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Purification and Partial Characterization of a Detergent and Oxidizing Agent Stable Alkaline Protease from a Newly Isolated *Bacillus subtilis* VSG-4 of Tropical Soil

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An extracellular detergent tolerant protease producing strain VSG-4 was isolated from tropical soil sample and identified as *Bacillus subtilis* based on morphological, biochemical characteristics as well as 16S-rRNA gene sequencing. The VSG-4 protease was purified to homogeneity using ammonium sulphate precipitation, dialysis and sephadex G-200 gel permeation chromatography with a 17.4 purification fold. The purified enzyme was active and stable over a broad range of pH (8.0-11.0, optimum at 9.0) and temperature (40°C to 60°C, optimum at 50°C). The thermostability of the enzyme was significantly increased by the addition $CaCl_2$. This enzyme was strongly inhibited by PMSF and DFP, suggesting that it belongs to the serine protease superfamily. The purified VSG-4 alkaline protease showed remarkable stability in anionic (5 mM SDS) and ionic (1% Trion X-100 and 1% Tween 80) detergents. It retained 97±2% and 83.6±1.1% of its initial activity after 1 h preincubation in the presence of 1% H_2O_2 and 1% sodium perborate, respectively. Furthermore, the purified enzyme showed excellent stability and compatibility with some commercial laundry detergents besides its stain removal capacity. Considering these promising properties, VSG-4 protease may find tremendous application in laundry detergent formulations.

***Keywords*:** *B. subtilis*, alkaline protease, purification, characterization, detergent stability

Proteases which catalyze the cleavage of peptide bonds in proteins are the class of enzymes having tremendous applications in both physiological and commercial fields (Mukherjee *et al*., 2008). Among various proteases, microbial proteases are of most significant, compared to that of others. Proteases are the most important industrial enzymes accounting for 60% of the total enzyme market (Ng and Kenealy, 1986) and microbial proteases represent approximately 40% of the total worldwide production of enzymes (Horkoshi, 1996). Proteases are classified as acid, neutral, and alkaline proteases on the basis of pH range in which their activities are optimum. Of these, alkaline proteases are particularly important because of their activity and stability at high pH and in the presence of surfactants and oxidizing agents (Gupta *et al*., 1999, 2002b; Haddar *et al*., 2009). They have ample biotechnological potential for industrial sectors like laundry detergents, leather processing, brewing, food, and pharmaceutical industries (Kembhavi *et al*., 1993).

Alkaline proteases are generated by a wide range of organisms, including bacteria, molds, yeasts, and mammalian tissues. Currently, a large proportion of commercially available alkaline proteases are derived from strains of *Bacillus* (Oberoi *et al.,* 2001; Gupta *et al*., 2002a; Joo *et al*., 2002).

Ideally, proteases used in detergent formulations should have high activity and stability within a broad range of pH and temperatures and should also be compatible with various detergent components along with oxidizing and sequestering agents (Kumar and Takagi, 1999; Oberoi *et al*., 2001). The most of the available commercial detergent proteases were reported to be stable at conditions of elevated temperatures and pH. However, the most of these enzymes are relatively unstable in the presence of non-ionic surfactants (Tween 80), anionic surfactants (SDS) and peroxide agents (H_2O_2), which are the common ingredients in modern bleach-based detergent formulations (Jaouadi *et al*., 2008). Therefore, it is desirable to search for new protease producers with novel properties like stability to pH, temperature, metal ions, and surfactants.

Thanjavur is the tropical district, situated at the southern part of Tamilnadu and well known for its rice production. In the present study, purification and characterization of an extracellular alkaline protease produced by *B. subtilis* (VSG-4), isolated from the soil sample of kitchen waste dumping site of Periyar Maniammai University, Thanjavur, Tamilnadu, India, have been reported. To our knowledge, there is no report on investigations of proteases from *B. subtilis* of tropical soil isolates.

However, we report for the first time a detergent tolerant and oxidant stable alkaline protease from *B. subtilis* VSG-4 isolated from tropical soil. The main research objective is to find the potential application of *B. subtilis* VSG-4 alkaline protease as a laundry additive.

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Materials and Methods

Bacterial strain isolation and identification

Soil samples were collected from the kitchen waste dumping site of Periyar Maniammai University, Thanjavur, Tamilnadu, India, and were diluted in sterile distilled water. The diluted samples were spread on casein nutrient agar plates, which contained (g/L): peptone, 5.0; beef extract, 3.0; yeast extract 3.0; NaCl, 5.0; and bacteriological agar, 15.0. Plates were incubated at 37°C for 24-48 h. The bacterial isolates showing high ratios of clear zone diameter to colony diameter were selected as potential protease producers for further experiments. The most promising isolate, VSG-4 was selected for further studies. VSG-4 was morphologically, biochemically characterized and further identified by 16S-rRNA amplification and nucleotide sequencing.

Crude enzyme production

Production of protease by VSG-4 was carried out in medium containing (g/L): starch, 10; beef-extract, 5; K_2HPO_4, 2; $MgSO_4$, 2; pH, 9.0. The medium was autoclaved at 121.6°C for 15 min. Production was carried out in Erlenmeyer flasks (1,000 ml) containing 500 ml of medium for 48 h at 37°C on a shaker at 160 rpm. After the completion of fermentation, the culture medium was centrifuged at 12,000 rpm for 15 min at 4°C and the cell-free supernatant was used for further studies.

Enzyme purification

Ammonium sulphate precipitation: The supernatant containing extracellular enzymes was fractionated by ammonium sulphate precipitation between 50-70% (w/v) of saturation and the pellet was collected after centrifugation at 20,000 rpm for 15 min. The pellet was resuspended in 0.1 M Tris-HCl buffer (pH 7.8) and dialyzed against the same buffer for 48 h and concentrated. All the steps were carried out at 4°C.

Sephadex G-200 gel filtration chromatography: The concentrated sample was applied on a Sephadex G-200 column (1.5×24 cm) for gel filtration and equilibrated with Tris-HCl buffer (pH 7.8). The column was eluted at flow rate of 30 ml/h with same buffer. Fractions with high protease activity were pooled, dialyzed and concentrated by lyophilization.

Polyacrylamide gel electrophoresis (SDS-PAGE) and zymography: The purity and molecular weight of purified protease were analyzed by SDS-PAGE as directed by Laemmli (1971) using 10% acrylamide. The molecular mass standards used were Rabbit muscle phosphorylase (97.4 kDa), ovalbumine (45 kDa), carbonic anhydrase (31 kDa), and soybean trypsin inhibitor (21.5 kDa).

Zymography was performed in connection with SDS-PAGE for the detection of proteolytic activity on polyacrylamide gel by the method of Haddar *et al*. (2009). The sample was not heated before electrophoresis. After electrophoresis, the gel was rinsed in 0.1 M glycine-NaOH buffer (pH 10.0) containing 2.5% Triton X-100 for 30 min with constant agitation to remove SDS. Triton X-100 was removed by washing with the same buffer. The gel was then incubated with 1% (w/v) casein in 0.1 M glycine-NaOH buffer (pH 10.0) for 20 min at 50°C. Finally, the gel was stained with 0.1% Coomassie Brilliant Blue R-250 in methanol:acetic acid:water (40:10:50, v/v/v) followed by destaining with methanol:acetic acid:water (5:10:85, v/v/v).

Determination of protein concentration

Protein concentration was determined by the method of Lowry *et al.* (1951) using bovine serum albumin (BSA) as standard and absorbance was measured at 280 nm.

Alkaline protease activity measurement

The procedure for alkaline protease activity measurement was adapted from Kembhavi *et al*. (1993) using casein as substrate. A 0.5 ml aliquot of the purified enzyme (preferably diluted) was mixed with 0.5 ml of 100 mM glycine-NaOH (pH 10.0) containing 1% casein and incubated for 30 min at 50°C. The reaction was terminated by the addition of 0.5 ml trichloroacetic acid (20%, w/v). The mixture was kept at room temperature for 15 min and then centrifuged at 12,000 rpm for 15 min to collect the supernatant. A blank was prepared in the same way, but 0.5 ml of 100 mM glycine-NaOH buffer was used instead of purified enzyme. The acid soluble material was estimated using spectrophotometer at 280 nm. A standard curve was prepared using solutions of 0-50 mg/L tyrosine. One unit of protease activity was defined as the amount of enzyme required to liberate 1 μg of tyrosine per minute under the experimental conditions used.

Characterization

Effect of pH on protease activity and stability: The effect of pH on the purified protease activity was determined at different pH values using casein (1%, w/v) as substrate. The pH values ranging from 7.0-13.0 were adjusted using the following buffer systems: phosphate (pH 7.0), Tris-HCl (pH 8.0 and 9.0), glycine-NaOH (pH 9.0-12.0), and Na_2CO_3 (pH 13.0). The ionic strength of each buffer was 0.1 M. Reaction mixtures were incubated at 37°C for 30 min and relative proteolytic activities were measured at 37°C.

To measure the enzyme stability, purified enzyme was diluted in different relevant buffer solutions mentioned above and pre-incubated at 37°C for 2 h and 15 h (Hadj-Ali *et al*., 2007). Afterwards, the residual proteolytic activities were determined under standard assay conditions.

Effect of temperature on protease activity and stability: The effect of temperature variations on purified enzyme activity was studied from 30°C to 70°C using casein as substrate for 30 min in 100 mM glycine-NaOH buffer, pH 9.0 and in the presence and absence of 5 mM $CaCl_2$. Relative protease activities were measured at 37°C.

To determine the enzyme stability, purified enzyme was incubated at different temperatures (40°C to 70°C) for 3 h at pH 9.0 in the presence of 5 mM $CaCl_2$ (Haddar *et al*., 2009). The residual activities were determined at every 30 min interval under standard assay conditions. The non-heated enzyme was considered as control (100%).

Effects of metal ions on protease activity: Effects of different metal ions on purified enzyme were investigated by supplementing monovalent (Na^+, K^+), divalent (Ca^{2+}, Mg^{2+}, Cu^{2+}, Mn^{2+}, Co^{2+}, and Zn^{2+}) and trivalent (Al^{3+}, Fe^{3+}) metal ions (all ions in chloride form and 5 mM final concentration) to the assay mixture. Mixtures were incubated at 37°C for 30 min (Haddar *et al*., 2009; Shah *et al*., 2010) and relative activities were calculated at temperature 50°C. The activity of the enzyme without metallic ions was considered as control (100%).

Effects of inhibitors on protease activity: The effects of various enzyme inhibitors on purified protease activity were studied using phenylsulphonyl fluride (PMSF), Iodoacetamide, di-isopropyl fluorophosphates (DFP), dithio-bis nitrobenzoic acid (DTNB), and ethylenediaminetetraacetic acid (EDTA). Purified enzyme was pre-incubated with inhibitors for 1 h at 37°C before adding substrate and then, the residual activity was measured under standard assay conditions using casein as the substrate. The activity of enzyme in the absence of inhibitors was considered as control (100%).

Table 1. Summary of purification steps of alkaline protease from *B. subtilis* VSG-4

Purification step	Total enzyme activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Recovery (%)
Culture Supernatant	48470	4765	10.1	1	100
$(NH_4)_2SO_4$ precipitation (61%)	34270	2670	12.8	1.18	70.7
Sephadex G-200	4150	23.5	176.5	17.4	8.5

All the steps were carried out at 4°C

Effect of detergents, oxidizing and bleaching agents on enzyme activities: Effects of detergents were studied with non-ionic detergents [Triton X-100, Tween 80, 1% (v/v)], anionic detergent (SDS, 5 mM), cationic detergent [cetyl trimethyl ammonium bromide (CTAB), 5 mM] and commercial detergents [Ariel, Surf excel, Tide and Rin; 1% (w/v)]. The influence of oxidizing and bleaching agent [hydrogen peroxide (H_2O_2), Sodium perborate 1% (v/v)] on purified protease activity was also studied. The endogenous proteases (if any) of commercial detergents were inactivated by heating the diluted detergents for 1 h at 65°C (Haddar *et al*., 2009) prior to the addition of enzyme. All the reaction mixtures were incubated for 1 h (Haddar *et al*., 2009; Shah *et al*., 2010) at 37°C. Relative protease activities were measured at 50°C using casein as substrate. The activity of enzyme without any additive was taken as control (100%).

Stain removal capacity of purified protease: Application of protease as a detergent additive was studied (Adinarayana *et al*., 2003) with a commercial detergent, Ariel. White cotton cloth pieces (5×5 cm) stained with blood was taken in separate beakers. Beakers were marked as A, B, and C. The following sets were prepared for experiment:

A. Beaker with distilled water (100 ml) + cloth stained with blood
B. Beaker with distilled water (100 ml) + cloth stained with blood + 1 ml of detergent (7 mg/ml).
C. Beaker with distilled water (100 ml) + cloth stained with blood + 1 ml of detergent (7 mg/ml) + 2 ml of enzyme solution (5,000 U/ml).

All the beakers were incubated at 37°C for 30 min. Then, the cloth pieces were taken out, rinsed with water and dried. Visual examination of various cloth pieces revealed the effectiveness of enzyme in removal of stains. The untreated cloth piece stained with blood was considered as control.

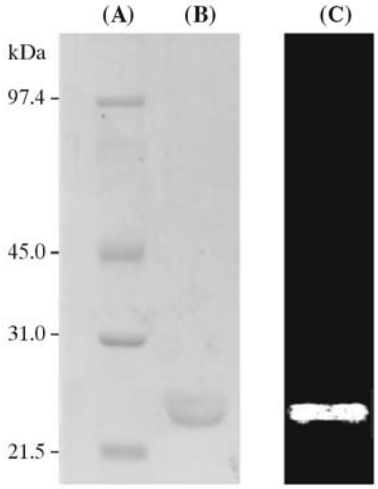

Fig. 1. SDS-PAGE and Zymogram of purified alkaline protease. Lanes: A, Protein molecular weight markers; B, Purified alkaline protease; C, Zymogram.

Results and Discussion

Bacterial strain identification

VSG-4 was a sporeforming, Gram-positive, catalase positive, aerobic, rod shaped motile bacteria. Morphological and biochemical characters of VSG-4 resembled Genus *Bacillus*. The comparative 16S rRNA partial gene sequence (470 base pair) analysis of VSG-4 showed 99% base homology with *B. subtilis* in BLAST search (www.ncbi.nlm.nih.gov). This sequence has already been submitted to NCBI GenBank (Accession no. HQ292067).

Purification of extracellular proteases

An extracellular alkaline protease was purified from the supernatant of *B. subtilis* VSG-4. The supernatant attained saturation at 61% (w/v) of ammonium sulphate. The protein pellet obtained after centrifugation, was dissolved in 0.1 M Tris-HCl (pH 7.8) and subjected to gel filtration on a sephadex G-200 column. A well-resolved single peak of enzyme activity was eluted from the Sephadex G-200 column (data not shown). The result of the purification steps of alkaline protease is summarized in Table 1. At the final step, a specific activity of 176.5 U/mg was achieved, with a yield of 8.5% of total protein and 17.4-fold purification. Similar procedure was applied by Adinarayana *et al.* (2003) for the purification of serine alkaline protease from a newly isolated *B. subtilis* PE-11 and achieved (21-fold purification with a yield of 7.5%) almost the same level of purification.

SDS-PAGE of purified alkaline protease

The homogeneity of the purified alkaline protease was checked by SDS-PAGE and a unique protein band was obtained for the purified enzyme. Based on the relative mobility, the molecular weight of the protein band was estimated to be 24 kDa (Fig. 1). The purity of the purified enzyme was also evaluated by zymogram (Fig. 1C). Zymogram staining activity showed a single clear band of casein hydrolysis against blue back ground, indicating the homogeneity of the purified protease.

Generally, the molecular weights of the alkaline proteases of *Bacillus* species lie within the range 15-38 kDa (Horikoshi, 1990; Adinarayana *et al*., 2003; Uchida *et al*., 2004; Kazan *et al*., 2005; Patel *et al*., 2006; Haddar *et al*., 2009; Shah *et al*., 2010). The molecular weight of VSG-4 protease-24 kDa-places it within the 15-38 kDa range.

Characterization of purified alkaline protease

Effect of pH on activity and stability: The pH activity profile of VSG-4 alkaline protease was determined using buffers of different pH values. The enzyme was highly active in the range

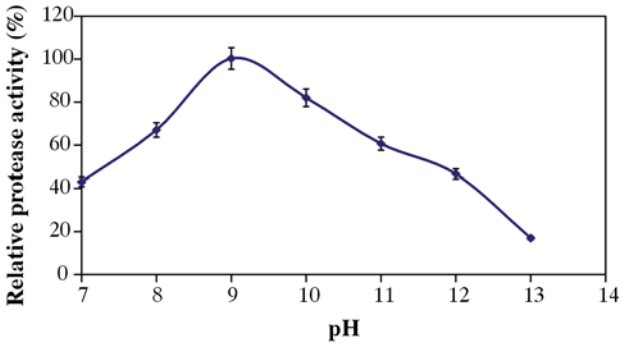

Fig. 2. Effect of pH on protease activity. The purified enzyme was incubated in buffers of different pH values (7.0-13.0) at 37°C for 30 min. Relative activity was measured at 37°C. Each value represents the mean of three independent determinations and error bars indicate the standard deviation.

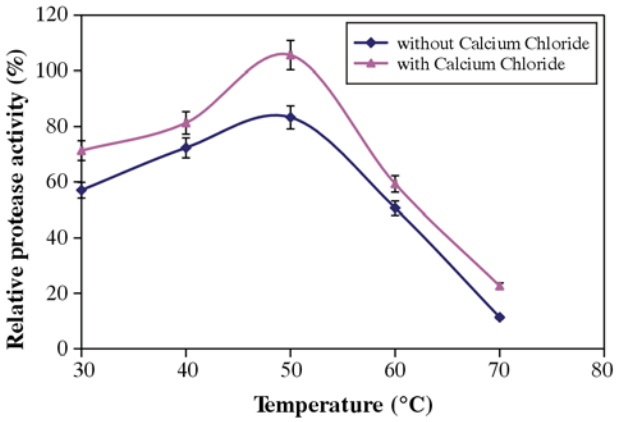

Fig. 3. Effect of temperature on protease activity. The purified enzyme was pre-incubated with substrate in the presence and absence of $CaCl_2$ at different temperatures (30-70°C) for 30 min. Relative protease activity protease activity was determined at 37°C. Each value represents the mean of three independent experiments and error bars indicate the standard deviation.

of pH 8.0-11 with an optimum at pH 9. The maximum protease activity was found to be at pH 9.0 (100%) using Tris-HCl buffer (Fig. 2) and at pH 10.0 it retained 82±1% activity. The sharp declines in the activity at pH lower than 9.0, suggests the alkaline nature of the enzyme. This enzyme showed better activity than *B. subtilis* DM-04 protease (Mukherjee *et al*., 2008) and *B. cereus* AK1871 protease (Shah *et al*., 2010). Many alkaline proteases isolated from *Bacillus* had high pH for their activity (Patel *et al*., 2006). Strain Ve1 and NH1 produced an alkaline protease which was active up to pH 11.0 (Patel *et al*., 2006; Hadj-Ali *et al*., 2007). Subtilisin Carlsberg of *B. licheniformis* showed maximum activity at pH 8.0-10.0 (Horikoshi, 1990).

The effect of pH on enzyme stability was determined by pre-incubating the purified enzyme in buffer solutions of various pH values for 2 h and 15 h. In both cases, enzyme showed higher stability at pH 9.0 and 10.0. In case of 15 h preincubation the enzyme retained 90.6±2.51% and 78.3±2.08% of its original activity at pH 9.0 and 10.0, respectively. It retained 100.3±1.52% and 92±1% of its original activity after 2 h of incubation at pH 9.0 and 10.0, respectively. Thus, 2 h incubation periods showed better stability than 15 h incubation. Purified protease lost about 18%, 39%, and 54% of its initial activity after 2 h of incubation at pH 11.0, 12.0, and 13.0, respectively. Our results differ from *B. subtilis* NCIM 2713 alkaline protease that was stable at pH range of 6.5-9.0 at 37°C for 3 h (Mane and Bapat, 2001). However, an earlier study reported the stability of *B. subtilis* PE-11 alkaline protease for 20 h between pH 8.0-10.0 (Adinarayana *et al*., 2003).

The maximum activity and stability at higher pH are excellent characteristics of an enzyme to use as laundry detergent additive (Kalisz, 1988), since the pH of laundry detergents is generally in the range of 9.0 to 12.0.

Determination of optimum temperature and thermal stability: The optimum temperature for purified alkaline protease activity was determined by varying reaction temperature from 30°C-70°C at pH 9.0. The temperature for optimum protease activity was 50°C (105.6±2.5%) and the enzyme activity gradually decreased at temperature beyond 50°C (Fig. 3). However, temperature tolerance of this enzyme is quite better than previously reported alkaline protease from a thermophilic *B. subtilis* DM-04, having optimum activity at 37-45°C (Mukherjee *et al*., 2008). The effect of $CaCl_2$ was observed on temperature profile of the enzyme. The addition of $CaCl_2$ had increased the enzyme activity. The stimulation of protease activity in presence of $CaCl_2$ has also been reported earlier (Oberoi *et al*., 2001; Adinarayana *et al*., 2003; Kazan *et al*., 2005).

The thermal stability profiles (Fig. 4) of purified alkaline protease showed that the enzyme was highly stable at 50°C. It retained 72.3±1.5% of the initial activity even after 3 h incubation at 50°C, while it retained 71.6±1.52, 100, 40±1.7, and 17±1.15% of its original activity after 1 h incubation at 40°C, 50°C, 60°C, and 70°C, respectively. Previously, two alkaline serine proteases purified from *B. mojavensis* A21, showed high stability at temperature below 50°C (Haddar *et al*., 2009). Beg and Gupta (2003) reported a thiol-dependent serine al-

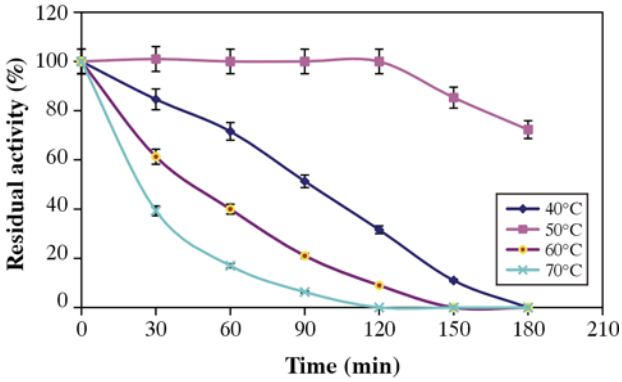

Fig. 4. Effect of temperature on protease stability. Purified enzyme was incubated with substrate in the presence of 5 mM $CaCl_2$ at temperatures between 40 to 70°C for 180 min at pH 9.0. Residual activities were determined at regular intervals under optimal conditions. Each value represents the mean of three independent experiments and error bars indicate the standard deviation.

kaline protease from *B. mojavensis* having 100% stability up to 50°C for 3 h; only a 40% loss of activity was observed at 60°C over the same incubation period. VSG-4 protease showed a low stability at 40°C even though optimum temperature for activity was 50°C. Similar result was reported by Arulmani *et al*. (2007) that a serine alkaline protease from *B. laterosporus*-AK1, showing higher stability at optimum temperature (75°C) for 30 min but less stability at lower temperature (50°C) over the same incubation period.

The thermal activity and stability of protease was enhanced in presence of 5 mM $CaCl_2$. This improvement in thermostability against thermal inactivation in the presence of $CaCl_2$ may be deciphered by the strengthening of interactions inside protein molecules and probably by the binding Ca^{2+} to autolysis site (Lee and Jang, 2001).

Effects of metal ions: The effect of various metal ions [Na^+, K^+, Ca^{2+}, Mg^{2+}, Zn^{2+}, Mn^{2+}, Cu^{2+}, Co^{2+}, Hg^{2+}, Al^{3+}, Cr^{3+} (5 mM)] on the activity of alkaline protease was studied. The s-block metals Ca^{2+} and Mg^{2+} stimulated protease activity by 50.6% and 14%, respectively (data not shown). The Mn^{2+}, Co^{2+} and monavalent (K^+, Na^+) ions had no effect on enzyme activity whereas trivalent ion Al^{3+} considerably inhibited the activity by 89%. However, the d-block metals Zn^{2+}, Cu^{2+}, Hg^{2+}, and Cr^{3+} inhibited the enzyme activity by 67.7%, 89.4%, 97.4%, and 93.7%, respectively. The enhancement of protease activity in presence of s-block metals (Ca^{2+}, Mg^{2+}) and reduction in the presence of d-block metals (Zn^{2+}, Cu^{2+}, Hg^{2+}, Cr^{3+}) has been reported by many researchers (Oberoi *et al*., 2001; Patel *et al*., 2006; Arulmani *et al*., 2007; Jaouadi *et al*., 2008; Shah *et al*., 2010). This result suggests that the concerned metal ions protected the enzyme against thermal denaturation and played vital role in maintaining active confirmation of the enzyme at high temperatures (Donaghy and Mckay, 1993). Generally, s-block metals bind poorly to ligands through ionic bond. As the bonding is mainly ionic, the metal ions are easily displaced. However, d-block elements preferentially bind to ligands through covalent bonds to give stable complexes and hence the enzyme gets irreversibly bound leading to poor activity (Shah *et al*., 2010).

Effects of inhibitors on alkaline protease activity: Proteases can be classified by their sensitivity to various inhibitors. In order to determine the nature of the purified enzyme, effects of various inhibitors on enzyme activity were determined. Enzyme activity was strongly inhibited by PMSF and DFP (at 5 mM) which are well known inhibitors of serine proteases, indicating involvement of serine in the catalytic activity. Enzyme retained 2.6±1.5% and 18±1.9% of its original activity after 1 h incubation in the presence of PMSF (2 mM) and DFP (2 mM), respectively. The enzyme activity was unaffected by Iodoacetamide and DTNB. This inhibition profile strongly suggests that extracellular VSG-4 protease belongs to serine proteinase super-family. Most alkaline proteases from *Bacillus* sp. are commonly inhibited by serine protease inhibitor PMSF (Joo *et al*., 2002; Adinarayana *et al*., 2003; Kazan *et al*., 2005; Arulmani *et al*., 2007; Jaouadi *et al*., 2008).

EDTA had marginal (15%) inhibitory effect on enzyme activity at 5 mM concentration, indicating that the enzyme had very little requirement for metal ions as cofactor. Similar reports on stability of alkaline proteases in the presence of EDTA have been made by Adinarayana *et al*. (2003), Kazan *et al.* (2005), and Jaouadi *et al*. (2008). The stability of enzyme in presence of chelating agent appears to be advantageous to use as detergent additives, since detergents contain high percentages of chelating agents, which serve as water softeners and also assist in stain removal. These agents specifically chelate metal ions making them unavailable in detergent solution, thus the activity of detergent enzymes will reduce significantly (Haddar *et al*., 2009).

Effects of surfactants, oxidizing, and bleaching agents on protease activity: A suitable detergent protease should be compatible and stable with all commonly used detergent compounds such as surfactants, oxidizing agents, bleaches and other additives, which might be present in the formulation (Gupta *et al*., 2002b). The present study showed that the enzyme was highly stable in the presence of non-ionic surfactants (Triton X-100 and Tween 80). Tween 80 (1%, v/v) stimulated the enzyme activity by 9%, whereas enzyme retained 94.3±3% activity after 1 h treatment with Triton X-100 (1%, v/v) at 37°C. The enzyme was highly stable in the presence of strong anionic surfactant (SDS). It retained 74±2.6% of its original activity even after 1 h incubation at 37°C with SDS (5 mM). These results are in agreement with the reported proteases from various *Bacillus* species, showing stability in the presence of various surfactants (Oberio *et al*., 2001; Beg and Gupta, 2003; Kazan *et al*., 2005; Patel *et al*., 2006; Jaouadi *et al*., 2008; Rai and Mukherjee, 2010). The purified VSG-4 protease was detergent stable at room temperature which is necessary in laundry uses. This effect of detergent on the enzyme can be correlated to their hydrophilic/lipophilic balance, which is defined as the way a detergent distributes between polar and non-polar phases (Shah *et al*., 2010). The stability towards SDS is important because SDS stable enzymes are scanty in market (Haddar *et al*., 2009).

Furthermore, VSG-4 alkaline protease showed stability against oxidizing and bleaching agents. It retained 97±2% and 83.6±1.1% of its original activity after 1 h of incubation at 37°C with H_2O_2 (1%, v/v) and sodium perborate (1%, v/v), respectively. In comparison, alkaline protease of *B. majavensis* showed 100% stability for 1 h at room temperature in the presence of H_2O_2 and sodium perborate (Beg and Gupta, 2003). These findings have a greater importance because oxidant, surfactant and bleachstable wildtype enzymes are rarely reported (Gupta *et al*., 1999).

Compatibility of purified enzyme with commercial detergents: The purified enzyme showed excellent compatibility and stability with all the tested commercial laundry detergents. The enzyme retained 100% of its activity in the presence of Ariel and 82.3±2.5% in Tide after 1 h incubation at 37°C. In comparison, alkaline protease from *B. mojavensis* A21 retained 99% of its initial activity after 1 h incubation at 30°C in the presence of Ariel at a concentration of 7 mg/ml (Haddar *et al*., 2009). In the presence of Surf excel and Rin, the enzyme retained 61±2% and 68±3.5% of its original activity, respectively. However, enzyme was found to be least stable in the presence of CTAB (5 mM); lost about 70% of its original activity. These findings are in agreement with several reports on alkaline proteases produced by *Bacillus* species, showing excellent stability with a long range of commercial solid detergents at concentration 7 mg/ml (Adinarayana *et al*., 2003; Hadj-Ali *et al*., 2007; Mukherjee *et al*., 2008; Haddar *et al*., 2009).

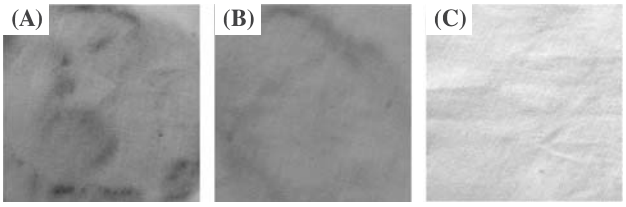

Fig. 5. Stain removal capacity of alkaline protease. Efficiency of alkaline protease amended with Ariel detergent in washing performance. (A) Blood stained cloth washed with water. (B) Blood stained cloth washed with Ariel detergent. (C) Blood stained cloth washed with combination of Ariel detergent and enzyme.

The obtained results clearly indicate that performance of enzymes in detergents depends on number of factors, including the detergents compounds since the proteolytic stability varied with each laundry detergents (Haddar *et al*., 2009). Therefore, partial loss of protease activity of VSG-4 in some of the detergents may be attributed to inhibitory effect(s) of component(s) of these detergents (Rai and Mukherjee, 2010).

Stain removal capacity of purified protease: The excellent laundry detergent stability of alkaline protease prompted us to evaluate its stain removal potency for application in commercial laundry detergent formulations. It was observed that the purified enzyme at a concentration of 5,000 U/ml with commercial detergent Ariel could remove blood stain from cotton cloths (Fig. 5). It has been recommended that proteases or the other hydrolytic enzymes to be used in detergent formulations should be effective at low levels ranging from 0.4% to 0.8% (Rai and Mukherjee, 2010) and therefore, it is reasonable to assume that VSG-4 is an ideal candidate for use in laundry detergent. Earlier reports made on addition of enzyme preparation with commercial detergent significantly enhanced the washing performances and removal of blood stains (Adinarayana *et al*., 2003; Arulmani *et al*., 2007; Rai and Mukherjee, 2010).

The efficient stain removal capacity as well as the ability to retain the protease activity even in the presence of metal chelator (EDTA) and other surfactants could have a greater advantage of this enzyme in commercial laundry detergent formulations. Besides, the enzyme was alkaline in nature and thermostable. However, the stability and compatibility of any component should not be the only pre-requisite for its inclusion in detergent formulation. To save energy from heating the water to be used for washing, the ability of the detergent components to perform wash function at lower temperature should also be addressed (Krik *et al*., 2002). There are only few reports published showing the activity of protease at room temperature (Rai and Mukherjee, 2010). The protease secreted by *B. subtilis* strain VSG-4 in the present study remains active at room temperature and at alkaline pH range favoring its inclusion in laundry detergents.

Conclusion

In the present study, a detergent and oxidizing agent stable alkaline protease produced by *B. subtilis* VSG-4 isolated from tropical soil, was purified and partially characterized. The purified enzyme was stable at alkaline pH and elevated temperature. It exhibited important properties like stability in the presence of various commercial laundry detergents, surfactants, oxidizing and bleaching agents. Considering these properties, it can be concluded that this enzyme may find potential application in laundry detergent formulations, pharmaceuticals and other industries. This would require further investigations on structure-function relationship, through site-directed mutagenesis and 3D structure determination.

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