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Purification and characterization of a solvent, detergent and oxidizing agent tolerant protease from *Bacillus cereus* isolated from the Gulf of Khambhat

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ABSTRACT

Twenty-eight organic solvent tolerant bacteria were isolated from crude oil contaminated samples, out of which, AK1871 isolate produced a solvent, detergent and oxidizing agent tolerant serine alkaline protease. Based on the morphological and biochemical characteristics, FAME analysis as well as 16S rRNA gene sequence, the isolate is identified as *Bacillus cereus*. A 58-fold purification of protease was achieved by a three-step purification procedure. This protease is active over a broad range of pH (6.0–9.0, optimum at 8.0); and temperature from 40 °C to 70 °C (optimum at 60 °C). Li^+, Ba^{2+}, K^+, Mg^{2+} and Mn^{2+} did not affect, while heavy metals like Cr^{3+}, Hg^{2+} and Cu^{2+} inactivated the enzyme. It is stable in the presence of non-ionic detergents (Triton X-100 and Tween 80), and oxidizing and bleaching agents (hydrogen peroxide). The protease exhibited noteworthy stability and activation in the presence of organic solvents with log *P* values equal to or more than 2.0. This protease could be used in detergent formulations, enzymatic peptide synthesis, biotransformation reactions and in the formulation of antifouling agent. This is the first report on the study of organic solvent tolerant protease from a marine organic solvent tolerant bacterium isolated from the Gulf of Khambhat.

1. Introduction

Proteases (EC 3.4.21) have weaved their own niche as an indispensible biocatalyst in industrial sectors of detergent, leather, pharmaceuticals, food, textile, silk, bakery, soy processing, meat tendering, brewery, protein processing, peptide synthesis, ultra filtration membrane cleaning, extraction of silver from used X-ray films as well as in basic research. [1,2]. Normally, proteases catalyze the hydrolysis reaction in aqueous conditions, but in water restricted media, the enzymatic action of protease reverts, leading to synthesis of peptides and esters [3,4]. Thus, microbial proteases that can mediate catalysis in non-aqueous solvents offer new possibilities such as shifting of thermodynamic equilibrium in favor of synthesis, increasing the solubility of hydrophobic substrates and products and facilitate the product recovery and improving thermal stability of enzymes [5].

Since most of the enzymes including proteases are not stable in organic solvents, several contemporary techniques such as site-directed mutagenesis [6], immobilization [7,8], chemical modification [9] and directed evolution [10] have been used to obtain organic solvent stable proteases [4,11]. However, exploring naturally organic solvent stable proteases shall mitigate some of the limitations encountered by above-mentioned modified proteases.

Organic solvent tolerant bacteria, classified under a relatively novel group of extremophiles with unique ability to live in the presence of organic solvents, have been isolated from soil and deep sea environment. They are classified under genera *Pseudomonas* [12–14], *Bacillus* [15], *Flavobacterium* [16] and *Rhodococcus* [17]. Since the organisms are tolerant by the virtue of various adaptations like toluene efflux pumps, cis–trans isomerisation of fatty acids, rapid membrane repair mechanisms, etc., the protease secreted by them are likely to be solvent stable [18–22]. Additionally, solvent-tolerant microorganisms catalyze biotransformation with the whole cells in two-phase solvent–water systems [23].

Many biofouling species, such as bacteria, diatoms, algal spores, and invertebrate larvae use protein and glycoprotein polymers for formation of biofilm [24–26]. As the paints contain solvents, a solvent stable protease might be able to cleave proteins and arrest the colonization of fouler.

Hitherto, major work on solvent stable protease has been explored from *Pseudomonas* sp. [5,13,18,27–34]. Presently, different *Bacillus* sp. are also studied for the production of organic solvent proteases [35], viz. *Bacillus cereus* [36], *Bacillus pumilus* [37], *Bacillus licheniformis* [38–40], *Bacillus sphaericus* [41] and *Bacillus subtilis* [42].

Gujarat has a coastline of about 1600 km and it is the second most industrialized state in India. Alang-Sosiya ship-breaking

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yard, one of the largest ship-breaking facilities in Asia, is situated on Gujarat coast. The ship-breaking process produces a lot of wastes including oil, asbestos, paint chips, heavy metals, plastic, glass and ceramics. Heavy load of pollution has greatly influenced the ecology of this area. This article describes isolation, purification and characterization of an organic solvent tolerant protease from *B. cereus*, isolated from an oil-contaminated site of Alang.

2. Materials and methods

2.1. Microorganisms

2.1.1. Isolation and characterization

The organic solvent tolerant bacterial strains were isolated from the oil contaminated soil and sea sediment of Alang off the Gujarat coast (N 21°23.561′, E 72°10.475′). The organic solvent-tolerant strains were screened by toluene enrichment {10% (v/v)} in modified Luria-Bertani (MLB) medium containing ($g l^{-1}$): tryptone, 10.0; yeast extract, 5.0; sodium chloride, 10; and magnesium sulphate, 0.5 [34]. Incubation was carried out with agitation at 120 rpm for 72 h. Cultures were acclimatized by repeated transfer in the same culture conditions with organic solvents. The grown microorganisms in the enrichment medium were diluted and spread on MLB plates and isolated colonies were further purified by repeated streaking. The purified colonies were screened qualitatively for their protease production on skim-milk agar (SMA) plate, which contained ($g l^{-1}$): tryptone, 5.0; yeast extract, 3.0; skimmed milk powder, 25.0; and bacteriological agar 15.0 [34]. The plates were incubated at 30 °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 the subsequent experiments. The promising isolates were maintained on Zobell Marine Agar 2216 (M384; HiMedia, India) ($g l^{-1}$): peptone 5.0, yeast extract 1.0, ferric citrate 0.10, sodium chloride 19.45, magnesium chloride 8.80, sodium sulphate 3.24, calcium chloride 1.80, potassium chloride 0.55, sodium bicarbonate 0.16, potassium bromide 0.08, strontium chloride 0.034, boric acid 0.022, sodium silicate 0.004, sodium fluorate 0.0024, ammonium nitrate 0.0016, disodium phosphate 0.008 (final pH at 25 °C 7.6 ± 0.2). The most promising isolate, AK1871, which is studied in detail, was isolated from crude oil contaminated sea sediment of Alang, West coast of India. Taxonomic characterization of AK1871 was done based on cultural, morphological and biochemical characteristics, FAME analysis and 16S rRNA gene sequencing.

2.1.2. Chromosomal DNA extraction and purification

The isolate AK1871, was grown in 10 ml of Marine Broth 2216 (M385; HiMedia, India) for 24 h at 35 °C. The biomass was harvested by centrifugation at 10,000 rpm for 10 min and washed twice in sterile Tris–EDTA buffer (10:1 molar ratio, pH 8.0) and the wet biomass obtained was used for DNA extraction. The extraction and purification of DNA was carried out by the phenol–chloroform extraction [43].

2.1.3. DNA amplification, sequencing and phylogenetic analysis

Two primers were selected for use in PCR amplification experiments, forward primer 50-AGA GTT TGA TCC TGG CTC AG-30 and reverse primer 50-AAG GAG GTG ATC CAG CC-30 (Sigma) [44]. The reaction mixture for PCR amplification (BioRad) contained 10× PCR buffer 5 μl, each dNTP 200 μM, Taq DNA polymerase 1.25 U, 0.5 μM of each primers and 50–100 ng of bulk DNA, 50 μl reaction mixer, 2 mM $MgCl_2$. Amplification was performed in a thermal cycler for an initial denaturation at 94 °C for 5 min followed by 35 cycles of 94 °C for 1 min, 58 °C for 1 min and 72 °C for 2 min and a final extension at 72 °C for 10 min. Sequencing of the amplified DNA was done at Macrogen, South Korea. The Basic Local Alignment Search Tool (BLAST) from NCBI was performed for nucleotide to find the closest match.

2.2. Enzyme production

The isolate AK1871 was grown in Erlenmeyer flasks (1000 ml) containing 500 ml of Zobell Marine Broth for 48 h at 35 °C on a shaker at 120 rpm. The culture was centrifuged at 10,000 rpm for 10 min at 4 °C and the supernatant was used as crude protease for further study.

2.3. Protease assay

Protease activity was assayed by following method of Sana et al. [45] using casein as a substrate. The reaction mixture containing 100 μl Glycine–NaOH buffer (100 mM, pH 8.5), 100 μl of 0.6% casein solution and 200 μl of enzyme solution was incubated at 40 °C for 30 min. The reaction was terminated by addition of 400 μl of 10% trichloroacetic acid solution and the non-hydrolyzed casein was removed by centrifugation at 10,000 rpm for 10 min. Peptide concentration of the supernatant was determined by Lowry's method [46] using tyrosine as a standard. One unit of enzyme is defined as the amount of enzyme required to produce color equivalent to 1.0 μg of tyrosine per minute at pH 8.6 at 40 °C.

2.4. Protein assay

Protein was measured by the method of Lowry [46] with bovine serum albumin as standard. During chromatographic purification steps, protein concentration of each fraction was estimated by measuring its absorbance at 280 nm.

2.5. Purification of protease

Cell free supernatant was subjected to 60% ammonium sulphate precipitation. After dialysis with glycine–NaOH buffer (pH 8.6), the dialysate was applied to DEAE-Cellulose column and fractionated by a step-wise elution using the same buffer having increasing concentration of NaCl (0.1–0.5 M). Fractions showing high caseinolytic activity were pooled, desalted, concentrated and fractionated on Sephadex G-200 with the same buffer. Fractions showing high protease activity were pooled and then concentrated by lyophilisation and purity was checked by SDS-PAGE.

2.6. Electrophoresis and zymography

SDS-PAGE was performed by the method of Laemmli [47] using 5% stacking gel and 10% resolving gel with Tris–glycine buffer, pH 6.8 and pH 8.8, respectively, containing 0.1% sodium dodecyl sulfate at 4 °C. The samples loaded were heated at 80 °C for 5 min. Following SDS-PAGE, the gel was cut into two to prevent over staining of markers by silver staining method to visualize the protein bands on the gel.

Substrate polyacrylamide gel electrophoresis (Substrate-PAGE) was performed by incorporating casein {0.1 (w/v)} into the separating gel before polymerization. After the electrophoresis, the gels were rinsed twice in 2.5% Triton X-100 for 30 min to remove SDS. The gel was then incubated in 0.2% (w/v) casein solution (0.05 M Tris, pH 7.6, 0.1 M NaCl). The gels were then stained with 0.1% Coomasie blue R-250 in methanol–acetic acid–water (40:10:50) followed by destaining with methanol–acetic acid–water (5:10:85).

2.7. Characterization of protease

2.7.1. Molecular weight determination by SDS-PAGE

SDS-PAGE was performed to determine the homogeneity and molecular mass of the purified protease using 5% stacking gel and 10% resolving gel according to the method of Laemmli [47] and electrophoresis was performed with 15 mA constant current. A standard molecular weight markers (100, 80, 70, 50, 40, 20, 15 and 10 kDa) (Genei; Bangalore, India) were used.

2.7.2. Effect of pH on activity of purified enzyme

The effect of pH on protease activity was studied by assaying purified protease at different pH. The pH of the reaction mixture was adjusted using one of the following buffers: 0.1 M citrate buffer (pH 5.0–6.0), 0.2 M Tris–HCl buffer (pH 7.0–9.0) and 0.2 M Glycine–NaOH buffer (pH 10.0–11.0).

2.7.3. Effect of temperature on activity of purified enzyme

To study the effect of temperature on activity of the purified enzyme, the reaction mixture was incubated at different temperatures ranging from 10 °C to 80 °C for 30 min and the activity was determined.

2.7.4. Effect of NaCl on activity of purified enzyme

To study the effect of NaCl on the protease activity, the purified enzyme was assayed in the presence of different concentrations of NaCl ranging from 0 M to 6 M.

2.7.5. Effect of metal ions on activity of purified enzyme

Effect of different metal ions on purified protease was studied using different metal ions like Ba^{2+}, Ca^{2+}, Cd^{2+}, Co^{2+}, Cr^{3+}, Cu^{2+}, Hg^{2+}, K^{+}, Li^{+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Pb^{2+} and Zn^{2+} (all ions in chloride form, final concentration of 5 mM) in the assay system, pre-incubated at 60 °C for 30 min and the relative activity was measured.

2.7.6. Effect of inhibitors

To study the effect of different inhibitors on the purified enzyme, aliquots were pre-incubated with different inhibitors, ethylenediaminetetraacetic acid disodium salt (EDTA, 5 mM), 1,10-phenanthroline (o-Phe, 5 mM), urea (5 mM), iodoacetamide (5 mM), phenylmethanesulfonylfluoride (PMSF, 5 mM) and protease inhibitor cocktail {AEBSF, Bestatin hydrochloride, E-64, Leupeptin hemisulphate, Pepstatin A, o-Phe (1% (v/v))} (Sigma–Aldrich P9599) at 60 °C for 30 min. Relative activity was compared with control (without inhibitors).

2.7.7. Effect of detergents, oxidizing, reducing and bleaching agents on activity of purified enzyme

Effect of detergents was studied with non-ionic detergent {Triton X-100, Tween 80, 1% (v/v)}, anionic detergent {sodium dodecyl sulphate (SDS), 5 mM}, cationic detergent {cetyl trimethyl ammonium bromide (CTAB), 5 mM}, and commercial detergents {Tide, Surf excel and Ariel; 1% (w/v)}. Influence of oxidizing–bleaching agent {hydrogen peroxide, 1% (v/v)}, reducing agent (glutathione, 5 mM) and disinfectant (sodium tetraborate, 5 mM) was also studied on protease activity. All mixtures were pre-incubated for 30 min at 60 °C.

2.7.8. Effect of different organic solvents on stability of purified enzyme

1.0 ml of the purified enzyme solution in 0.2 M Glycine–NaOH buffer (pH 8.6) was mixed with 0.5 ml of different organic solvents (with 0.3% Tween 80 as emulsifier) and left for 144 h at 30 °C with constant shaking. Relative protease activity, with respect to control, was measured at different time intervals of 1 h, 48 h, 96 h and 144 h.

2.7.9. Substrate specificity

Substrate specificity of the enzyme was tested for substrates such as casein, BSA and wheat gluten at 1% (w/v) concentration.

Table 1
Morphological and biochemical characteristics of AK1871.

Properties	Growth pattern	Properties	Growth pattern
Shape	Rod	Acid from	
Colony	Frosted glass appearance Undulate margin	D-Glucose	+
Hydrolysis of		L-Arabinose	–
Casein	+	D-Xylose	–
Gelatin	+	D-Mannitol	–
Starch	+	Dulcitol	–
Lipase	+	Inositol	–
Catalase	+	Lactose	–
		Maltose	+
		Sorbitol	–
		Sucrose	+
		Trehalose	+

(+) = positive; (–) = negative.

3. Results

3.1. Characterization of the bacterium

Organic solvent tolerant bacterium, AK1871, is Gram-positive, aerobic, rod-shaped and motile. It fermented glucose, maltose, sucrose and trehalose and also produced enzymes like amylase, lipase, gelatinase, caseinase and catalase. According to *Bergey's Manual® of Systematic Bacteriology* [48], the biochemical characters of AK1871 resembled Genus *Bacillus* (Family *Bacillaceae*, Order *Bacillales*, Class *Bacilli*, Phylum *Firmicutes*) (Table 1).

The Fatty Acid Methyl Ester analysis of AK1871 was conducted using MIDI Sherlock® Microbial Identification System. Isolate AK1871 possessed iso-$C_{15:0}$ (42.05%), summed feature 3 ($C_{16:1\omega}7c/_{16:1\omega}6c$; 8.33%), iso-$C_{13:0}$ (5.00%), $C_{14:0}$ (4.02%), iso-$C_{16:0}$ (4.0%), $C_{16:0}$ (4.60%), iso-$C_{17:1\omega}5c$ (4.41%), iso-$C_{17:1}$ (7.89%) as the major cellular fatty acids. The RTSBA library matches from Sherlock® software showed Similarity Index (SI) value of 0.649 with *B. cereus*.

The comparative 16S rRNA gene sequence (1371 bp) analysis of AK1871 showed 99% homology with *B. cereus*. This sequence was submitted to NCBI GenBank (Accession no. FJ573187). Thus, through a polyphasic identification approach, the isolate is identified as *B. cereus*.

Table 2
Purification profile of protease.

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Recovery (%)
Crude enzyme	6805.6	977.6	6.96	1.00	100
Ammonium sulphate precipitation	2620.8	23.0	113.94	16.37	38.51
DEAE ion exchange	2052.4	7.6	271.48	39.00	30.16
Sephadex Gel-filtration	732.6	1.8	406.09	58.34	10.76

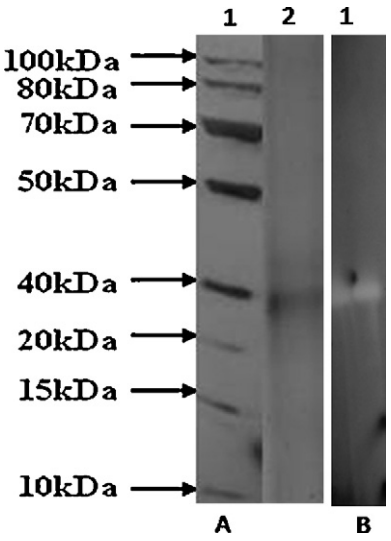

Fig. 1. (A) SDS-PAGE of purified protease. Lane 1, molecular weight markers; Lane 2, purified protease. (B) Substrate–PAGE of purified protease. Lane 1, purified protease (See text for details).

3.2. Enzyme purification and molecular weight

Protease purification is summarized in Table 2. Approximately 58-fold purification of the crude enzyme was achieved with a recovery of approximately 10.76%. Purified protease migrated as a single band in SDS-PAGE under reducing conditions, suggesting its homogeneity (Fig. 1). Zymogram activity staining showed a band of clear zone of proteolytic activity against the blue background (Fig. 1). The apparent molecular mass of the purified protease as revealed by SDS-PAGE is about 38 kDa (Fig. 1).

3.3. Effect of pH on activity of protease

Fig. 2 illustrates the relative protease activity at different pH values ranging from 5.0 to 11.0. The optimum pH for the purified protease was 8.0 with 60–90% activity over a broad range of pH, i.e. 6.0–9.0.

3.4. Effect of temperature on enzyme activity

The purified protease exhibited activity over a wide temperature range of 40 °C to 70 °C, optimum being at 60 °C as observed in Fig. 3. It retained almost 70% and 60% activity at 50 °C and 70 °C, respectively.

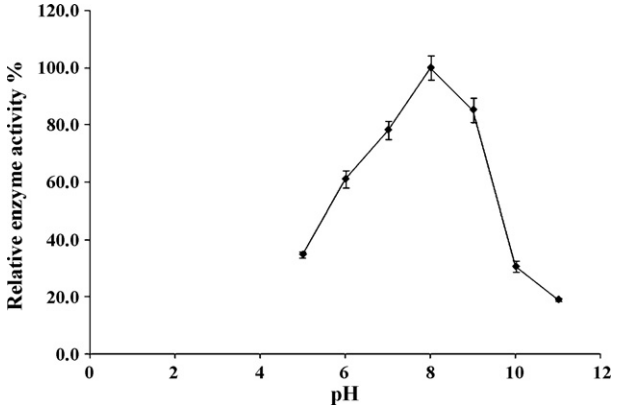

Fig. 2. Effect of pH on protease activity. The buffers used were 0.1 M citrate buffer (pH 5.0–6.0), 0.2 M Tris–HCl buffer (pH 7.0–9.0) and 0.2 M Glycine–NaOH buffer (pH 10.0–11.0). The activity at pH 8.0 is taken as 100%. Each value represents the mean of three independent determinations. Error bars indicate the standard deviation.

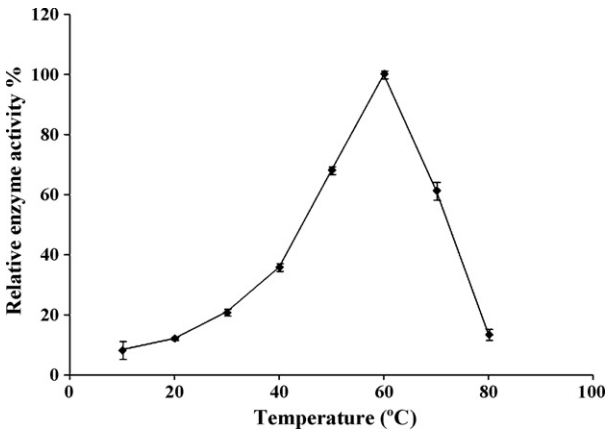

Fig. 3. Effect of temperature on protease activity. The purified enzyme was incubated with the substrate at different temperatures. The activity at 60 °C has been taken as 100%. Each value represents the mean of three independent determinations. Error bars indicate the standard deviation.

3.5. Effect of sodium chloride concentration on protease activity

The enzyme showed optimum activity in the absence of sodium chloride but it could tolerate 0.5 M NaCl with 65% relative activity. Higher concentration of NaCl inhibited protease activity (Fig. 4).

3.6. Effect of metal ions on activity of enzyme

The effect of various metal ions (final concentration 5 mM) on activity of enzyme was studied and the results are shown in Table 3. Ca^{2+} increased the activity of enzyme by 50%, while Ba^{2+}, K^{+}, Mg^{2+}, Li^{+} and Mn^{2+} ions did not show either positive or negative effect on the enzyme activity. Cr^{3+}, Cd^{2+}, Zn^{2+}, Cu^{2+}, Hg^{2+} and Pb^{2+} suppressed the enzyme activity considerably.

3.7. Effect of inhibitors

As observed in Table 4, effect of specific protease inhibitors on enzyme activity is evident. The activity of protease was marginally affected in the presence of alkylating agent like iodoacetamide and a ligand like o-phe. Its activity was unaffected in the pres-

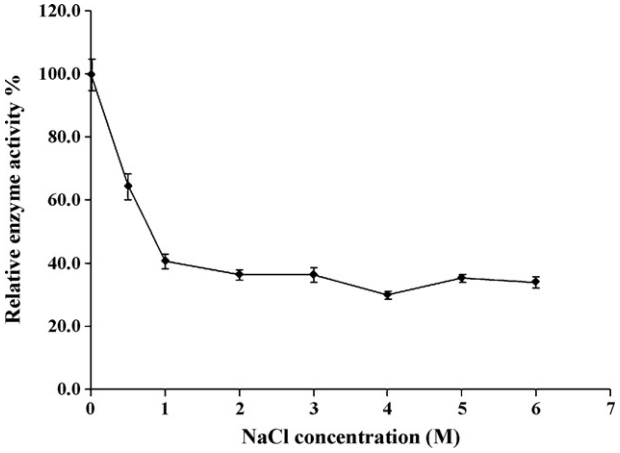

Fig. 4. Effect of NaCl on activity of protease. Percentage relative activity has been calculated on the basis of activity of protease without NaCl (100%). Each value represents the mean of three independent determinations. Error bars indicate the standard deviation.

Table 3
Effect of metal ions on the enzyme activity.

Metal ions (5 mM)	Relative enzyme activity (%)	Metal ions (5 mM)	Relative enzyme activity (%)
None	100	Mixture[a]	21.2 ± 0.9
Ba^{2+} ($BaCl_2$)	97.3 ± 4.8	Ca^{2+} ($CaCl_2$)	147.8 ± 6.6
Cd^{2+} ($CdCl_2$)	17.7 ± 0.7	Co^{2+} ($CoCl_2$)	78.8 ± 2.7
Cr^{3+} ($CrCl_3$)	8.0 ± 0.3	Cu^{2+} ($CuCl_2$)	6.2 ± 0.18
K^+ (KCl)	92.9 ± 5.6	Hg^{2+} ($HgCl_2$)	1.8 ± 0.08
Mg^{2+} ($MgCl_2$)	108.0 ± 3.4	Li^+ (LiCl)	101.8 ± 4.8
Ni^{2+} ($NiCl_2$)	61.9 ± 2.8	Mn^{2+} ($MnCl_2$)	91.1 ± 3.8
Zn^{2+} ($ZnCl_2$)	20.3 ± 1.1	Pb^{2+} ($PbCl_2$)	37.2 ± 1.5

Each value represents the mean of three independent determinations. (±) Standard deviation.

[a] Mixture of Ba^{2+}, Ca^{2+}, Cd^{2+}, Co^{2+}, Cr^{3+}, Cu^{2+}, Fe^{2+}, Hg^{2+}, K^+, Li^+, Mg^{2+}, Mn^{2+}, Ni^{2+}, Pb^{2+}, and Zn^{2+}.

Table 4
Effect of various protease inhibitors on protease activity.

Inhibitors	Concentration (mM)	Relative enzyme activity (%)
None	–	100
EDTA	5	107.2 ± 4.8
1,10 Phenanthroline	5	77.5 ± 3.9
Urea	5	57.7 ± 2.6
Iodoacetamide	5	82.0 ± 2.9
PMSF	5	16.2 ± 0.6
Cocktail[a]	1% (v/v)	103.6 ± 4.3

Each value represents the mean of three independent determinations. ± indicate the standard deviation.

[a] Cocktail components: AEBSF, Bestatin hydrochloride, E-64, Leupeptin, Pepstatin A, o-Phe.

ence of metal chelator like EDTA which indicated that the enzyme is metal independent. Moreover, the enzyme activity was unaffected by the cocktail of Bestatin hydrochloride (an aminopeptidase and metalloprotease inhibitor), pepstatin A (reversible acid protease inhibitor) and E-64 (a non-competitive irreversible cysteine protease inhibitor), whereas it showed considerable inhibition in presence of PMSF, which is an irreversible serine protease inhibitor. This indicated that protease of the present study is a serine protease.

3.8. Effect of detergents, oxidizing, reducing and bleaching agents on enzyme activity

In the presence of non-ionic detergents like Tween 80, protease activity was increased by 25%, whereas reverse trend with 15% decrease was observed with Triton X-100 (Table 5). Additionally, cationic and anionic detergents like CTAB and SDS significantly reduced the protease activity by 80% and 55%, respectively. The presence of commercial detergents, Surf excel, Ariel and Tide

Table 5
Effect of detergents, oxidizing, reducing and bleaching agents on protease activity.

Additives	Concentration	Relative enzyme activity (%)
None	–	100
Triton X-100	1% (v/v)	84.7 ± 3.3
Tween 80	1% (v/v)	125.2 ± 4.5
CTAB	5 mM	18.0 ± 0.6
SDS	5 mM	46.8 ± 1.9
Tide	1% (w/v)	55.0 ± 2.4
Surf excel	1% (w/v)	1.8 ± 0.06
Ariel	1% (w/v)	26.1 ± 1.0
Hydrogen peroxide	1% (v/v)	103.6 ± 4.2
Sodium tetraborate	5 mM	49.6 ± 2.0
Ammonia	1% (v/v)	40.7 ± 1.7
Glutathione	5 mM	84.7 ± 3.2

Each value represents the mean of three independent determinations. (±) Standard deviation.

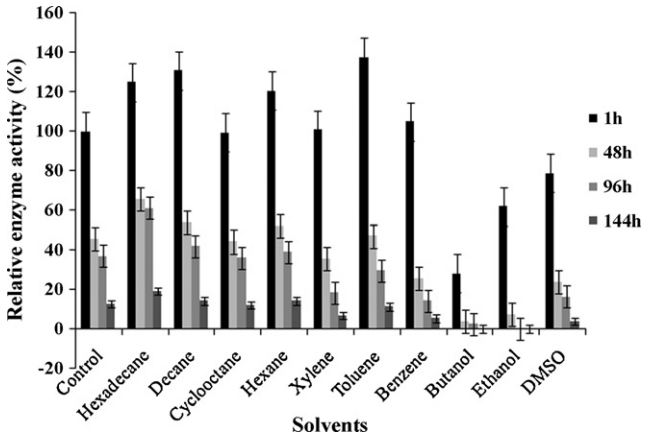

Fig. 5. Effect of organic solvents on activity and stability of purified protease. Purified protease was incubated at 30 °C with constant shaking in the absence or presence of various solvents at 33% (v/v) for 144 h. Relative activity was calculated on the basis of activity of protease without solvent as 100. Each value represents the mean of three independent determinations. Error bars indicate the standard deviation.

reduced the protease activity drastically by 98%, 75% and 45%, respectively. Protease activity was reduced marginally by 15% with reducing agent, glutathione, while the enzyme retained its original activity in presence of oxidizing and bleaching agent, hydrogen peroxide. Enzyme activity dropped to almost half in presence of a weak disinfectant, sodium tetraborate and an antimicrobial agent, ammonia solution.

3.9. Effect of organic solvents on protease stability

In the presence of water immiscible solvents except butanol, the protease activity increased as compared to water miscible solvents. Here, in the presence of decane, hexadecane and hexane, the protease activity was higher as compared to control, throughout the period of incubation (144 h). In case of cyclooctane and toluene, the activity was higher than the control, until 1 h of incubation, which was at par with control on further incubation whereas in case of benzene, enhanced activity was observed after 1 h and then marginal decrease was observed on further incubation. On the contrary, the protease activity was decreased in the presence of water miscible solvents like ethanol and DMSO with about 70% and 80% activity, respectively, after 1 h incubation (Fig. 5).

3.10. Substrate specificity of enzyme

Protease showed specificity towards casein, as compared to bovine serum albumin and wheat gluten where the activity was only 20% as compared to casein.

4. Discussion

Organic solvent tolerant bacteria are a novel and unique group of extremophilic microorganisms that thrive in the presence of very high concentrations of organic solvents [12]. In general, organic solvents are extremely toxic to bacteria, as they disrupt the cell membrane, by altering with the structural and functional integrity of the cell [49,50].

Twenty-eight solvent-tolerant bacteria were isolated from oil contaminated sea sediment samples by toluene enrichment method. Out of seventeen protease producing strains, isolate AK1871, *B. cereus*, produced a solvent, detergent, oxidizing and bleaching agent tolerant alkaline serine protease. 58-fold purification of protease was achieved by a three-step purification process

with 10.76% recovery. During ion exchange chromatography, the enzyme was eluted as a loosely bound fraction.

The purified protease exhibited activity over a broad range of pH, 6.0–9.0, with optimum activity at pH 8.0 and about 80% activity at pH 7.0 and 9.0. Organic solvent-stable proteases from *Pseudomonas aeruginosa* PST-01 [30], *P. aeruginosa* PseA [32] and *B. cereus* BG1 [36], were reported to have optimal pH between 8.0 and 9.0 while activity was completely lost at pH values over 10.0.

AK1871 protease exhibited activity over temperature range of 40–70 °C, with optimum at 60 °C. This is in agreement with the report of Reddy et al. [51], who have reported organic solvent and detergent tolerant protease from *Bacillus* sp. RKY3 having same temperature optima. Ghorbel et al. [36] also have reported protease from *B. cereus* having temperature optima of 60 °C in the presence of Ca^{2+} which otherwise is 50 °C. Higher temperature optima of 70 °C for protease of *Pseudomonas* sp. is reported by Rahman et al. [52] whereas lower temperature optima of 45 °C and 50 °C are reported by Rahman et al. [37] and Sareen and Mishra [39], respectively.

The protease of the present study was marginally affected by the presence of 1% non-ionic detergents like Triton X-100 and Tween 80, whereas, SDS (anionic) exhibited inhibitory effect. These results are in agreement with the reported proteases from *Pseudomonas* as well as *Bacillus* sp. [32,39,42,51]. This effect of detergent on the enzyme can be correlated to their hydrophilic/lipophilic balance (HLB), which is defined as the way a detergent distributes between polar and nonpolar phases [53]. Triton X-100 with HLB of 13.5 is less detrimental as compared to SDS with a HLB of 40. Besides, the protease was stable in the presence of oxidizing and bleaching agent like hydrogen peroxide which matches with the reports of Sana et al. [45] and Reddy et al. [51]. This makes it suitable for its use in the detergent industry.

Various reports on effect of metal ions on protease activity suggested enhancement in activity in presence of Ca^{2+} and Mg^{2+}, whereas drop in activity in presence of Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+} and Hg^{2+} [32,36,39,45,51]. Similar trend was observed in the present study where increase in the AK1871 protease activity in the presence of Li^{+}, Ca^{2+} and Mg^{2+} (s-block metals) and reduction in presence of Cr^{3+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+} and Hg^{2+} (d-block element) was observed. Generally, s-block metals bind poorly to ligands and form mainly ionically bound complexes with donor ligands. As the bonding is mainly ionic, the metal ions are easily displaced. Usually d-block elements preferentially bind to ligands to give stable complexes through covalent bonds [54] and hence the enzyme gets irreversibly bound leading to poor activity. Similar effects have earlier been demonstrated wherein the activation was increased with an increase in the ionic radii of the cation [55]. Ca^{2+} has the largest ionic radii (0.099 nm) among the divalent metal ions studied, whereas ionic radii for other ions like Mn^{2+}, Zn^{2+}, Cu^{2+}, and Mg^{2+} are 0.082 nm, 0.075 nm, 0.073 nm and 0.072 nm, respectively. In spite of small ionic radii of Mg^{2+}, its activation effect on the enzyme activity is in agreement with the results of Towatana et al. [56]. Another reason for an increase in activity in the presence of Ca^{2+} might be due to stabilization of enzyme in its active conformation by acting as an ion bridge via a cluster of carboxylic groups and thereby maintaining the rigid conformation of the enzyme molecule [39,57].

AK1871 protease was inhibited by serine protease inhibitor PMSF, whereas it seemed almost unaltered by metal-chelating reagents such as EDTA and o-Phe. Therefore, the AK1871 protease was classified under serine protease family and is metal independent.

The present study indicated that the purified protease was stable in a few water immiscible solvents throughout the period of incubation as compared to water miscible solvents. Reddy et al. [51] reported marginal increase in the activity in presence of benzene, hexane and toluene which is in concurrence with the present report. On the contrary, Geok et al. [5], Rahman et al. [52] and Ogino et al. [30] reported inactivation of protease in the presence of benzene, toluene, xylene and hexane and enhancement in activity of protease in the presence of hexadecane and decane [5,52]. Protease stability in the presence of water miscible solvents like ethanol, acetone and DMSO is reported by Sana et al. [45], while inactivation is reported by Tang et al. [34] and Li et al. [40], except DMSO which did not show any adverse effect on protease activity. In the present study also, protease activity was reduced significantly in the presence of ethanol and by 20% in the presence of DMSO after 1 h of incubation. Usually, presence of organic solvent reduces the structural flexibility of enzyme which is required for optimal catalysis. Here, the activation of protease in the presence of organic solvents indicates the ability of enzyme to resist denaturation by formation of multiple hydrogen bonds with water for structural flexibility and conformation mobility [58]. DMSO serves as a highly solvating organic media for homogenous organic-aqueous mixtures to catalyze kinetic and equilibrium-controlled synthesis [59]. Thus, the stability of this protease in organic solvents of log *P* values ranging from 2.0 to 8.8, and having 80% activity in DMSO (−1.35) up to 1 h, suggest its probable application in peptide synthesis.

In search of new antifouling coatings in place of TBT, Dobretsov et al. [60] showed potential of crude proteases from *Pseudoalteromonas* species to reduce larval attachment. Since paints contain solvents, a formulation containing solvent stable protease, like AK1871 might be useful in formulation of antifouling agent.

5. Conclusion

In the present study, an organic solvent, detergent and oxidizing agent tolerant protease produced by *B. cereus* was purified and characterized. The purified protease exhibited important properties, such as higher activity in the presence of a wide range of organic solvents, oxidizing and bleaching agent, at alkaline pH and elevated temperature. Based on these properties, this enzyme may find its application in the detergent industry, in catalytic reactions in non-aqueous media and also in formulation of antifouling agent.

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