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Full Length Research Paper

An oxidant, detergent and salt stable alkaline protease from *Bacillus cereus* SIU1

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A novel soil bacterium, *Bacillus cereus* SIU1 was earlier isolated from non-saline, slightly alkaline soil of Eastern Uttar Pradesh, India. The isolate *B. cereus* SIU1 was grown in modified glucose yeast extract (modified GYE) medium at pH 9.0 and 45°C. It produced maximum protease at 20 h incubation. The enzyme was stable at pH 9.0 and 55°C. It was fully stable at 0.0 to 3.0% and moderately stable at 4.0 to 10.0% (w/v) NaCl concentrations. Whereas PMSF, EDTA and ascorbic acid were inhibitory, cysteine and β-mercaptoethanol enhanced protease activity. Calcium, magnesium, manganese and copper at 1 mM concentration increased the enzyme activity. Hydrogen peroxide, sodium perborate, sodium lauryl sulphate, Triton X100 and Tween 80 significantly increased the activity, while protease remained fairly stable (52 to 98%) at 0.1 and 1.0% concentrations of commercial detergents. The halotolerant thermoalkaline protease of *B. cereus* SIU1 was highly active and stable in the presence of several modulators, oxidants and detergents, revealing its possible use in several commercial and biological applications.

Key words: *Bacillus cereus* SIU1, thermoalkaline protease, PMSF, EDTA, Hydrogen peroxide, Triton X100, Tween 80.

INTRODUCTION

Proteases are enzymes of utmost importance as they are invariably found in all forms of life including prokaryotes, protists, plants and animals. This group of enzymes constitutes ~66% of total enzymes employed in various industries (Gupta et al., 2002). Among these, alkaline proteases have vast applications principally in the food, detergent, leather and pharmaceutical industries (Rao et al., 1998). Alkaline protease producing bacteria are widely distributed and can be found almost every where. Among them, bacilli are most potential candidates for thermoalkaline protease production (Gupta et al., 2002).

Bacterial serine proteases are well known for their high activity at alkaline pH and broad substrate range. Their optimum temperature for activity ranges near 35 to 80°C (Rao et al., 1998; Gupta and Khare, 2007). This efficiency of bacterial alkaline proteases supports them as potential enzymes for various processes. Proteases having halotolerance are endowed with capacity to work in the presence of high salt concentrations (Joo and Chang, 2005; Thumar and Singh, 2007). This property of proteases is desirable for their application under saline conditions.

Alkaline proteases stable in presence of various metal ions, denaturants, surfactants, oxidants and detergents are like goldmine from their industrial application point of view. Such types of proteases have been studied by several researchers (Joo et al., 2003; Banik and Prakash, 2004; Gupta and Khare, 2007; Zambare et al., 2007; Sareen and Mishra, 2008). Oxidant and pH stability of a protease are most desirable markers for possible application in commercial detergents and bleaches to improve the washing efficiency. Highly thermoalkaline proteases appear to have better washing properties. The use of proteases in detergents is known to solve the problem of removing proteinaceous dirt from fabric, and is one of the early applications of proteases. If proteases applied in detergents are alkaline as well as

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thermostable, the washing becomes easy at 50 to 60°C (Garg and Johri, 1999).

In our earlier study, we isolated and characterized a soil isolate *Bacillus cereus* SIU1, which is Gram positive rod, strict aerobe, motile and endospore former. It exhibited positive catalase and oxidase activity and is able to grow over a wide pH range of 5 to 12, temperature (15 to 55°C) and NaCl up to 10% (w/v) concentrations. Hydrolysis of casein and gelatin under alkaline conditions revealed its capability to produce alkaline protease (Singh et al., 2010). In view of this, the study was envisaged with the aim to characterize the protease produced by *B. cereus* SIU1 for the properties essentially required in an enzyme to be effectively employed in detergent industry.

MATERIALS AND METHODS

Bacterial growth and protease production

The bacterium was originally isolated from non-saline, slightly alkaline soil of district Pratapgarh, Uttar Pradesh, India (Singh et al., 2010). It was grown in modified glucose yeast extract (modified GYE) broth containing (gl^{-1} distilled water): glucose, 10.0; peptone, 10.0; yeast extract, 5.0 and NaCl, 5.0. Briefly, 1.0 ml of mother culture of 0.5 absorbance (A_{620}; 1 cm cuvette) containing $3.4x10^7$ cfu ml^{-1} was inoculated in 99 ml of aforementioned medium (pH 9, adjusted after autoclaving using sterilized 1 M Na_2CO_3 solution in distilled water) in Erlenmeyer flasks and incubated at 45°±1°C on an incubator shaker (120 rpm) for 24 h. The culture was centrifuged at 16,000 *g* (4°C) for 5 min and cell-free supernatant was employed for ammonium sulphate precipitation (30 to 75%, w/v). The protein precipitate was obtained by centrifugation at 12,000 *g* (4°C) for 10 min, dissolved in minimum volume of 50 mM sodium carbonate-bicarbonate buffer (pH 9) and dialyzed thoroughly against the same buffer for 24 h. The dialyzed protein was employed for protease characterization.

Analytical methods

Enzyme assay

The proteolytic activity was assayed by casein digestion method of Anson (1938). 1 ml of enzyme was incubated with 3.0 ml of casein (1% (w/v) in 100 mM sodium carbonate-bicarbonate buffer; pH (9) at 55±1°C. The reaction was stopped after 10 min by addition of 3.0 ml of 10% (w/v) trichloro acetic acid (TCA). The mixture was centrifuged at 16,000 *g* (4°C) for 10 min and supernatant used to estimate the amount of free tyrosine. One unit of enzyme activity is defined as the amount of enzyme that liberates 1.0 µg of tyrosine min^{-1}.

Protein estimation

The cell-free supernatant and ammonium sulphate fraction were subjected to estimate the amount of protein present as per Lowry et al. (1951) using BSA as standard.

Combined effect of temperature and pH on protease

Activity

The enzyme was subjected to protease activity assay at temperatures 35, 45, 55 and 65°±0.5°C. The assay of enzyme activity at each temperature was performed at pH ranging 3 to 12 using suitable buffers that is, citric acid-sodium citrate buffer (pH 3 to 6), sodium phosphate buffer (pH 7, 8), sodium carbonate-bicarbonate buffer (pH 9 to 11) and glycine-NaOH buffer (pH 12).

Stability

The enzyme stability at various pH and temperature was assessed using enzyme diluted with equal volume of each buffer of pH 3 to 12 and incubated at 35, 45, 55 and 65°±1°C for 30 min. The residual protease activity was assayed at optimized pH 9 and temperature 55±1°C.

Effect of NaCl on protease stability

The effect of NaCl on protease stability was assayed using NaCl solution of 0.0 to 12.0% (w/v) concentrations. Enzyme was diluted with equal volume of each solution and incubated for 30 min at 55±1°C. Subsequently, the residual protease activity was assayed under standard conditions.

Effect of modulators and divalent cations on protease stability

The enzyme was subjected to study the effect of phenyl methyl sulfonyl fluoride (PMSF, a specific serine protease inhibitor), ethylene diamine tetra acetic acid (EDTA), β-mercaptoethanol, ascorbic acid (vitamin C), cysteine, divalent cations Ca^{2+}, Mg^{2+}, Zn^{2+}, Fe^{2+}, Ni^{2+}, Co^{2+}, Cu^{2+}, Mn^{2+} and Hg^{2+} on protease stability. Solution of each modulator was prepared in 1.0 to 5.0 mM concentration range. Calcium chloride, magnesium sulphate, zinc chloride, ferrous sulphate, nickel chloride, cobaltous chloride, cupric chloride, manganous chloride and mercuric chloride solutions were employed in three different concentrations (0.1, 1.0 and 10 mM). The enzyme was diluted separately with each solution in equal volume and incubated for 30 min at 55±1°C. Thereafter, residual protease activity was assayed under standard conditions.

Effect of surfactants and oxidants on protease stability

Various surfactants (Triton X-100, Tween 80, sodium lauryl sulphate) and oxidants (H_2O_2, sodium perborate) were employed to study their effect on protease stability. Their solutions were prepared at 0.1, 1.0, 5 and 10% (w/v) concentrations in distilled water. Enzyme was incubated with each solution for 30 min at 55±1°C and assayed for residual protease activity under standard conditions.

Effect of commercial detergents on protease stability

Commercial detergents (Rin, Surf, Ariel, Tide, Wheel, Nirma, More and Ghari) were employed to study their effect on protease stability. Detergent solutions were prepared at 0.1, 1.0, 5 and 10% (w/v) concentrations in distilled water. Enzyme was mixed with equal volume of each solution, incubated for 30 min at 55±1°C and assayed for residual protease activity under standard conditions.

Statistical analysis

The experiments were performed twice, each in triplicate. For statistical analysis, standard deviation for each experimental result was calculated using the Microsoft excel.

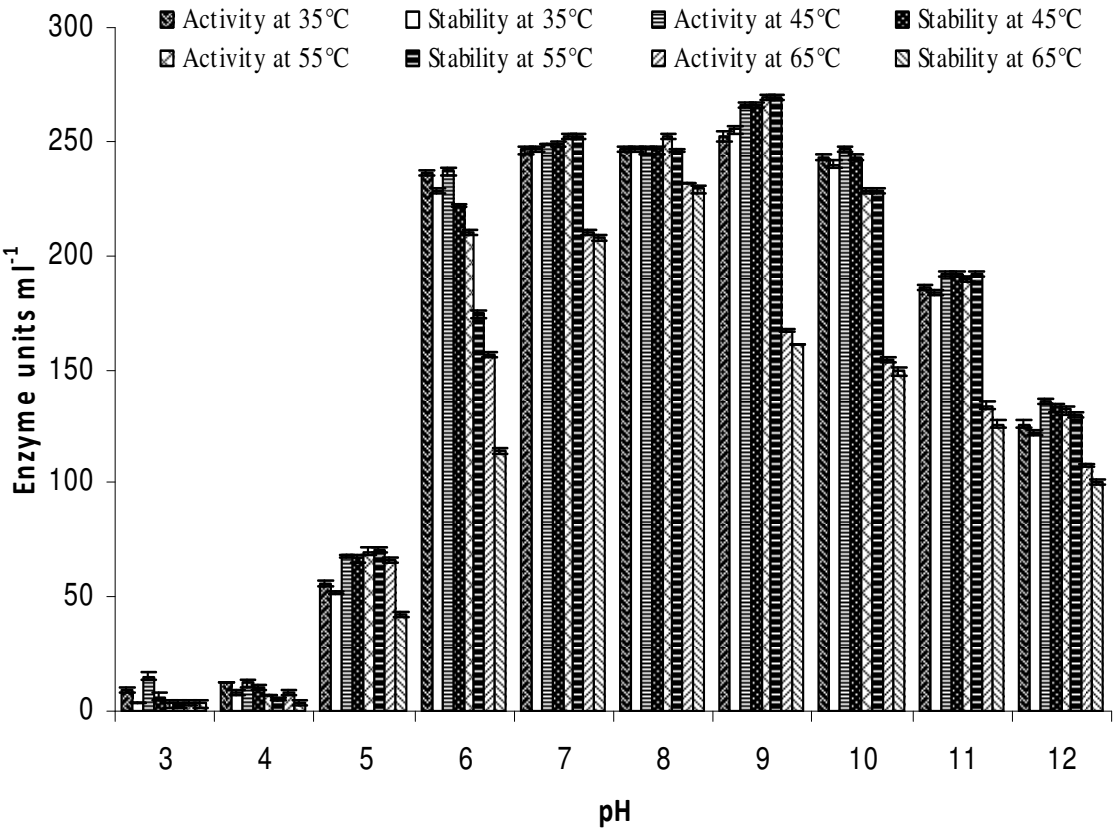

Figure 1. Combined effect of pH and temperature on protease activity and stability.

RESULTS AND DISCUSSION

The isolate *B. cereus* SIU1 produced 88 protease Uml^{-1} at 20 h incubation in un-optimized modified GYE broth (Singh et al., 2010). The protein content and specific activity in crude enzyme was 4.2 mg ml^{-1} and 20.95, respectively. Ammonium sulphate fractionation enhanced 3 times protease activity to 265 Uml^{-1} with a specific activity and total recovery of 31.92 and 83.3%, respectively. The partially purified protease was thereafter subjected to its characterization. The genus *Bacillus* has proven its ability as a source of almost every enzyme which is required in bulk. Likewise, for the production of thermoalkaline protease also, *Bacillus* sp. is an important candidate for industries. Several workers have reported production of thermoalkaline proteases from *Bacillus* sp. An alkaline protease from *Bacillus* sp. was produced by Gupta et al. (1999) having activity in broad pH range (5 to 12) at 60°C. Joo and Chang (2005) have reported an oxidant and SDS-stable alkaline protease from a halotolerant *Bacillus clausii* I-52. The pH 9.0 and temperature 45°C for protease production by our *B. cereus* isolate is in agreement with other workers, reporting enzyme production in wide pH and temperature range of 7 to 12 and 37 to 80°C, respectively (Olajuyigbe and Ajele, 2005; Miyaji et al., 2006; Abusham et al., 2009).

Combined effect of temperature and pH on enzyme activity and stability

The protease activity was more than 65% (172 to 265 units) at 35 to 55°C in the pH range of 6 to 10. At 65°C, the activity reduced at every pH studied, but was still fairly high (66 to 231 units) except at pH 3 and 4. At pH 11 and 12 also, the protease activity was good at temperature range of 35 to 55°C, but largely reduced at pH 4 and 5 and almost zero at pH 3. Hence, it can be inferred that the protease was active (55 to 265 units) in broad pH and temperature range of 5 to 12 and 35 to 65°C, respectively. However, enzyme activity was maxi-mum (265 units) at pH 9 and 45 to 55°C (Figure 1). The stability studies on protease also revealed fairly stable (15 to 100%) in the pH range of 5 to 12. However, it exhibited good stability (65 to 100%) in the pH and temperature range of 7 to 11 and 35 to 55°C, respectively with maxima at pH 9 and 45 to 55°C. The stability decreased (20 to 99%) at 65°C when treated for 30 min at complete pH range (3 to 12) under study (Figure 1). The protease stability reduced drastically to the tune of 90 to 99% at pH 4 in complete temperature range of 35 to 65°C and approached nearly zero at pH 3.

In this study, combined effect of pH and temperature indicated the suitability of protease as it was 40 to 100%

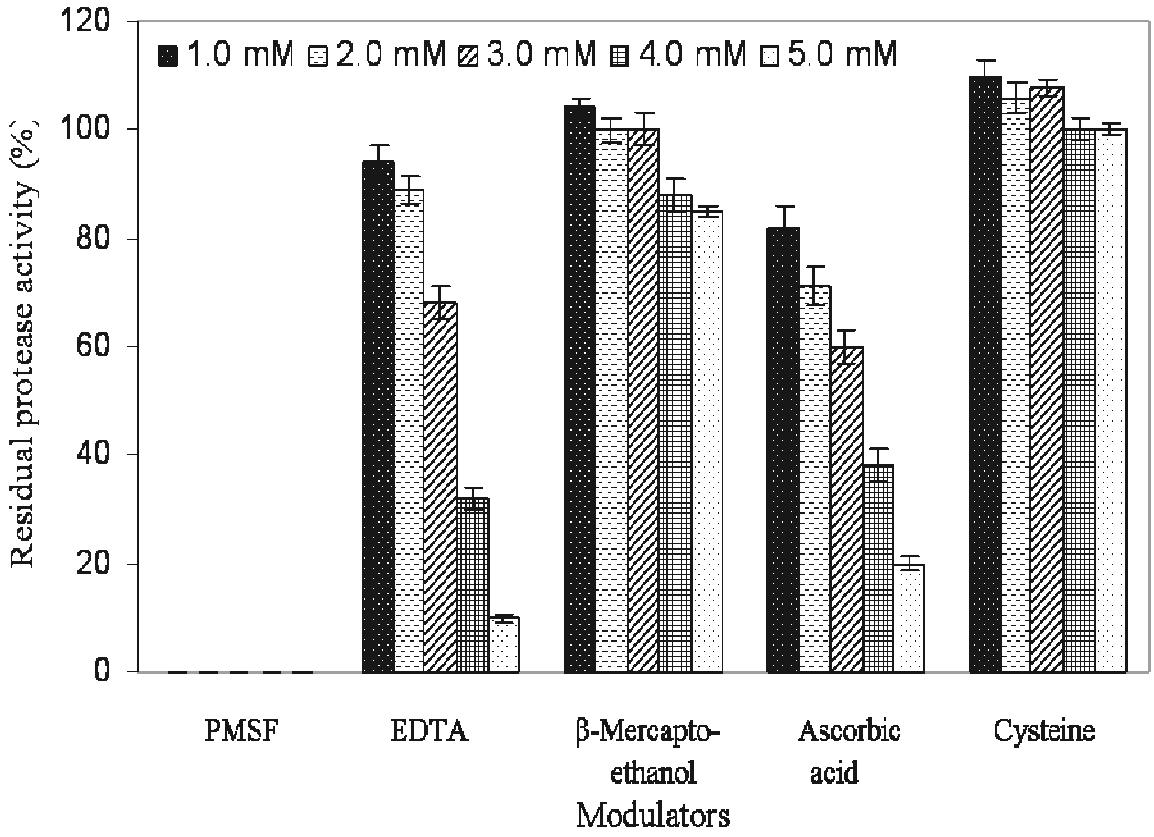

Figure 2. Effect of various modulators (1 to 5 mM) on protease stability.

active and stable in pH range of 6 to 12 at every temperature studied (Figure 1) with an optimum pH of 9.0 and temperature 45 to 55°C. Alkaline proteases active in broad pH (7 to 12) and temperature (10 to 80°C) range have also been reported by several workers (Guangrong et al., 2006; Qiuhong et al., 2006). Manachini et al. (1998) also reported an alkaline protease active in a broad pH (7.5 to 11) and temperature (10 to 80°C) range with an optimum pH 9 and 45°C.

Effect of NaCl on protease stability

The protease was almost fully stable and active up to 3% (w/v) NaCl concentrations. Further increase in salt concentration resulted in gradual decrease, and retained 92, 84, 68, 52 and 48% activity at 4, 5, 7, 9 and 10% salt level, respectively. Higher than 10% NaCl resulted in drastic decrease with residual activities of 18 and 6% at 11 and 12% (w/v) salt concentrations, respectively.

The effect of common salt revealed moderately to highly halotolerant nature of enzyme, which was independent of NaCl requirement. This finding is interesting as the isolate is from non saline environment. Joshi et al. (2007) have reported a *B. cereus* isolate from a lake having protease active up to 5% NaCl concentration, but lost its activity with increasing salinity. Joo and Chang (2005) have also reported an alkaline protease from a halo-tolerant *B. clausii* I-52, active in a broad range of NaCl concentration, but most active only at 1% NaCl. The stability in the presence of high salt concentration is a very important characteristic as NaCl is used as core component in granulation of protease prior to addition in detergents. The quality of ground water available in different Indian geoclimatic regions is saline (Tyagi et al., 2003). The salinity is detrimental for washing properties of the detergent; hence, the presence of halo-toletant alkaline protease in detergents is certain to make washing easy and convenient.

Effect of modulators on protease stability

In this study, PMSF, EDTA and ascorbic acid were inhibitory for protease activity. PMSF completely inhibited the enzyme at all concentrations employed, whereas the extent of inhibition increased with elevated levels of EDTA and ascorbic acid. At 5 mM concentration of EDTA and ascorbic acid, the residual activity was 10 and 32%, respectively. β-Mercaptoethanol and cysteine did not affect the protease activity, thereby indicating its stability in the presence of reducing agents (Figure 2). Since

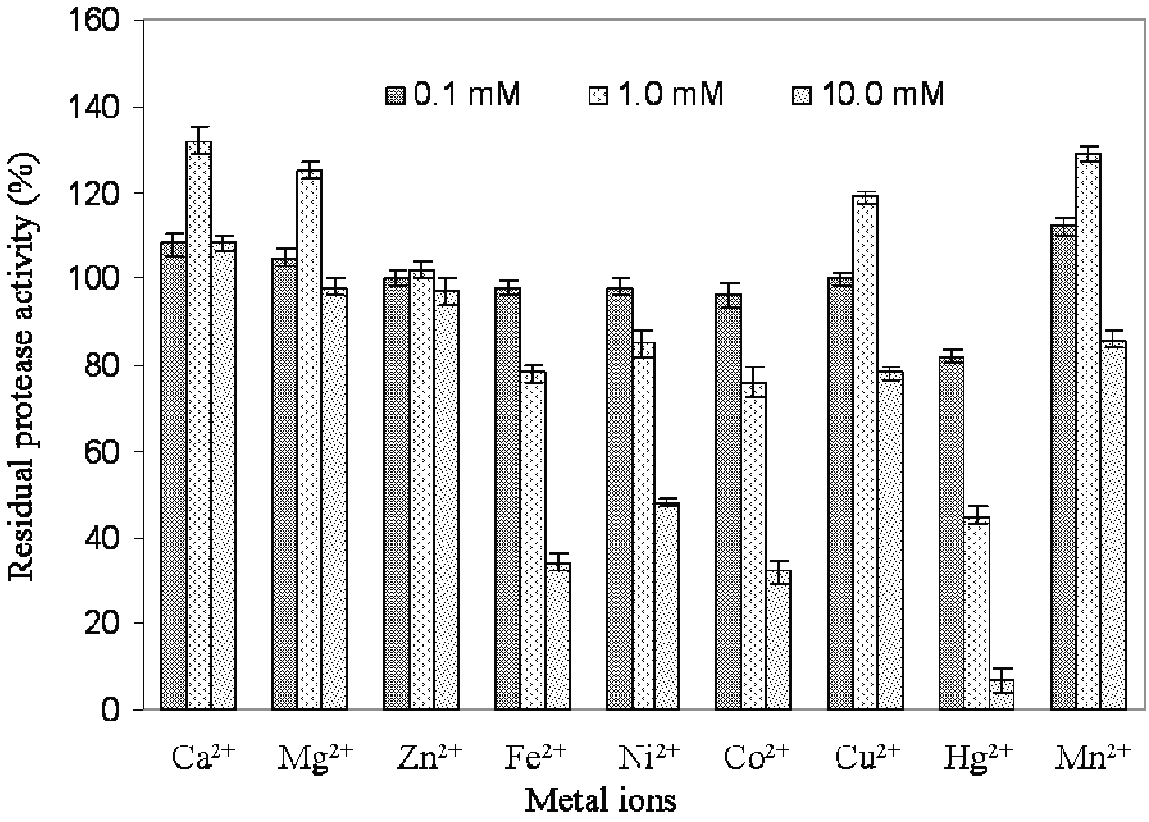

Figure 3. Effect of various metal ions (0.1 to 10 mM) on protease stability.

protease activity was completely blocked by PMSF even at 1.0 mM concentration (Figure 2), this enzyme must be an alkaline protease with serine residue at its active site. It is well known that PMSF sulphonates essential serine residue at active site of the protease, thereby completely blocking the activity (Adinarayana et al., 2003). Inhibition by EDTA reveals that this protease needs metal ion(s) for optimal activity. As EDTA removes metal ion(s) from active site by chelation process, activity of enzyme decreases significantly. In the presence of ascorbic acid (vitamin C), protease activity decreased, which may be due to highly acidic environment (pH 4.0) imposed by ascorbic acid. This is further corroborated by our finding that acidic pH (3 to 4) is detrimental to protease activity and stability (Figure 1). Presence of β-mercaptoethanol and cysteine did not affect protease activity, thereby revealing its monomeric nature. Figure 3 reveals that the presence of calcium, magnesium, manganese and copper at 1 mM concentration increased the protease activity up to 132, 125, 119 and 129%, respectively. Other metal ions reduced the activity to variable extent. At 10 mM concentration, mercury reduced the activity down to 7%. Figure 3 further shows that, 0.1 mM concentration of zinc, iron, nickel and cobalt did not remarkably affect the protease activity. However, at 1 mM concentration iron, nickel and cobalt retained the residual activity up to 78, 85 and 76%, while at even 10 mM; the residual activities were 34, 48 and 32%, respectively. Zinc did not have such an inhibitory effect on protease activity both at 1.0 and 10 mM concentrations. In general, cations are known to induce enzyme secretion, activity and also increase thermostability. But, at higher concentrations iron, nickel, cobalt and mercury reduced the protease activity in our study. However, calcium, magnesium, copper and manganese exhibited positive modulating effect, thereby indicating certain metal ion(s) requirement of this protease. Paliwal et al. (1994) have also reported positive effect of calcium, magnesium and manganese on protease activity from *Bacillus* sp. Kuddus and Ramteke (2009) reported an alkaline metallo-protease which was stimulated by Mn^{2+}. Studies by several other researchers have also demonstrated that presence of different metal ion(s) is necessary for enhanced protease activity (Adinarayana et al., 2003; Qiuhong et al., 2006; Gupta and Khare, 2007).

Effect of oxidants and surfactants on protease stability

The protease from *B. cereus* SIU1 reflected considerable stability not only in the presence of nonionic surfactants Triton X100 and Tween 80, but also towards strong anionic detergent sodium lauryl sulphate (SLS). The

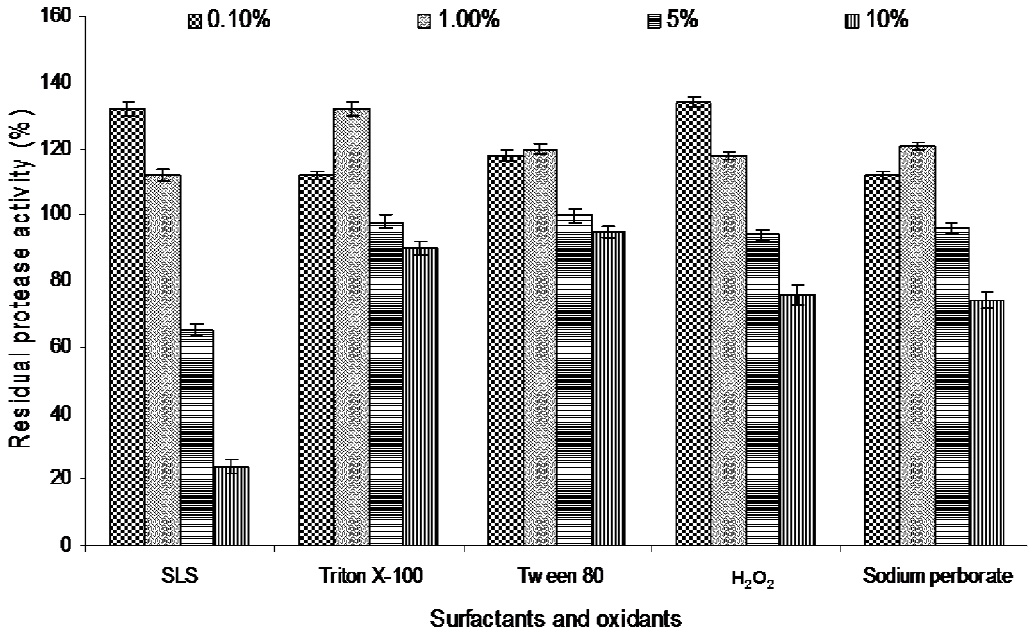

Figure 4. Effect of various surfactants and oxidants at 0.1 to 10% (w/v) on protease stability.

surfactants and SLS at 0.1 and 1% (w/v) concentration enhanced the enzyme activity up to 132%. Even at very high concentrations (5 and 10%) of Triton X100 and Tween 80, the enzyme was active up to 90 to 100%. Conversely, such an elevated concentration of SLS was detrimental to protease stability (Figure 4). Oxidants like H_2O_2 and sodium perborate did not affect the protease stability at 0.1 and 1.0% concentration, but their increased concentration (5 and 10%) resulted in decreased (74 to 96%) protease stability (Figure 4). It is interesting to note that our protease was not only stable but also its activity significantly enhanced (up to 132%) in the presence of surfactants and oxidants at 0.1 and 1.0% (w/v) concentrations. Further, at 5 and 10% concentrations, except SLS (residual activity 65 and 24%, respectively) all other detergents and oxidants did not affect the protease at large extent (Figure 4). This property is very important for its use as a detergent additive. Commercially, available proteases (Subtilisin Carlsberg, Subtilisin BPN', Alcalase, Esparase and Savinase) though have great stability in the presence of detergents, most of them are unstable in the presence of oxidants and bleaches. Therefore, search for a newer bleach stable, thermoalkaline protease is desirable. Oxidant and surfactant stable proteases have been reported by other workers also from *Bacillus* sp. (Gupta et al., 1999; Joo et al., 2003).

Effect of commercial detergents on protease stability

The protease was suitably stable to the extent of 88 to 100% in the presence of 0.1% (w/v) commercial detergents. Upon 10 fold increase (at 1%) in commercial detergents' concentration, the protease retained 78 to 84% activity depending on the individual detergent employed. Furthermore, 50 fold increases (at 5%) in commercial detergents' concentration, the protease still retained residual activity in the range of 22 to 41%, thereby indicating its highly stable nature (Figure 5). Even at 10% (100 fold increase) detergent level, the residual protease activity was 10, 12, 14 and 8% in Rin, Surf, Ariel and Wheel, respectively.

Stability pattern of protease in the presence of commercial detergents (Figure 5) also reveals its possible commercial application in detergent formulations. Generally, most of the manufacturers recommend the use of detergents at 0.1 to 0.2% (w/v) range for washing purposes. Accordingly, the halotolerant thermoalkaline protease from *B. cereus* SIU1 is highly stable in the aforementioned recommended range. Detergent stable proteases have been studied by several groups of workers with varying levels of activity in the presence of different detergents (Joo and Chang, 2005; Kuddus and Ramteke, 2009). Detergent stability of an alkaline protease is an important property for its industrial use, as they are currently supplemented in detergent formulations for better washing efficiency.

Conclusions

A novel thermoalkaline protease produced by a *B. cereus* SIU1 was found considerably stable in the presence of detergents, surfactants and NaCl. Owing to these desirable properties, this protease may have potential

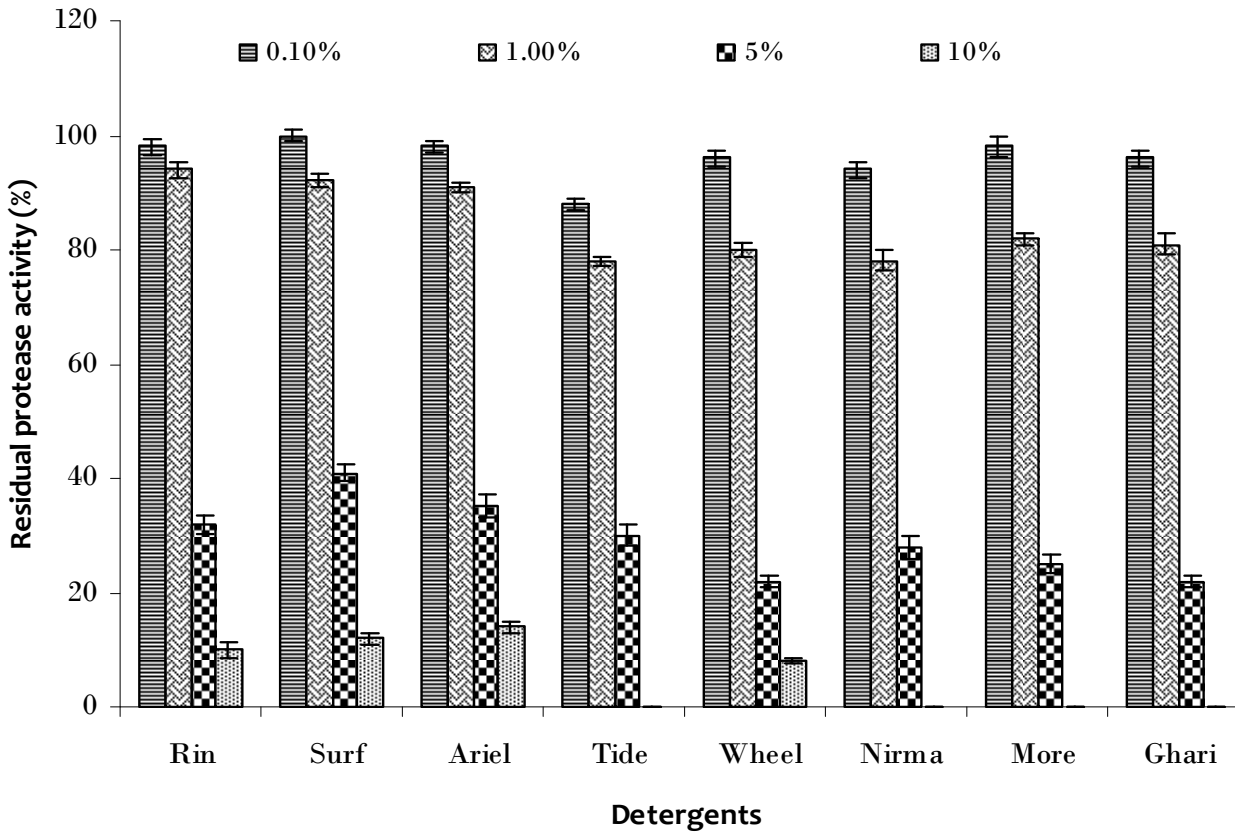

Figure 5. Effect of various commercial detergents at 0.1 to 10% (w/v) on protease stability.

use in industries such as detergent, food, pharmaceutical, leather, agriculture, etc. as well as molecular biology techniques. The oxidant and detergent stability facilitates it to withstand the effect of bleach, detergents and other oxidizing agents and makes it highly suitable for washing purpose. The organism appears to have greater potential for enhanced protease production by optimization of physical and nutritional factors. Further studies on biochemical and structural characteristics of protease are under investigation.

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