein was removed by centrifugation (13500 rpm) and concentrations of released peptides were measured by reading the absorbance at 280 nm. Activity with casein as substrate was considered to be 100%.

Analytical technique

Assay for proteolytic activity: Alkaline protease activity was measured by a modified method of Anson-Hagihara as described earlier (Sen *et al.*, 2009a).

Protein concentration measurement: The total protein contents of the samples were determined according to the method described by Lowry *et al.* (1951) using bovine serum albumin (Sigma) as standard.

RESULTS

Screening and isolation of microorganisms producing alkaline protease: A total of 67 strains were isolated from the soil collected in the premises of tannery industry. Among them, 23 isolates were screened based on the diameter of clear zone due to gelatin hydrolysis on the agar plates and the production of extracellular alkaline protease. The strain (designated as SVB1), which exhibited maximum zone of clearance shown in Fig. 1 was used in the present study. This microorganism have shown growth in a wide range of pH (6.2-11.0) and temperature (20-36°C) as well as shown ability to produce alkaline protease at similar conditions (Sen *et al.*, 2009b).

Digestion of natural proteins: Insoluble coagulated egg white and coagulated blood were converted to soluble form after incubation with the alkaline protease as apparent from Fig. 2. Poultry feather was also found to be solubilized with the culture broth (supernatant).

Identification of the SVB1: The partial 16S rRNA nucleotide sequence of SVB1 (~1.5 kb) was determined and submitted to GenBank (accession no. EU533950).

Enzyme purification and molecular weight: About 18.8-fold purification and near homogeneity of the enzyme was achieved by acetone precipitation (40-60%) followed by cation-exchange chromatography in CM-650 TOYOPEARL® column and results are shown in Table 1. The specific activity and % recovery of the purified enzyme was found to be 711.43 U mg⁻¹ protein and 52.3%, respectively.

Appearance of single band in SDS PAGE as well as zymography indicated that the purified alkaline protease was a monomeric protein with molecular mass of 85 kDa (Fig. 3).

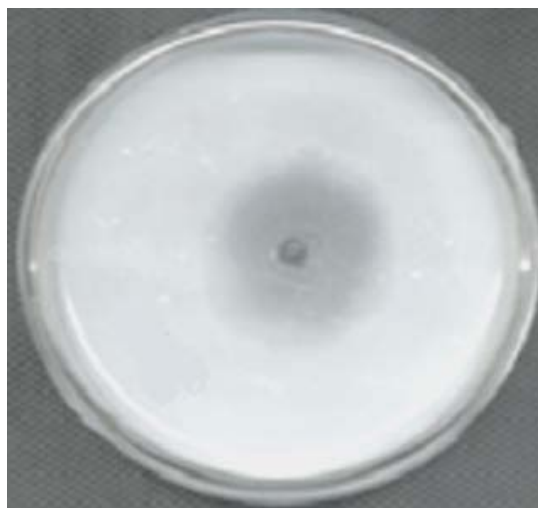


Fig. 1: Alkaline protease activity of SVB1 on gelatin agar plate

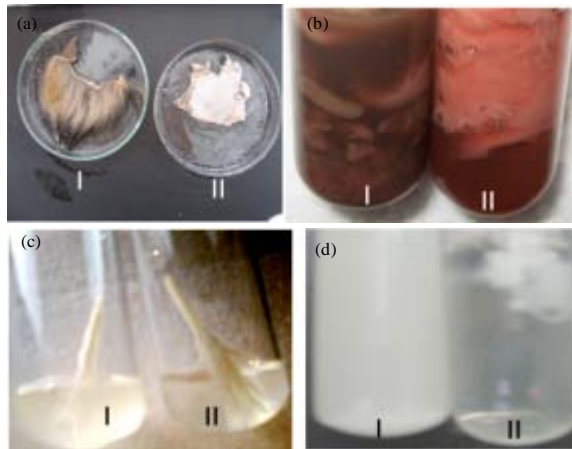


Fig. 2(a-d): Alkaline protease activity on various natural proteins: (a) goat skin dehairing (I: control, II: test), (b) clotted blood (I: control, II: test), (c) poultry feather (I: test, II: control) and (d) egg white (I: test, II: control)

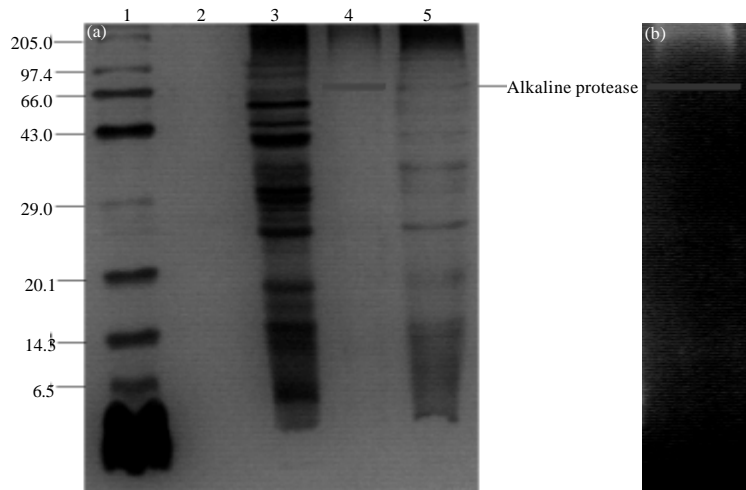


Fig. 3(a-b): Silver stained SDS-PAGE of the purified protease from *B. pseudofirmus* SVB1. Lane 1: molecular mass markers (sizes in kDa), lane 2: gel loading buffer, lane 3: crude supernatant, lane 4: purified protease after CM-650 TOYOPEARL® cation-exchange column, lane 5: 40-60% acetone fraction of the crude supernatant; (b) Ezeebblue® stained zymography on SDS-PAGE of the purified protease after CM-650 TOYOPEARL® cation-exchange column

Table 1: Purification of alkaline protease from *Bacillus pseudofirmus* SVB1

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U mg protein ⁻¹)	Recovery (%)	Fold purification
Crude extract	370000	9868	37.49	100.0	1.00
Acetone precipitation (40-60%)	290820	2439	119.24	78.6	3.18
Ion exchange (Toyopearl)	193510	272	711.43	52.3	18.77

Characterization

Determination of the pH optimum and stability: The enzyme produced by *Bacillus pseudofirmus* SVB1 showed its optimum activity at pH 10.0. Any further variation of the pH of the reaction mixture resulted in reduced catalytic activity as shown in Fig. 4a and even more drastically towards alkalinity. However, the purified enzyme retained 50% relative activity at pH as high as 12.0 or as low as pH 7 which inferred its robust nature in a pH range of 7.0-12.0.

Enzyme stability studies at the broad pH range of 5.0 to 12.0 for 10-60 min indicated that the enzyme activity varied with incubation time and pH as evident from Fig. 4b. Incubation of this enzyme at a pH range of 8.0-10.0 for 1 h did not show any reduction in the activity (Fig. 4b). At pH 5 and 6 the rate of deactivation was higher than at pH 7. At pH 12, 40% reduction in activity was observed after 1 h of incubation.

Determination of the optimum temperature and thermal stability: The protease activity of the purified enzyme was measured at different temperatures that ranged from 15 to 50°C as shown in Fig. 5a. Optimum activity of the purified enzyme was found at 40°C. About 40% of the activity was lost after incubation at 50°C.

The profiles from stability study carried out at a temperature range from 30 to 50°C are presented in Fig. 5b. The purified enzyme retains its full activity for 30 min in the temperature range of 30 to 35°C and the loss of activity was nominal even after 60 min of incubation. However, the loss of enzyme activity was more prominent and drastic when incubated at 45 or 50°C (retaining only 25% residual activity after 1 h).

Estimation of kinetic parameters (V_{max} and K_m): The rate of alkaline protease catalyzed reactions was determined at different initial concentrations of substrate (Casein). A plot was drawn between the rates of alkaline protease catalyzed reaction (V) versus the initial casein (substrate) concentration ($[S]$) (Fig. 6a). V_{max} and K_m values were evaluated from Lineweaver and Burk (1934) double reciprocal plot as presented in Fig. 6b. The K_m and V_{max} were determined to be 1.83 and 533.83 U mL⁻¹, respectively.

Effect of various metal ions: Influence of various metal ions on the activity of alkaline protease from SVB1 is presented in Table 2. It is quite evident that though Ca²⁺, Mg²⁺ and Mn²⁺ ions

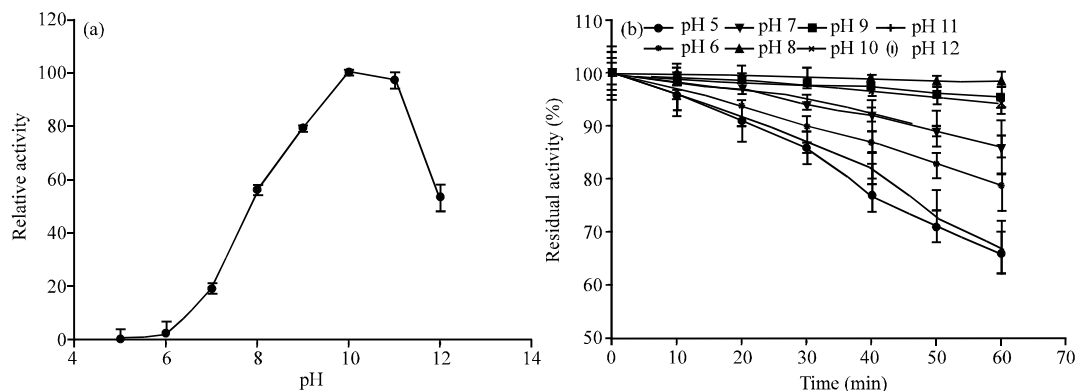


Fig. 4(a-b): Effect of pH on (a) activity (b) stability of the purified alkaline protease from *Bacillus pseudofirmus* SVB1

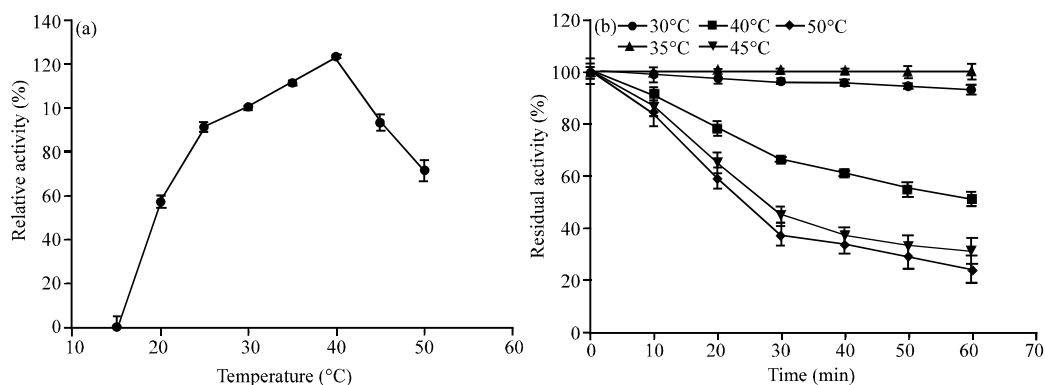


Fig. 5(a-b): Effect of temperature on (a) activity in the temperature range of 15-50°C and (b) stability of the purified alkaline protease from *Bacillus pseudofirmus* SVB1 in the temperature range of 10-60°C

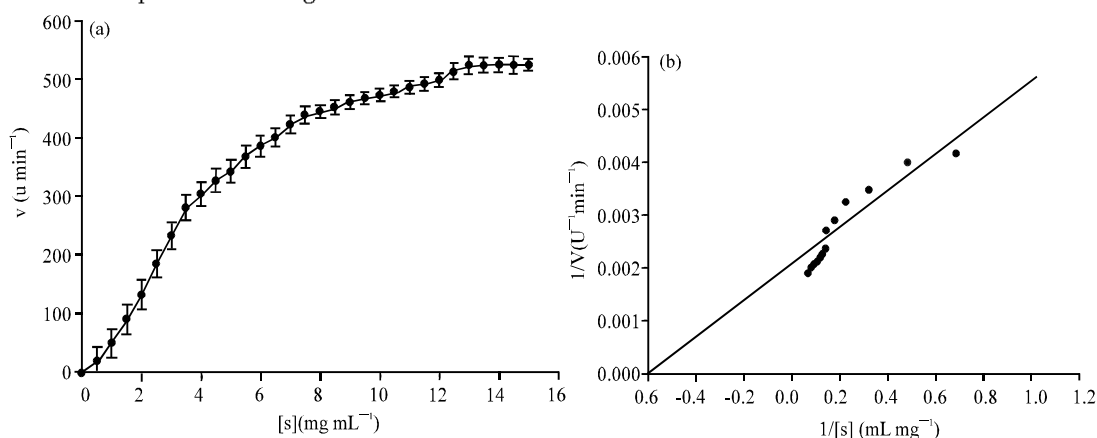


Fig. 6(a-b): Plot of initial velocity of reaction (V) vs. initial substrate concentration [S] and (b) Lineweaver-Burk double reciprocal plot of 1/V vs. 1/[S]

Table 2: Influence of different metal ions on activity* of alkaline protease from *Bacillus pseudofirmus* SVB1

Metal ion	Concentration (mM)	Relative activity (%)
Control	--	100±2
Na ⁺ (NaCl)	10	102±1
Mg ²⁺ (MgCl ₂)	10	113±5
Ca ²⁺ (CaCl ₂)	10	132±5
Mn ²⁺ (MnCl ₂)	10	117±3
Zn ²⁺ (ZnCl)	10	92±1
Cu ²⁺ (CuCl ₂)	10	78±4
Cd ²⁺ (CdCl ₂)	10	91±1
Hg ²⁺ (HgCl ₂)	10	86±4
Fe ³⁺ (FeCl ₃)	10	43±5

*100% of activity correspondent to 0.5 U of enzyme in absence of any metal ion

regulated the enzyme activity positively. Enzyme is also stable and retained more than 90% of its original activity in presence of metal ions such as Na⁺, Zn²⁺ and Cd²⁺. Unlike reported by Aftab *et al.* (2006). The enzyme was inhibited with Cu²⁺ and Fe³⁺ ions and retained 78 and 43% of its original activity.

Effect of protease inhibitors: Proteases are classified based on their sensitivity to various inhibitors (North, 1982). To know the nature of the alkaline protease produced by *B. pseudofirmus*, the enzyme activity in the presence of different protease inhibitors (1.0 or 5.0 mM) were analyzed. It was observed that EDTA (metalloprotease inhibitor), iodoacetamide (cysteine protease inhibitor) and pepstatin A (aspartic protease inhibitor) showed no or minimal effect on alkaline protease activity (Table 3). However, PMSF (serine protease inhibitor) almost completely inhibited the enzyme activity even at very low concentration (1.0 mM) and completely at 5.0 mM concentration.

Effect of surfactants/detergents and oxidizing agents: The purified enzyme showed stability in the presence of all compounds selected in this study as shown in Table 4. In fact the non-ionic detergents, Triton X-100 and Tween-20 enhanced its activity by 13 and 25%, respectively. In the presence of strong anionic surfactant SDS (1%), the enzyme retained 71% of its initial activity.

Effect of various organic solvents: The enzyme was stable in presence of all the organic solvents tested after 30 min incubation as given in Table 5. In organic solvents such as isoctane, n-decane, n-dodecane and acetone (25%, v/v) retained much of the activity of purified alkaline protease by 51, 45, 39 and 63%, respectively.

Substrate specificity: Substrate specificity of the purified protease was examined using various natural protein substrates. The alkaline protease showed a high level of hydrolytic activity against casein and moderate hydrolysis of gelatin and egg albumin and very low affinity towards BSA (Table 6). The enzyme is highly specific towards casein followed by gelatin and egg albumin with 68, 43% relative activity, respectively.

Table 3: Effect of different inhibitors on activity* of alkaline protease from *Bacillus pseudofirmus* SVB1

Inhibitors	Relative activity at inhibitor concentration of		Type
	1 mM	5 mM	
Control	100±5	100±5	----
PMSF	3±1	0±0	Serine protease inhibitor
Iodoacetamide	98±4	94±4	Cysteine protease inhibitor
EDTA	100±4	96±4	Metallo-protease inhibitor
Pepstatin A	97±2	91±3	Aspartic protease inhibitor

*100% of activity correspondent to 0.5 U of enzyme in absence of any inhibitor

Table 4: Influence of different surfactants/detergents on the activity* of alkaline protease from *Bacillus pseudofirmus* SVB1

Surfactants/detergents	Relative activity (%)
Control	100±1
Triton X-100	113±2
Tween-20	125±2
SDS	71±5
H ₂ O ₂	89±4
Nirma	91±3
Surf excel	95±2
Rin	98±2
Ariel	87±5

*100% of activity correspondent to 0.5 U of enzyme with casein as substrate in absence of any surfactants/detergents

Table 5: Influence of different organic solvents on activity* of alkaline protease from *Bacillus pseudofirmus* SVB1

Organic solvents	Relative activity (%)
Control	100±3
Isooctane	51±4
Decane	45±4
Dodecane	39±5
Acetone	63±3

*100% of activity correspondent to 0.5 U of enzyme with casein as substrate in absence of any organic solvent

Table 6: Substrate specificity of alkaline protease from *Bacillus pseudofirmus* SVB1*

Substrate added	Relative activity (%)
Casein	100±2
Gelatin	68±3
BSA	17±5
Egg albumin	43±4

*100% of activity correspondent to 0.5 U of enzyme with casein as substrat

DISCUSSION

In the screening experiment, gelatin was used as the sole source of carbon and nitrogen in the agar plates at pH 10.0 to screen alkaline protease producers. Hydrolysis of gelatin at pH 10.0 has been performed by alkaline protease, which solubilize gelatin by digesting peptide bonds. Hence, it was confirmed that all the organisms grown on the gelatin agar plates were producing extracellular alkaline protease.

The isolate that showed maximum zone of clearance was related to the *Bacillus* lineage, closely clustering with *B. pseudofirmus* (GenBank accession no. AB029256.1) along with *Bacillus* sp. (GenBank accession No. AB043857.1) being the nearest homolog as was evident from the phylogenetic tree. Digestion of natural proteins envisaged its potential in different keratinous waste treatment or treatment of industrial waste water rich in proteinaceous compound.

The molecular mass observed in the present study was in accordance with few literature reports where the molecular masses of alkaline proteases from *Bacillus* genus have been found to be above 70 kDa, 75 kDa (Jung *et al.*, 2007); 80 kDa (Ghosh *et al.*, 2008); 90 kDa (Kato *et al.*, 1992); 86.29 kDa (Arulmani *et al.*, 2007). There are some reports of alkaline protease with molecular mass as low as 15-20 kDa (Soliman *et al.*, 2009).

The effect of pH on the stability of the enzyme is shown in Fig. 4b. About 85% activity was retained when incubated at pH 5.0. These findings were in accordance with several earlier reports showing pH optima between 8.0 and 10.5 for proteases from *Vibrio fluvialis* VM10 (Venugopal and Saramma, 2006), *Salinivibrio* sp. (Amoozegar *et al.*, 2007), *gamma-proteobacterium* (Sana *et al.*, 2006), *A. clavatus* ES1 (Hajji *et al.*, 2007) and some *Bacillus* sp. (Beg and Gupta, 2003; Horikoshi, 1990). Hajji *et al.* (2007) reported that purified alkaline protease from *Aspergillus clavatus* ES1 was stable in pH range of 7 to 9. Optimum pH for *Bacillus pseudofirmus* alkaline protease was found to be pH 10-11 (Patel *et al.*, 2006).

The result from the optimum temperature experiments showed that alkaline protease from SVB1 was active over a wide range of temperature (from 20 to 50°C) due to mesophilic nature of the enzyme.

Thermal stability study indicated that the better stability of alkaline protease from SVB1 than that reported by Mitra and Chakrabartty (2005). Proteolytic activity in this study decreased

dramatically when the temperature increased above 40°C which retained only 30 and 25% of activity at 45 and 50°C, respectively (Fig. 5b). This was similar to the results reported by Pawar *et al.* (2009) where about 95 and 70% activity of *Bacillus* protease were retained at 40 and 50°C, respectively compared to that of its optimum temperature of 30°C. The proteases obtained from *B. pumilus* (Feng *et al.*, 2001; Huang *et al.*, 2003) which lost its 53% activity at 50°C from its optimal temperature of 45°C and thus was inferior to protease from SVB1 in this study. Another serine protease was reported to retain the activity at a pH range of 7.5 to 9.5 and temperature range of 30 to 45°C, resulting in the relative activity of higher than 80% (Yossan *et al.*, 2006). These properties of the enzyme are particularly suitable for laundry detergents and it is effective under more alkaline conditions and at moderately wide temperature stability than the important detergent enzyme subtilisin Carlsberg, which showed maximum activity at pH values of 8-10 and at a temperature of 60°C (Horikoshi, 1990).

The low K_m and high V_{max} values inferred that the high affinity and efficient catalytic role of the enzyme. These values were comparable to the literature reported values of alkaline proteases, haloalkaliphilic *Bacillus* sp. (K_m of 2 mg mL⁻¹ and V_{max} of 289 U min⁻¹) (Gupta *et al.*, 2005a), *Haloalkaliphilic bacterium* sp. AH-6 (K_m of 2.5 mg mL⁻¹ and V_{max} of 625 U min⁻¹) (Dodia *et al.*, 2008) and *Pseudomonas aeruginosa* PseA (K_m of 2.69 mg mL⁻¹ and V_{max} of 3.03 mmol min⁻¹) (Gupta *et al.*, 2005b).

The nature of the Lineweaver and Burk double reciprocal plot as evident from Fig. 6b obtained in this study was very much similar to that reported by Padron-Pereira *et al.* (2009) except that their R² value (0.85) was lower than that obtained in this study (0.93). Similarly, R² obtained for calculating K_m and V_{max} for different enzymes in the literature are lower than that achieved in the present study (0.70: Evans *et al.*, 2009; 0.62: Linton and Greenaway, 2004). Thus, a good fitting of the equation suggested that Michaelis-Menten kinetics was followed by this alkaline protease for protein hydrolysis reaction.

The Ca²⁺ ion dependent activity improvement might be attributed to its involvement in stabilization of the enzyme molecular structure which has also been reported for some other proteases derived from *Bacillus* sp. (Towatana *et al.*, 1999; Takii *et al.*, 1990; Kobayashi *et al.*, 1996; Sana *et al.*, 2006; North, 1982; Anvari and Khayati, 2011). Complete inactivation of the enzyme in presence of PMSF suggested that the protease produced by the *B. pseudofirmus* belongs to serine protease group (Anvari and Khayati, 2011).

The proteases belonging to *Bacillus* genus are known to be unstable against the oxidants and bleaching agents (Anwar and Saleemuddin, 2000). However, the enzyme under investigation showed very little inhibition in presence of 1% hydrogen peroxide and retained 89% of its activity. Similar kind of observation was reported by Habib *et al.* (2011). This experimental observation suggested that the purified enzyme was stable to all tested cationic, anionic, non-ionic and to the different commercially available detergents.

Hydrophobic solvents are usually superior to hydrophilic solvents, because the latter have a greater tendency to strip tightly bound water from the enzyme molecules essential for catalytic activity. If organic solvents are used as media in the synthetic reaction, the reaction equilibrium of hydrolytic enzymes can be shifted towards completion of the reverse of hydrolysis. Therefore, proteases, which are naturally stable in the presence of organic solvents, could be very useful for synthetic reactions. There are very few reports available on organic solvent tolerant alkaline protease. The alkaline protease from SVB1 is superior to other alkaline proteases reported in the literature (Thumar and Singh, 2009; Rahman *et al.*, 2006). The Stability of the purified enzyme

in the presence of surfactants and bleach (Triton X-100, SDS and H₂O₂), commercial detergents (Nirma®, Surf excel®, Rin® and Ariel®), heavy metals (Cd and Hg) and organic solvents (isooctane, decane and dodecane) inferred that the purified enzyme seems to be a potential candidate for industrial applications. The enzyme had shown broad substrate specificity.

CONCLUSION

A new alkaline protease of 85 kDa was purified upto 18.77 fold from *Bacillus pseudofirmus* SVB 1 with 52.3% yield. The enzyme showed optimum pH in the range of 8-11. The optimum temperature for the alkaline protease was found to be 40°C. The enzyme showed high stability in the temperature range of 40-50°C. The K_m and V_{max} were found to be 1.83 mg mL⁻¹ and 533.83 U mL⁻¹, respectively. The enzyme activity of alkaline protease from *B. pseudofirmus* SVB1 was not much affected in the presence of different metal ions. The enzyme was also stable in presence of detergents, surfactants and organic solvents.

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