INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print), 2222-5234 (Online)
http://www.innspub.net
Vol. 8, No. 2, p. 47-65, 2016

RESEARCH PAPER OPEN ACCESS

Production, purification and characterization of detergent-stable, halotolerant alkaline protease for eco-friendly application in detergents' industry

Maliha Ahmed, Ramla Rehman, Aisha Siddique, Fariha Hasan, Naeem Ali*, Abdul Hameed

Microbiology Research Laboratory (MRL), Department of Microbiology, Quaid-i-Azam University, Islamabad, Pakistan

Key words: Alkaline Protease, *Bacillus cereus*, 16s rRna sequencing, Halotolerant, Washing Test.

http://dx.doi.org/10.12692/ijb/8.2.47-65 Article published on February 20, 2016

Abstract

An alkalophilic, halotolerant bacterial strain ASM1 isolated from agricultural soil was found to be capable of producing extracellular protease enzyme. Proteolytic strain was identified as *Bacillus cereus* and nucleotide sequence has been submitted in NCBI database under accession number KJ600795. Optimum enzyme production in terms of specific activity 9.58 U/mg of total protein was obtained at 35°C; pH, 9.0; 1 % glucose as C-source and 35 g/l beef extract as N-source after 48 hours of incubation in a defined medium inoculated with 2% inoculum size. Bacterial isolate was capable of tolerating up to 12.5% NaCl without requiring salt for physiological activities. Bacterial crude enzyme was purified by 6 folds with 25% yield and specific activity of 57.9 U/mg protein by two step purification i.e. ammonium sulfate precipitation and gel-filtration chromatography. Thermostability studies revealed retention of 60% proteolytic activity upto 55°C. Moreover enzyme remained stable in the pH range of 6-11. PMSF (phenylmethylsulfonyl fluoride) inhibited enzyme activity categorizing the enzyme as a serine protease. Enzyme remained stable in presence of 8 different metals, however activity declined in the presence of 20 mM Fe^{2+} ions. Enzyme retained substantial stability in the presence of solvents, surfactants, commercially available detergents, and NaCl. Enzyme exhibited efficacious de-staining of fixed blood stains in the washing test at room temperature, without requiring additional energy. This particular type of protease enzyme is of immense importance due to its alkaline-halotolerant profile at mesophilic temperature range which is a great deal for revolutionizing detergents' industry.

* **Corresponding Author:** Naeem Ali ✉ naeemali2611@gmail.com

Introduction

Proteases are hydrolytic enzymes that catalyze hydrolysis of proteins by addition of water across the peptide bonds into smaller polypeptides and free amino acids (Beg and Gupta, 2003). Proteases are ubiquitous in nature playing important physiological roles, in all domains of life (Barrett *et al.,* 2001; Burhan *et al.,* 2003). Microbial proteases constitute one of the three commercially significant groups of enzymes, contributing more than 60% of share in the global enzyme market (Chu, 2007; Huang *et al.,* 2003; Jayakumar *et al.,* 2012; Jon, 2008). Proteases constitute a very diverse group of biocatalysts with members having different substrate specificities; nature of catalytic sites; evolutionary relationship in amino acids' sequence; catalytic mechanisms and varying activity-stability profiles on broad range of temperature and pH (Rai and Mukherjee, 2010; Rao and Narasu, 2007; Rawlings *et al.,* 2012).

Bacterial bio-factories hold much more temptation for exploitation than other enzyme producers due to the ease of handling and production in a limited time and space with less complicated purification steps. Besides that bacteria are susceptible to artificial genetic manipulations and are able to survive under diverse and extreme environmental conditions (Burhan *et al.,* 2003; Khademi *et al.,* 2013; Rao and Narasu, 2007; Rao *et al.,* 1998). Genus *Bacillus* is considered as the most significant source of bulk amounts of industrially important neutral and alkaline proteases which are highly stable at temperature and pH extremes (Beg and Gupta, 2003; Gupta and Khare, 2007; Venugopal and Saramma, 2006; Yang *et al.,* 2000).

Proteases active and stable in the alkaline pH range are referred as alkaline proteases. Active site of alkaline protesaes may contain serine residues or metal ions (Khan, 2013). Alkaline proteases with serine residues on catalytic site are referred as Serine Alkaline Proteases (SAPs). Optimum pH for production and activity of serine proteases ranges between pH 7.0-12.0. Some SAPs are endowed with additional characteristic of halotolerance which makes them perfect tool for utilization in various industrial processes (Joo and Chang, 2005; Joshi *et al.,* 2007; Maurer, 2004; Purohit and Singh, 2011; Singh *et al.,* 2010). Stability studies in presence of salts, metal ions, surfactants, oxidants and solvents help in prospecting probable use of enzyme in industry (Gupta and Khare, 2007; Joo *et al.,* 2003; Zambare *et al.,* 2014). Alkaline proteases are majorly used as additives in the commercial detergents (Maurer, 2004). Different industries especially leather and detergent industries require efficacious, environment friendly and economical approaches for degradation of unwanted proteins (Hameed *et al.,* 1996; Huang *et al.,* 2003; Wang *et al.,* 2007).

Protease production can be enhanced by optimization and manipulation of fermentation methods and conditions; cloning and modulation of genes expression and protein engineering (Gupta *et al.,* 2002a; Gupta *et al.,* 2002b). To achieve high protease production rates, understanding of strategies for protease production and broad range application in the industrial processes hold central importance. Aim of this study was to isolate, characterize and optimize proteolytic strain present in soil biome for enhanced enzyme production. Moreover, biochemical characterization and stability studies of the enzyme were aimed to determine possible eco-friendly application of enzyme in detergent industry.

Materials and methodology

Chemicals

BSA (bovine serum albumin) was obtained from BDH (UK). Different media components i.e. Casein, Peptone, Gelatin, Yeast Extract and Malt Extract were obtained from Oxoid. Beef extract and Tyrosine were purchased from Sigma-aldrich (UK). All solvents used in this study were of analytical grade and were obtained from Sigma-aldrich (UK).

Isolation and screening of proteolytic strain

Soil sample was collected from an agricultural land near the cattle dock site in Shehzad Town, Islamabad Pakistan. Sample was serially diluted in 1 N saline and

plated on nutrient agar. Isolated pure colonies were screened for proteolytic activity on 1% casein agar plates by simple plate assay (Vermelho *et al.,* 1996). After incubation, cultures with proteolytic activity were selected by the size of zone of hydrolysis produced on flooding plates with glacial acetic acid (Natt, 2000). Strain with maximum proteolytic activity was sub-cultured on 1% casein agar plate and proteolytic activity was reconfirmed by flooding with TCA ((Medina and Baresi, 2007). Proteolytic strains were screened for amylase and cellulase production on 1% starch and CMC nutrient agar plates (Sazci *et al.,* 1986).

Identification and characterization of selected proteolytic strain

Strain with highest proteolytic activity was characterized by microscopic and phenotypic examination. Moreover strain was checked for Oxidase production, Catalase production, H_2S production, Indole production, Urease production, Mobility, Sugars fermentation, Citrate utilization and MRVP tests by already reported methods for biochemical characterization (Bergey *et al.,* 1994).

Molecular characterization of the selected proteolytic strain was carried out by 16s rRNA sequencing after DNA extraction. Bacterial cells were separated from nutrient broth with saturated growth through centrifugation in the late exponential phase at 14000 x g for 10 mins and pellet was re-suspended in 567µl of TE buffer. Then 30µl of SDS (10% w/v) and 3µl of proteinase K (20 mg/ml) were added prior to incubation at 37^0 C for 1 hr. 100µl of NaCl (5M) and 80µl of CTAB/NaCl (10% w/v CTAB, 0.7 M NaCl) was added, followed by thorough mixing and incubation at 65°C for 10 mins. DNA was extracted with equi-volume chloroform/iso-amyl alcohol (24:1) and centrifuged for 5 mins at 14000 x g, which resulted in appearance of white interface showing precipitation of CTAB–protein/polysaccharide complex. Aqueous phase was transferred to fresh tube and equal volume of phenol/chloroform/iso-amyl alcohol (25:24:1) was added followed by centrifugation at 14000 x g for 5 mins. Supernatant was transferred to a fresh tube and 600µl of isopropanol (i.e. 0.6 Vol.) was added followed by overnight incubation at 37°C to precipitate out DNA. DNA pellet was washed with 70% ethanol to wash out residual CTAB and centrifuged at 14000 x g for 2 mins. Supernatant was discarded and pellet was re-suspended in 100µl of TE buffer and stored at -20°C until next step. DNA was amplified by using universal primers 518F 5'CCAGCAGCCGCGGTAATACG3', and 800R 5'TACCAGGGTATCTAATCC3'. Sequencing was performed on automated DNA sequencer (Applied Bio-Systems model 3100) by using Big Dye terminator cycle sequencing kit v.3.1 (Applied Bio-Systems, USA) at Macrogen DNA Sequencing Services, Korea. Sequenced data was examined using NCBI-BLAST, MAFFT and Mega 6 programs. Phylogenetic analysis was performed by comparing sequence with already available sequences in NCBI database. After molecular characterization, DNA sequence was submitted in NCBI database.

Determination of initial protease specific activity of the selected strain

A small experiment was conducted to analyze initial proteolytic potential of the selected bacterial strain. Casein nutrient broth medium (1% - w/v) with pH 7.0 was inoculated with 1% (v/v) of 24 hr old inoculum of pure strain prior to incubation at 37°C for 72 hrs in a continuous shaking incubator at 150 rpm. After 72 hrs of incubation, samples were drawn out to assay specific activity of sample (proteolytic activity/ protein content). Method of Kunitz was modified to assay proteolytic activity, while Lowry's method was used for estimation of total protein content (Kunitz, 1947; Lowry *et al.,* 1951).

Protease assay

Method of Kunitz was modified to assay proteolytic activity of the selected strain (Kunitz, 1947). Assay was performed after every 24 hrs up in 72 hrs of experiment. Clear supernatant was obtained by centrifuging culture broth at 10000 x g for 30 mins. Supernatant was assayed for proteolytic activity by using soluble casein as enzyme substrate. 200µl of crude extract was added to 400µl of substrate (1% casein in 0.02M Tris-HCl buffer with pH 8.8)

followed by 30 mins incubation at 37°C. After incubation, 1 ml of TCA (10%) was added to terminate the enzyme-substrate reaction and mixture was centrifuged at 10000 x g for 5 mins at 4°C to precipitate out undigested substrate (casein). Finally 400µl of NaOH (1.8 N) solution was added as a chromogen. Optical density of the reaction mixture was measured at 420 nm on spectrophotometer. Control was prepared in the same way except that enzyme was not added. Readings were taken in triplicates and their average was used to evaluate "Proteolytic Activity Unit" (U/ml). One unit of protease for the studies was defined as the fraction of enzyme that produces 1 µg of Tyrosine in one minute under standard assay procedure.

Optimization of culture conditions for enhanced protease production

Culture medium conditions were optimized for maximum enzyme production by conducting different shake flask experiments. 24 hr old inoculum was used throughout the optimization studies. Parameters like pH, incubation temperature, inoculum size and shaking conditions were maintained at 7.0, 37°C, 1 % and 150 rpm respectively, unless mentioned.

Best culture medium composition for maximum protease production was selected by using five different defined media: M1 (Yeast Extract: 35g/l; Peptone: 10 g/l; NaCl: 5 g/l and Dextrose: 2%), M2 (Gelatin: 20 g/l; Casein: 6 g/l and Glycerol: 20%), M3 (Malt Extract: 35 g/l; Peptone: 10 g/l and NaCl: 5 g), M4 (Beef Extract: 35 g/l; Peptone: 10g/l and NaCl: 5g/l) and M5(Gelatin: 10 g/l; Glucose: 10 g/l; Yeast Extract: 20 g/l; K_2HPO_4: 0.3%; KH_2PO_4; 0.1% and $MgSO_4.7H_2O$: Trace amount). After selection of best medium for protease production, different sets of experiments were conducted to check effect of various culture conditions such as pH (4.0 to 11), temperature (15°C to 65°C), supplementary carbon sources (1% w/v of glucose; sucrose, starch and glycerol) and size of inoculum (0.5 to 5% v/v; approximately 10^3 viable cells/mL of culture broth). After optimization of culture medium composition and conditions, an experiment was conducted on selected parameters to check optimal time of incubation for enzyme production. Samples were drawn out every 24 hrs to observe growth absorbance (280 nm) and specific activity of sample (proteolytic activity/ protein content).

Studies on halotolerance of proteolytic strain

To check halotolerance, effect of different concentrations of sodium chloride (NaCl) was checked on growth and enzyme production by adding different amount of NaCl ranging from 2.5 to 15% (w/v) with 2.5 units interval in selected media prior to autoclaving. Samples were drawn out, every 24 hrs to measure growth and specific activity.

Protease production under optimized culture conditions and purification

Strain was cultured in a shake flask on optimized conditions for maximum protease production. 10 ml of 24 hrs old culture was added into a 1000 ml Erlenmeyer flask containing 500 ml of optimized medium. Fermentation broth was incubated for 48 hrs at 37°C in a shaker incubator. Cell free broth was harvested by centrifuging culture broth at 10,000 x g for 10 mins and was proceeded for protease purification. All purification steps were performed at 4°C to avoid loss of proteolytic activity during purification procedure.

Ammonium sulfate precipitation

Crude enzyme of volume 500 ml was subjected to ammonium sulfate precipitation. Solid ammonium sulfate was added to crude enzyme with gentle stirring at 4°C until extract reached to a saturation of 80% (w/v). After centrifugation at 10,000 x g for 10 mins, precipitates were collected and dissolved in 25 ml of 20 mM Tris-HCl buffer (pH 8.8). Precipitated protein was dialyzed against same buffer overnight at 4°C. Proteolytic activity and protein content of dialyzed sample was assayed prior to storage at -20°C.

Gel Filtration Chromatography Sephadex G-75-120

To purify protein, size exclusion gel filtration chromatography using column (10/50 mm) packed with Sephadex G 75-120 was used. 2 ml of partially

purified enzyme sample was applied on the gel column and Tris HCl buffer (20 mM; pH 8.8) was used for protein elution. 18 protein fractions each with a volume of 3 ml were collected at a flow rate of 1.0 ml/5 min by automatic fraction collector, Advantec SF-100. Absorbance (280 nm and specific activity (proteolytic activity/ protein content) of each fraction was assayed and fractions with maximum activity were pooled and lypholized.

Biochemical Characterization and Stability Studies of Enzyme

Biochemical characterization and stability studies of purified enzyme were conducted to have a brief insight of the chemical nature of protein and possible applications of enzyme.

Effect of temperature and pH on protease activity and stability

Optimum temperature of protease activity was determined by incubating purified protease in 20 mM Tris HCl buffer at a temperature range of 5°C to 100°C for 1 hr. Thermal stability studies were conducted by pre-incubating purified enzyme at a temperature range of 5°C to 100°C for 1 hr prior to assaying residual activity. Optimum pH for protease activity was determined by incubating purified protease with casein dissolved in different buffers. Buffer systems (20 mM concentration) used for this purpose were: Acetate buffer (pH 4 and 5); Phosphate buffer (pH 6 and 7); Tris-HCl buffer (pH 8 and 9); Sodium hydroxide/di-sodium hydrogen phosphate buffer (pH 10 and 11). pH stability studies of protease were established by incubating equal-volume of enzyme and buffers at 37°C for 1 hr. Post-incubation residual activity of the enzyme was quantified by the standard assay procedure.

Effect of protein modulators (inhibitors and activators)

Effect of different protein modulators was evaluated to find out the possible enzyme-protein family and to establish catalytic properties of enzyme. Different concentrations (10mM, 20mM and 30mM) of modulators: PMSF (phenylmethylsulfonyl fluoride- serine protease inhibitor); EDTA (Ethylenediaminetetraacetic acid- metalloprotease inhibitor); idoacetic acid (cysteine protease inhibitor), ascorbic acid (aspartic protease activator) β-mercaptoethanol and cysteine were incubated in equal volume with enzyme for 1 hr at 37°C.

Effect of Metal ions on protease activity and stability

To check effect of different metal ions (Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Hg^{2+}, Mg^{2+}, Mn^{2+}, Ni^{+} and Zn^{2+}) on enzyme different concentrations (10mM and 20mM) of metal salts ($CaCl_2$, $CoCl_2$, $CuCl_2$, $FeSO_4$, $HgCl_2$, $MgCl_2$, $MnCl_2$, $NiCl_2$, $ZnCl_2$) were incubated with equal volume of enzyme for 1 hr at 37°C.

Effect of NaCl, detergents, surfactants and solvents on protease stability

Effect of NaCl on protease activity was checked by incubating equi-volume salt solution (0.0 to 12.0 % w/v with 2.0 unit interval) and enzyme, followed by standard assay procedure. To investigate compatibility of detergents with protease enzyme, three different percentages (1.0 %, 5.0 % and 10.0 %) of commercially available detergents (Surf Excel, Express Power and Bonus) were incubated with enzyme for 1 hr at 37°C. Effect of various surfactants was evaluated by incubating 1% solution of surfactants (Triton X-100, Tween 80 and Sodium dodecyl sulfate) with equal volume of enzyme for 1 hr at 37°C. Solvent stability studies were performed by incubating enzyme and organic solvent (analytical grade) in a ratio of 3:1. Solvents used for solvent stability studies were Acetone, Benzene, Cyclohexane, DMSO, Ethanol, Lactic Acid, Propanol and Xylene.

Application of Proteases- Destaining/Washing Test

To check potential of enzyme as a detergent additive, white cotton-cloth pieces (2x2 inches) were stained with blood. After overnight drying at room temperature, stains were fixed with 1% formaldehyde solution. After that cloth pieces were dried in oven at 60^0 C for 1 hr and were subjected to 3 different treatments: 50 ml hot distilled water (Temp: 75°C); 46 ml distilled water + 4 ml surf excel (10mg/ml) and 45 ml distilled water + 4 ml surf excel (10mg/ml) + 1

ml of purified enzyme. After 15 mins of shaking incubation at respective temperatures, results were noted down on the basis of visual examination.

Results and discussion

Isolation and qualitative screening of protease producing bacteria in soil

Twenty strains were isolated on nutrient agar plates, from the agricultural soil sample. On primary screening for proteolytic activity, 13 strains showed clear zones of hydrolysis. Hydrolysis zones with size greater than 3 mm were considered as significant zones of hydrolysis, as diameter of zone of hydrolysis is related to the enzyme production by the strains (Vermelho *et al.,* 1996)**.** Zones with diameters ranging in 5-22 mm were produced by strains isolated from agricultural soil. Bacterial isolate ASM1 showed biggest zone of hydrolysis i.e. 22 mm on flooding of 1% casein agar plates with glacial acetic acid (Fig. 1a). Proteolytic activity of all isolated strains was reconfirmed by another plate assay i.e. flooding of 1% casein nutrient agar plates with trichloroacetic acid (Fig. 1b). On basis of production of biggest zone of hydrolysis in both plate assays isolate ASM1 was selected for further studies.

Table 1. Overall Purification Scheme: showing sequential purification fold and percentage yield at different purification stages.

Purification Stage	Protease Activity (U/ml)	Total Protein (mg/ml)	Specific Activity (U/mg)	Purification Fold	Yield (%)
Crude enzyme extract	5.46	0.57	9.58	1	100
$(NH_4)_2SO_4$ precipitates	12.30	0.54	22.77	2.37	94
Purified enzyme	8.41	0.145	57.9	6.04	25

To evaluate additional physiological potential of isolated bacterial strains, screening for amylase and cellulase production was performed. Out of 13 proteolytic strains, 11 strains including ASM1 were found to be capable of producing additional extracellular enzymes i.e. Cellulases and Amylases. Microbial strain ASM1 is capable of producing more than one industrially important enzyme (Fig. 2a & 2b). Results of enzymatic studies of the strain ASM1 were found to be quite satisfactory as the proteolytic strains with an additional property of amylase and cellulase production may find out application in detergents and food industry (Kuhad *et al.,* 2011; Singh *et al.,* 2007).

Identification and characterization of selected proteolytic strain

Growth pattern and characteristics of isolated colonies of selected isolate i.e. ASM1 were noted. Colony was medium in size; creamish in color; circular; opaque; smooth and raised. Gram staining of 24 hrs old culture of ASM1 revealed moderate sized Gram positive rods under oil immersion lens. Culture was found to be positive for oxidase production, catalase production, citrate utilization, motility, Methyl red and Voges Proskauer test. On sequencing, a sequence of 1119 nucleotide base pairs was obtained for strain ASM1. Analysis of 16S rRNA sequence of isolate ASM1 revealed 99% homology with *Bacillus cereus* strains available in NCBI database. Nucleotide sequence of the reported strain is available in NCBI database under accession number KJ600795.

Determination of initial protease specific activity of the selected strain

To determine initial proteolytic potential of the selected bacterial strain a small experiment was conducted. Casein nutrient broth medium (1% - w/v) with pH 7.0 was inoculated with 1% (v/v) of 24 hr old inoculum of pure strain followed by incubation at 37°C for 72 hrs in a continuous shaking incubator at 150 rpm. Specific activity of 1.82 U/mg of protein was assayed after 72 hrs of shake flask fermentation of production media under un-optimized conditions. Optimization and manipulation of various factors has been reported to occupy central importance for high

production of industrially important proteases (Reddy *et al.,* 2008).To enhance the protease production, medium composition and culture conditions were optimized by conducting different shake flask experiments.

Table 1. Effect of NaCl, Detergents, Surfactants and Solvents on residual activity of Protease.

Factors	Levels		Residual Activity
NaCl	2%		110%
	4%		102%
	6%		97%
	8%		93%
	10%		75%
	12%		52%
Detergents	Surf Excel	1%	119%
		5%	89%
		10%	35%
	Express Power	1%	126%
		5%	95%
		10%	49%
	Bonus	1%	103%
		5%	84%
		10%	43%
Surfactants	SDS		108%
	Triton X-100		91%
	Tween-80		103%
Solvents	Acetone		113%
	Benzene		97%
	Cyclohexane		93%
	DMSO		97%
	Ethanol		74%
	Lactic Acid		66%
	Propanol		77%
	Xylene		83%

Optimization of culture conditions for enhanced protease production

Different parameters for maximum protease production were optimized: Media composition; pH; Temperature; Carbon source; Size of Inoculum and Time of incubation. Optimum protease production was assayed in M4 medium (with beef extract as substrate for enzyme) with a specific activity of 8.4 U/mg of protein after 24 hrs of incubation (Fig. 3 a). Beef extract has been reported earlier as a good substrate for high proteolytic activity of many proteolytic strains including *Bacillus* species (Johnvesly and Naik, 2001; Yang and Lee, 2001; Mukherjee *et al.,* 2008; Naidu and Devi, 2005;). Maximum bacterial growth was observed in M2 medium (with Gelatin and Caesin as enzyme substrates) after 48 hours of incubation. Enzyme production was found to be not related to the growth in conducted experiments. Co-relation coefficient of the data shows that growth and specific activity are independent variables and protease production is not related to the growth of *Bacillus cereus* ASM1 strain (r = 0.09257885).

In pH optimization studies, *Bacillus cereus* strain ASM1 was found to be growing on a wide pH range i.e. 6.0-10.0. Growth and specific activity on a broad pH range i.e. 5-9 has been reported earlier by *Bacillus cereus* strain BM1 (Mienda and Huyop, 2013). Growth maxima of strain was observed at pH 6.0 after 72 hrs of incubation while maximum enzyme production was assayed at pH 9.0 after 48 hrs of incubation (Fig. 3 b). Growth and specific activity maxima lied at different pH (r = 0.296), concluding protease produced by *Bacillus cereus* ASM1 as a non-growth related product as reported earlier for enzyme

produced by other *Bacillus* sp. (Genckal and Tari, 2006). In temperature optimization studies, maximum growth and specific activity were assayed at 35°C. Significant growth and enzyme activity was shown on temperature range of 25-45°C, as this strain was isolated from a mesophilic environment. Growth and enzyme production successively decreased after 45°C which may be attributed to the risk of denaturation of proteins with increasing temperature. Bacterial inoculum sizes in range of 0.5 to 5% were used to check their effect on protease production. An increase in growth was observed with time in all sizes of inoculum. Effect of size of inoculum on growth of *B. cereus* ASM1 strain was not much significant, as approximately equal amount of growth absorbance was observed at all inoculation concentrations after a particular time period which is in agreement with previously reported study (Genckal and Tari, 2006). Maximum enzyme activity was observed with an inoculum size of 2% (v/v) after 48 hrs of shaking incubation with specific activity of 10.25 U/mg of protein. High surface area to volume ratio which leads to higher nutrient availability might be a fair reason for increased enzyme production on inoculation with smaller inoculum volumes (Shafee *et al.,* 2005). Low specific activity was observed with 5% inoculum size. High inoculum concentration leads to decline in enzyme production due to increase in cell burden I n medium, leading to oxygen limitation and/or quick exhaustion of nutrients and substrate for proteolytic activity. Nevertheless some work has been reported that witnessed high enzyme production with high inoculum concentrations (Nadeem *et al.,* 2006).

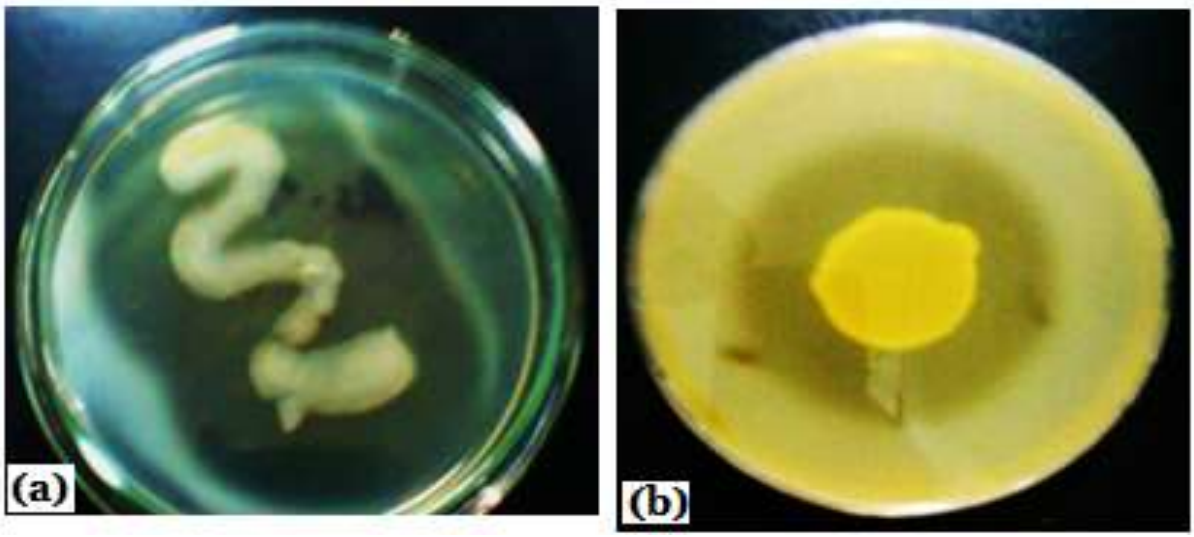

Fig. 1. Zones of Hydrolysis produced by isolate ASM1 on 1% Casein Agar plate (a) with Glacial acetic acid flooding (b) with Trichloroacetic acid flooding.

To enhance protease production, optimized medium was supplemented with 1% of different carbon sources (glucose, sucrose, starch and glycerol). Maximum specific activity i.e. 8.8 U/mg of protein was assayed after 24 hrs of time of incubation with glucose as a carbon source. After 48 hours of incubation, growth and specific activity drastically deceased due to the exploitation and depletion of the easily utilizable carbon source i.e. glucose. Enhancement in protease production on using glucose as a carbon source may be due to the constitutive nature of some part of enzyme as reported earlier (Mukherjee *et al.,* 2008). Many earlier reports have described enhanced alkaline protease production by *Bacillus* sp. in the presence of glucose as a carbon source (Akcan and Uyar, 2011; Kotlar, 2009; Mukherjee *et al.,* 2008). After 48 hrs of incubation, sucrose showed maximum activity of 7.50 U/mg of total protein followed by glycerol which showed 7.32 U/mg activity. Starch emerged as an effective source for growth of ASM1. Increase in growth/protease production by *B. cereus* in presence

of starch is in accordance with previously reported studies (Johnvesly and Naik, 2001; Naidu and Devi, 2005; Uyar *et al.,* 2011; Zambare *et al.,* 2014). Glycerol was found to be stimulating high protease activity after 48 and 72 hrs, which is in agreement with previously reported study (Sharma and De, 2011).

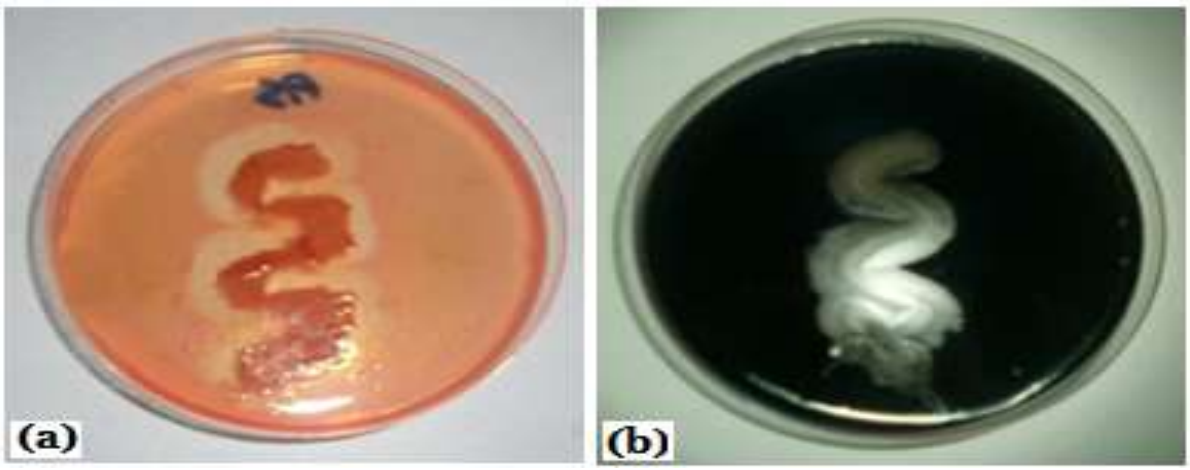

Fig. 2. Zones of hydrolysis produced by isolate ASM1 on selective media for enzymes production (a) Cellulase (b) Amylase.

After optimization of all of the parameters, protease production was carried out for 96 hrs on optimized conditions. Maximum enzyme production by *B. cereus* ASM1 was assayed after 48 hrs of incubation on optimized parameters with a specific activity of 9.58 U/mg of protein. Beyond 48 hrs, decrease in enzyme production was observed which might be attributed to depletion of nutrients from the medium. Moreover auto-digestion and proteolytic attack by other proteases due to prolonged incubation has been reported to cause a reduction in enzyme productivity and associated activity (Shafee *et al.,* 2005; Uyar and Baysal, 2004). Overall, enzyme production was increased to 9.58 U/mg of protein from 1.82 U/mg of protein which concludes that optimization studies increased enzyme production by 5 folds.

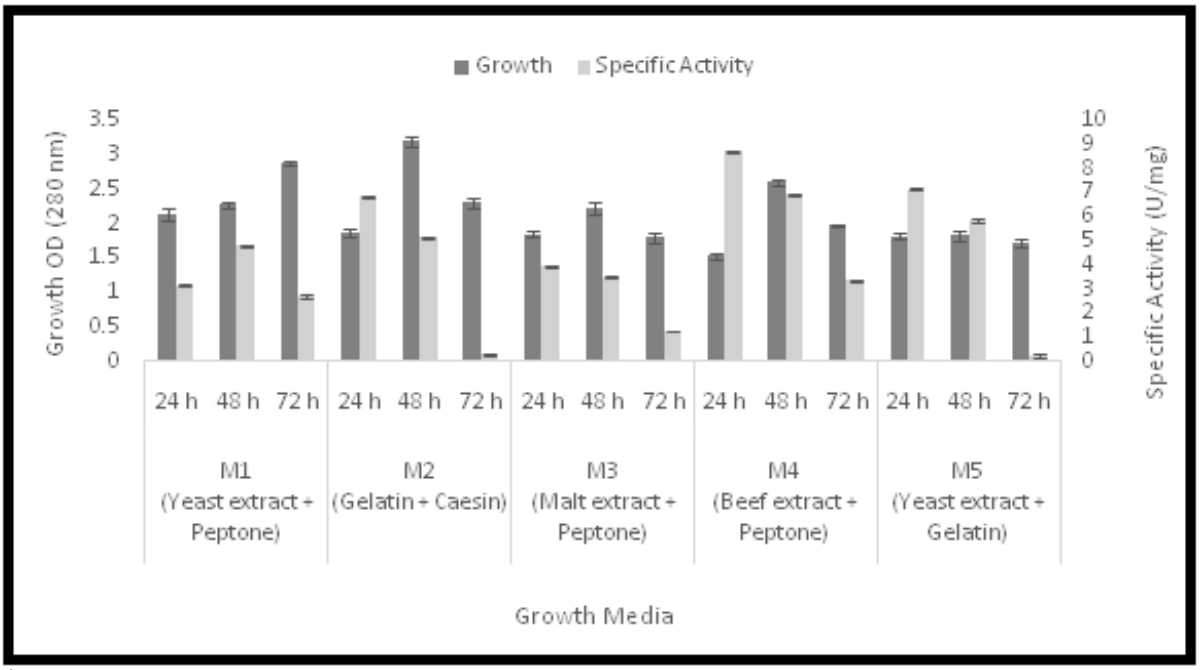

Fig. 2a. Effect of different defined growth media on growth and protease specific activity.

Studies on halotolerance of proteolytic strain

Isolate *B. cereus* ASM1 was isolated from non-saline agricultural soil. Growth of strain was observed up to 12.5% NaCl concentration while specific activity comparatively reduced on addition of NaCl in the optimized growth medium (Fig. 4). Maximum specific activity 3.81 U/mg of protein was assayed in growth medium with 2.5% NaCl concentration after 48 hrs of incubation. 15% NaCl concentration showed inhibitory effect on both growth and specific activity. Decrease in enzyme specific activity showed that *B. cereus* strain ASM1 doesn't require NaCl for optimal enzyme production, but growth in medium with salt concentration up to 12.5% showed halotolerance of the strain. A similar result was reported for another *B. cereus* strain capable of growing and tolerating up to 10% salt concentration without requiring salt for physiological activities (Singh *et al.,* 2010). Alkaline protease produced by *B. cereus* ASM1 holds an additional property of halotolerance that makes this enzyme a potential candidate for usage in various industrial processes including detergents' formulation (Joo and Chang, 2005).

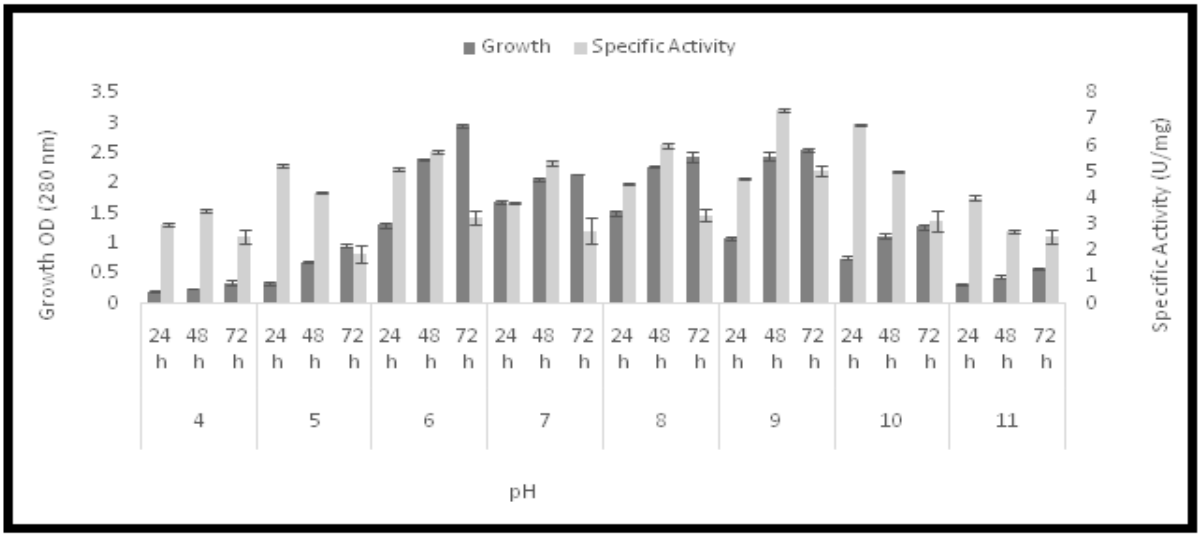

Fig. 3b. Effect of pH on Growth and Protease Specific Activity.

Purification of Protease

Crude enzyme produced on optimized parameters showed specific activity of 9.58 U/mg of total protein. Protease enzyme was partially purified by 80% ammonium sulfate precipitation. Precipitates showed specific activity of 22.77 U/mg of total protein with 94% yield and 2.37 purification folds. On using Sephadex G-75-120 for gel filtration chromatography, enzyme was purified by 6 folds with 25% yield holding a specific activity of 58 U/mg of total protein (Table 1).

Biochemical Characterization and Stability Studies of Enzyme

In order to have a brief insight of chemical nature of enzyme and possible applications, biochemical characterization and stability studies were conducted. Effect of pH, temperature, protein modulators, metal ions, NaCl concentration, solvents, detergents, and surfactants on the residual activity was assessed.

Effect of temperature and pH on protease activity and stability

In temperature stability studies, purified enzyme retained more than 80% residual activity up to 55°C. Optimal proteolytic activity was assayed at 35°C. Activity and stability of enzyme drastically decreased after 60°C leading to suppression of residual activity (Fig. 5). A proper decreasing trend was observed in the residual activity with increase in temperature which is in accordance with previously reported protease produced by *Pseudomonas aeruginosa* which was stable up to 50°C (Boopathy *et al.,* 2013). Increase in the temperature leads to decrease in the

residual activity which may be due to conformational changes and denaturation of protein on higher temperatures (Adinarayana *et al.,* 2003; Niyonzima and More, 2014).

Proteolytic *Bacilli* have been reported to be producing two types of extracellular proteases i.e. neutral/metalloproteases having optimal activity at pH 7.0 and alkaline proteases having optimal pH range between pH 9.0 and 11.0 (Ghorbel *et al.,* 2003; Rao *et al.,* 2009). Enzyme retained more than 80% activity on a pH range of 7.0 to 10.0 (Fig. 5 b). Optimal activity of enzyme was assayed at pH 9.0 which hints that enzyme produced by *Bacillus cereus* ASM1 belongs to alkaline class of proteases. Minimal activity was shown by enzyme at pH extremes i.e. 4.0 and 11.0, but more drastic decline in activity was observed in the acidic range i.e. activity decreased to just 41% of the activity in control. Alkaline proteases with broad pH activity profile and optimum activity between pH 9.0 and 11.0 have been reported earlier (Nilegaonkar *et al.,* 2007; Rao *et al.,* 2009). In pH stability studies, enzyme retained stability at a broad range of pH i.e. 6.0-11.0 with more than 80% residual activity. A slight decrease in residual activity was observed at acidic pH for purified enzyme while a slight elevation of 1-5% was observed in residual activities at alkaline pH range which indicates high stability of enzyme in the alkaline pH range. Alkaline serine protease produced by *Bacillus laterosporus*-AK1 with maximum activity at pH 9.0 with stability at pH 7.0-12.0 has been reported earlier (Arulmani *et al.,* 2007). Loss in enzyme stability and associated activity could be due to modification of three dimensional structure associated with alteration in ionization state of amino acids at active site of the enzyme under non-suitable pH conditions (Uyar *et al.,* 2011). High activity and stability in a broad range of pH with maxima in alkaline pH makes the enzyme a candidate for utilization in the detergents industry and cleaning of ultra-filtration membranes.

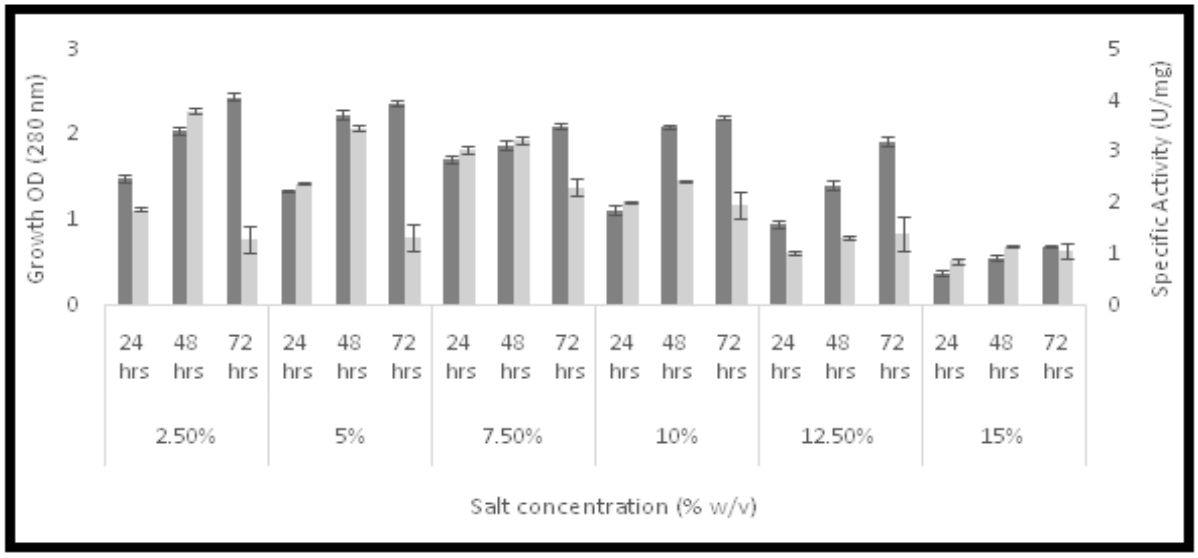

Fig. 3. Effect of different NaCl concentrations on growth and specific activity.

Effect of protein modulators (inhibitors and activators)

Effect of different protein modulators such as PMSF, EDTA, idoacetic acid, ascorbic acid, β-mercaptoethanol and cysteine was studied to have an insight into chemical nature of enzyme. Enzyme activity was significantly reduced in the presence of PMSF (Figure 6) which is a serine protease inhibitor known for sulfonating serine residues in catalytic site of enzyme which leads to reduction in enzymatic activity. Idoacetic acid didn't affect activity significantly, excluding the probability of enzyme to be a member of cysteine protease family. On increasing concentration of ascorbic acid from 10 mM to 30 mM proteolytic activity dropped from 90% to 54%. Ascorbic acid has been reported as an inducer

for aspartic class of proteases creating acidic environment to support activity of aspartic proteases hence decreasing activity of serine alkaline proteases. With increase in concentration of EDTA, proteolytic activity was slightly suppressed that may be due to role of some metal ions in the stable conformation of enzyme which is in accordance with previously reported findings for proteases produced by *Geobacillus sp.* (Zhu *et al.,* 2007). Proteolytic activity was not much affected by β-mercaptoethanol and cysteine demonstrating its monomeric nature (Singh *et al.,* 2013).

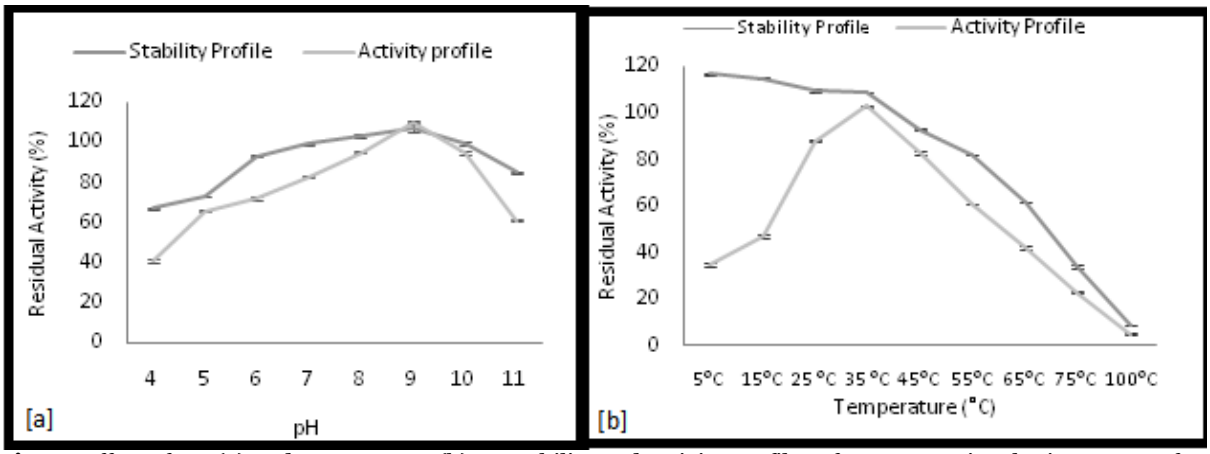

Fig. 4. Effect of pH (a) and temperature (b) on stability and activity profiles of enzyme on incubation at 37°C for 1hr.

Effect of Metal ions on protease activity and stability

Presence of Ca^{2+} ions in the incubate increased residual activity to 116% (Fig. 4). Calcium dependent serine proteases are less studied with few reports in the literature (Kobayashi *et al.,* 1996; Rao *et al.,* 2009; Sana *et al.,* 2006; Syngkon *et al.,* 2010). However a few reports with Calcium dependent proteases produced by *Bacillus* species have been reported earlier (Haddar *et al.,* 2009; Sehar and Hameed, 2011). Enhancement of activity in the presence of Ca^{2+} ions shows role of ions in stable confirmation of enzyme. Presence of different concentrations of Co^{2+} had no significant effect on the residual activity. Enzyme activity slightly reduced in the presence of Cu^{2+}, Hg^{2+}, Mg^{2+}, Mn^{2+}, Ni^{+} and Zn^{2+} ions without significant inhibitory effect. Presence of 10mM and 20mM Fe^{2+} ions showed inhibitory effect on enzyme activity reducing residual activity to 24% and 1% that may be due to binding of ferrous ions adjacent to active binding site of enzyme.

Effect of NaCl, Detergents, Surfactants and Solvents on stability of enzyme

Residual activity decreased with increasing concentration of NaCl in the mixture. Maximum residual activity 110% was assayed in the presence of 2% NaCl (Table 2). Residual activity decreased with increasing concentration of NaCl. More than 90% activity was retained up to 8% NaCl concentration verifying halotolerance of enzyme without requiring NaCl for protease activity and production. This discovery is interesting as the protease producer *Bacillus cereus* ASM1 strain was isolated from a non-saline environment. Another *Bacillus cereus* strain which retained 100% activity up to 10% salt concentration, highest concentration of halotolerance discovered so far for *B. cereus* was also isolated from non-saline environment (Mienda and Huyop, 2013).

To investigate stability and compatibility of detergents with protease enzyme, 3 different concentrations (1%, 5% and 10% w/v) of three available detergents were incubated with enzyme for 1 hr at 37°C. Enzyme retained more than 100% activity in the presence of 1% detergents (Table 2). More than 80% proteolytic activity was retained in presence of 5% detergents showing compatibility of enzyme with detergents. Protease stability studies have been already reported in a number of works with varying

amount of stability and activity of the proteases in the presence of a range of commercially available detergents (Joo and Chang, 2005; Kuddus and Ramteke, 2009; Kumar and Bhalla, 2004; Rao *et al.,* 2009). Stability and activity of enzyme in the presence of detergents show the enzyme as potential candidate for utilization in detergent industry as a detergent additive for efficacious stain removal.

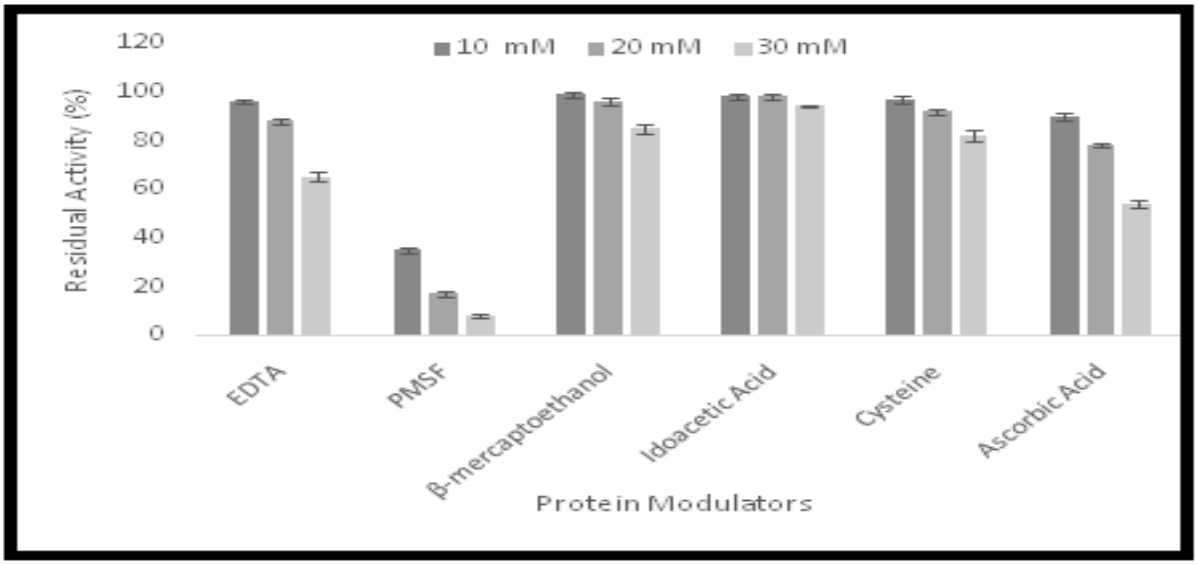

Fig. 5. Effect of different protein modulators (activators and inhibitors) on residual activity of purified protease enzyme.

Enzyme was found to be capable of retaining more than 90% activity in the presence of surfactants: SDS; Triton X-100; Tween-80 with maximum activity in presence of SDS (Table 2). An increase of 8% activity was assayed in the presence of 1% SDS, which might be due to dissociation of protein substrate in the presence of SDS that contributes in proteolytic activity of enzyme. Similar results were reported in a study where enzyme's relative activity increased upto 132 % in the presence of 0.1% SLS-Sodium Lauryl Sulfate (SDS). Increase in concentration of SLS to 1% led to a decline in activity to 110% yet maintaining higher activity than control (Singh *et al.,* 2013). High stability in the presence of SDS makes alkaline protease of *B. cereus* ASM1 a good candidate for utilization in detergent industry as a stable detergent-additive.

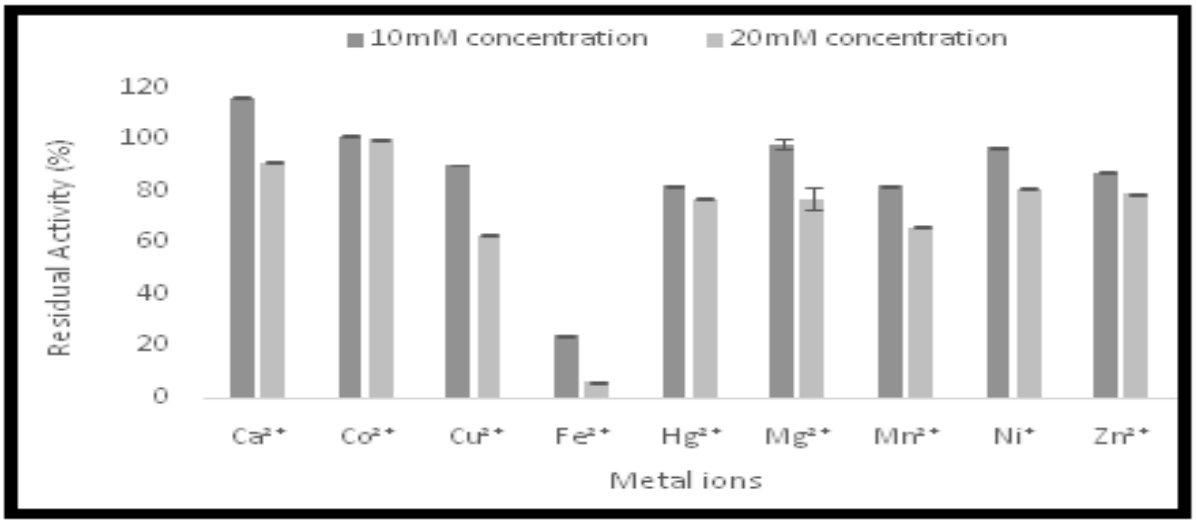

Fig. 6. Effect of different concentrations of metals ions on enzyme activity.

In solvent-stability studies, maximum residual activity 113% was retained in the presence of acetone followed by DMSO, benzene, cyclohexane, xylene, propanol and ethanol (Table 2). Least residual activity of 66% was observed in the presence of lactic acid. Maximum activity was shown in the presence of acetone which may be due to positive interactions between the serine residues on catalytic site and carbonyl group in acetone. Least activity was assayed in the presence of lactic acid which may be due to the creation of acidic environment leading to repression of activity.

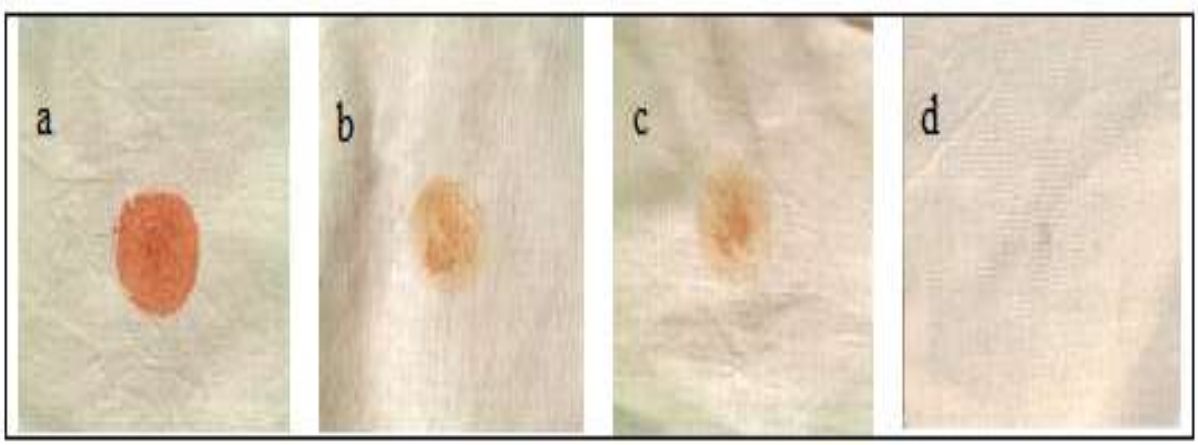

Fig. 7. Washing Test Results: (a) Control-Without Treatment; (b) Hot Distilled Water-Temp: 75°C); (c) Distilled Water + Detergent-Temp: 37°C ; (d) Distilled Water + Detergent + Enzyme-Temp: 37°C).

Application of Protease-Washing Test

Washing test was performed to check efficacy of purified protease as a detergent additive. Visual comparison was used to compare the results. Visual comparison of incubated cloth pieces showed highest stain removal in the in the cloth soaked in the combination of enzyme and detergent solution at room temperature after 10 minutes of incubation (Fig. 8). Most of the alkaline proteases capable of stain removal are thermotolerant in nature. To save energy, discovery of proteases with ability to perform efficient stain removal at lower temperatures, is of great importance in the enzyme-detergent industry (Kirk *et al.,* 2002; Mukherjee *et al.,* 2008). This protease was found to be showing high efficiency in stain removal at room temperature which is an additional potential for an alkaline protease to be used as detergent additive. There are few reports published showing stain removal activity of alkaline protease at room temperature (Mukherjee *et al.,* 2008; Venugopal and Saramma, 2006).

Conclusions

Bacillus cereus ASM1 strain isolated from agricultural soil could produce alkaline proteases. In optimization studies, enzyme production was increased by 5 folds and in most of the media conditions enzyme was found to be non-growth related product. Enzyme was purified by 6 folds with a yield of 25% by two step purification process employing ammonium sulfate precipitation and gel filtration column chromatography. Protease produced by *B. cereus* ASM1 is a member of alkaline serine class of proteases which was confirmed by repression of around 80% activity in the presence of PMSF and high activity-stability profile in alkaline pH range. Enzyme retained good amount of activity and stability at a broad range of temperature. Enzyme was found to be stable in different detergents, solvents and surfactants. On the basis of halotolerant and alkaline profile enzyme was tested in blood stain removal, which gave satisfactory results. This particular serine protease may prove to be a perfect tool for usage in detergent industry as a detergent additive due to its mesophilic, halotolerant, eco-friendly, surfactant-stable and alkaline nature.

Acknowledgement

Authors are thankful to Microbiology Research Lab (MRL), Quaid-i-Azam University, Islamabad Pakistan for providing all facilities and chemicals required to conduct this study.

References

Adinarayana K, Ellaiah P, Prasad DS. 2003. Purification and partial characterization of thermostable serine alkaline protease from a newly isolated Bacillus subtilis PE-11. AAPS PharmSciTech **4(4),** E56.
http://dx.doi.org/ 10.1208/pt040456.

Akcan N, Uyar F. 2011. Production of extracellular alkaline protease from Bacillus subtilis RSKK96 with solid state fermentation. Eurasia J Biosci **5**, 64-72.

Arulmani M, Aparanjini K, Vasanthi K, Arumugam P, Arivuchelvi M, Kalaichelvan PT. 2007. Purification and partial characterization of serine protease from thermostable alkalophilic Bacillus laterosporus-AK1. World Journal of Microbiology and Biotechnology **23(4),** 475-481.
http://dx.doi.org/10.1007/s11274-006-9249-7.

Barrett AJ, Rawlings ND, O'Brien EA. 2001. The MEROPS database as a protease information system. Journal of structural biology **134(2-3),** 95-102.
http://dx.doi.org/ 10.1006/jsbi.2000.4332.

Beg QK, Gupta R. 2003. Purification and characterization of an oxidation-stable, thiol-dependent serine alkaline protease from< i> Bacillus mojavensis</i>. Enzyme and Microbial Technology **32(2),** 294-304.

Bergey D, Holt J, Krieg P. 1994. Bergey's Manual of Determinative Bacteriology. 1994. Williams and Wilkins, Baltimore, MD, USA.

Boopathy NR, Indhuja D, Srinivasan K, Uthirappan M, Gupta R, Ramudu KN, Chellan R. 2013. Statistical medium optimization of an alkaline protease from Pseudomonas aeruginosa MTCC 10501, its characterization and application in leather processing. Indian journal of experimental biology **51(4),** 336-342.

Burhan A, Nisa U, Gökhan C, Ömer C, Ashabil A, Osman G. 2003. Enzymatic properties of a novel thermostable, thermophilic, alkaline and chelator resistant amylase from an alkaliphilic Bacillus sp. isolate ANT-6. Process Biochemistry **38(10),** 1397-1403.
http://dx.doi.org/10.1016/S0032-9592(03)00037-2.

Chu WH. 2007. Optimization of extracellular alkaline protease production from species of Bacillus. Journal of industrial microbiology & biotechnology **34(3),** 241-245.

Genckal H, Tari C. 2006. Alkaline protease production from alkalophilic< i> Bacillus</i> sp. isolated from natural habitats. Enzyme and Microbial Technology **39(4),** 703-710.

Ghorbel B, Sellami-Kamoun A, Nasri M. 2003. Stability studies of protease from Bacillus cereus BG1. Enzyme and Microbial Technology **32(5),** 513-518.

Gupta A, Khare S. 2007. Enhanced production and characterization of a solvent stable protease from solvent tolerant Pseudomonas aeruginosa PseA. Enzyme and Microbial Technology **42(1)**, 11-16.

Gupta R, Beg Q, Khan S, Chauhan B. 2002a. An overview on fermentation, downstream processing and properties of microbial alkaline proteases. Applied microbiology and biotechnology **60(4),** 381-395.

Gupta R, Beg Q, Lorenz P. 2002b. Bacterial alkaline proteases: molecular approaches and industrial applications. Applied microbiology and biotechnology **59(1),** 15-32.

Haddar A, Bougatef A, Agrebi R, Sellami-Kamoun A, Nasri M. 2009. A novel surfactant-stable alkaline serine-protease from a newly isolated

Bacillus mojavensis A21. Purification and characterization. Process Biochemistry **44(1),** 29-35.

Hameed A, Natt M, Evans, C. 1996. Production of alkaline protease by a new Bacillus subtilis isolate for use as a bating enzyme in leather treatment. World Journal of Microbiology and Biotechnology **12(3),** 289-291.

Huang Q, Peng Y, Li X, Wang H, Zhang Y. 2003. Purification and characterization of an extracellular alkaline serine protease with dehairing function from Bacillus pumilus. Current microbiology **46(3),** 0169-0173.

Jayakumar R, Jayashree S, Annapurna B, Seshadri S. 2012. Characterization of thermostable serine alkaline protease from an alkaliphilic strain Bacillus pumilus MCAS8 and its applications. Applied biochemistry and biotechnology **168(7),** 1849-1866.

Johnvesly B, Naik G. 2001. Studies on production of thermostable alkaline protease from thermophilic and alkaliphilic Bacillus sp. JB-99 in a chemically defined medium. Process Biochemistry **37(2),** 139-144.

Jon S. 2008. Global Industry Analysts. www.theprofitdepot.com.

Joo HS, Chang CS. 2005. Oxidant and SDS-stable alkaline protease from a halo-tolerant Bacillus clausii I-52: enhanced production and simple purification. Journal of applied microbiology **98(2),** 491-497.

Joo HS, Kumar C, Park GC, Paik S, Chang CS. 2003. Oxidant and SDS-stable alkaline protease from Bacillus clausii I-52: Production and some properties. Journal of applied microbiology **95(2),** 267-272.

Joshi GK, Kumar S, Sharma V. 2007. Production of moderately halotolerant, SDS stable alkaline protease from Bacillus cereus MTCC 6840 isolated from lake Nainital, Uttaranchal state, India. Brazilian Journal of Microbiology **38(4),** 773-779.

Khademi F, Abachi S, Mortazavi A, Ehsani M, Tabatabaie M, Malekzadeh F. 2013. Screening and isolation of substitute-rennet producing thermophilic phycomycetes, by modified Warcup method and improved selective medium. IOSR Journal of Agriculture and Veterinary Science **2**(1): 25-31.

Khan F. 2013. New microbial proteases in leather and detergent industries. Innovative research in chemistry **1(1),** 1-6.

Kirk O, Borchert TV, Fuglsang CC. 2002. Industrial enzyme applications. Current opinion in biotechnology **13(4),** 345-351.

Kobayashi T, Hakamada Y, Hitomi J, Koike K, Ito S. 1996. Purification of alkaline proteases from a Bacillus strain and their possible interrelationship. Applied microbiology and biotechnology **45(1-2),** 63-71.

Kotlar CE, Sansevro R, Roura SI. 2009. Characterization of Bacillus cereus isolated from fermented cabbage and conventional optimization of extracellular protease production. The Internet Journal of Microbiology **8**(1).

Kuddus M, Ramteke PW. 2009. Cold-active extracellular alkaline protease from an alkaliphilic Stenotrophomonas maltophilia: production of enzyme and its industrial applications. Canadian journal of microbiology **55(11),** 1294-1301.

Kuhad RC, Gupta R, Singh A. 2011. Microbial Cellulases and Their Industrial Applications. Enzyme Research **2011**, 10. http://dx.doi.org/10.4061/2011/280696.

Kumar D, Bhalla T. 2004. Purification and characterization of a small size protease fran1 Bacillus sp. APR-4. Indian journal of experimental biology **42,** 515-521.

Kunitz M. 1947. Isolation of a crystalline protein

compound of trypsin and of soybean trypsin-inhibitor. The Journal of general physiology **30(4),** 311-320.

Lowry OH, Rosebrough NJ, Farr AL, Rall RJ. 1951. Protein measurement with the Folin phenol reagent. J biol Chem **193(1),** 265-275.

Maurer KH. 2004. Detergent proteases. Current opinion in Biotechnology **15(4),** 330-334.

Medina P, Baresi L. 2007. Rapid identification of gelatin and casein hydrolysis using TCA. Journal of microbiological methods **69(2),** 391-393.

Mienda BS, Huyop F. 2013. Characterization of Bacillus cereus BM1 with Protease Activity. Research in Biotechnology **4(3),** 07-19.

Mukherjee AK, Adhikari H, Rai SK. 2008. Production of alkaline protease by a thermophilic Bacillus subtilis under solid-state fermentation (SSF) condition using Imperata cylindrica grass and potato peel as low-cost medium: characterization and application of enzyme in detergent formulation. Biochemical Engineering Journal **39(2),** 353-361.

Nadeem M, Baig S, Qurat-ul-Ain S, Qazi J. 2006. Microbial production of alkaline proteases by locally isolated Bacillus subtilis PCSIR-5. Pakistan Journal of Zoology (Pakistan) **38**, 109-114.

Naidu KSB, Devi KL. 2005. Optimization of thermostable alkaline protease production from species of Bacillus using rice bran. African Journal of Biotechnology **4(7),** 724-726.

Natt MA. 2000. Studies on the production and use of microbial enzymes in leather processing. Unpublished thesis.

Nilegaonkar S, Zambare V, Kanekar P, Dhakephalkar P, Sarnaik S. 2007. Production and partial characterization of dehairing protease from Bacillus cereus MCM B-326. Bioresource technology **98(6),** 1238-1245.

Niyonzima FN, More S. 2014. Purification and properties of detergent-compatible extracellular alkaline protease from Scopulariopsis spp. Preparative biochemistry & biotechnology **44(7),** 738-759. http://dx.doi.org/10.1080/10826068.2013.854254.

Purohit MK, Singh SP. 2011. Comparative analysis of enzymatic stability and amino acid sequences of thermostable alkaline proteases from two haloalkaliphilic bacteria isolated from Coastal region of Gujarat, India. International journal of biological macromolecules **49(1),** 103-112.

Rai SK, Mukherjee AK. 2010. Statistical optimization of production, purification and industrial application of a laundry detergent and organic solvent-stable subtilisin-like serine protease (Alzwiprase) from Bacillus subtilis DM-04. Biochemical Engineering Journal **48(2),** 173-180.

Rao CS, Sathish T, Ravichandra P, Prakasham RS. 2009. Characterization of thermo-and detergent stable serine protease from isolated Bacillus circulans and evaluation of eco-friendly applications. Process Biochemistry **44(3),** 262-268.

Rao K, Narasu ML. 2007. Alkaline protease from Bacillus firmus 7728. African Journal of Biotechnology **6(21)**, 2493-2496.

Rao MB, Tanksale AM, Ghatge MS, Deshpande VV. 1998. Molecular and biotechnological aspects of microbial proteases. Microbiology and molecular biology reviews **62(3),** 597-635.

Rawlings ND, Barrett AJ, Bateman A. 2012. MEROPS: the database of proteolytic enzymes, their substrates and inhibitors. Nucleic acids research **40(D1),** D343-D350.

Reddy L, Wee YJ, Yun JS, Ryu HW. 2008.

Optimization of alkaline protease production by batch
Burman and response surface methodological approaches. Bioresource Technology **99(7),** 2242-2249.

Sana B, Ghosh D, Saha M, Mukherjee J. 2006. Purification and characterization of a salt, solvent, detergent and bleach tolerant protease from a new gamma-Proteobacterium isolated from the marine environment of the Sundarbans. Process Biochemistry **41(1),** 208-215.

Sazci A, Erenler K, Radford A. 1986. Detection of cellulolytic fungi by using Congo red as an indicator: a comparative study with the dinitrosalicyclic acid reagent method. Journal of Applied Bacteriology **61(6),** 559-562.

Sehar S, Hameed A. 2011. Extracellular alkaline protease by a newly isolated halophilic Bacillus sp. Global J Biotechnol Biochem **6(3),** 142-148.

Shafee N, Aris SN, Rahman R, Basri M, Salleh AB. 2005. Optimization of environmental and nutritional conditions for the production of alkaline protease by a newly isolated bacterium Bacillus cereus strain 146. J Appl Sci Res **1**(1), 1-8.

Sharma N, De K. 2011. Production, purification and crystallization of an alkaline protease from Aspergillus tamarii [EF661565. 1]. Agric. Biol. JN Am **2(7)**, 1135-1142.

Singh A, Kuhad RC, Ward OP. 2007. Industrial application of microbial cellulases. Lignocellulose Biotechnologgy: Future Prospects: 345-358.

Singh SK, Tripathi V, Garg S. 2013. An oxidant, detergent and salt stable alkaline protease from Bacillus cereus SIU1. African journal of Biotechnology **10(57),** 12257-12261.

Singh SK, Tripathi VR, Jain RK, Vikram S, Garg SK. 2010. An antibiotic, heavy metal resistant and halotolerant Bacillus cereus SIU 1 and its thermoalkaline protease. Microbial cell factories **9(59),** 1-7.

Syngkon A, Elluri S, Koley H, Rompikuntal PK, Saha DR, Chakrabarti MK, Bhadra RK, Wai SN, Pal A. 2010. Studies on a novel serine protease of a ΔhapAΔprtV Vibrio cholerae O1 strain and its role in hemorrhagic response in the rabbit ileal loop model. PloS one **5(9),** e13122.

Uyar F, Baysal Z. 2004. Production and optimization of process parameters for alkaline protease production by a newly isolated Bacillus sp. under solid state fermentation. Process Biochemistry **39(12),** 1893-1898.

Uyar F, Porsuk I, Kizil G, Yilmaz EI. 2011. Optimal conditions for production of extracellular protease from newly isolated Bacillus cereus strain CA15. EurAsian Journal of BioSciences **5**, 1-9.

Venugopal M, Saramma A. 2006. Characterization of alkaline protease from Vibrio fluvialis strain VM10 isolated from a mangrove sediment sample and its application as a laundry detergent additive. Process Biochemistry **41(6),** 1239-1243.

Vermelho AB, Meirelles MNL, Lopes A, Petinate SDG, Chaia AA, Branquinha MH. 1996. Detection of extracellular proteases from microorganisms on agar plates. Memórias do Instituto Oswaldo Cruz **91(6),** 755-760.

Wang H, Liu D, Liu Y, Cheng C, Ma Q, Huang Q, Zhang Y. 2007. Screening and mutagenesis of a novel Bacillus pumilus strain producing alkaline protease for dehairing. Letters in applied microbiology **44(1),** 1-6.

Yang JK, Shih L, Tzeng YM, Wang SL. 2000. Production and purification of protease from a Bacillus subtilis that can deproteinize crustacean wastes☆. Enzyme and Microbial Technology **26(5),**

406-413.

Yang SS, Lee CM. 2001. Effect of culture media on protease and oxytetracycline production with mycelium and protoplasts of Streptomyces rimosus. World Journal of Microbiology and Biotechnology **17(4),** 403-410.

Zambare V, Nilegaonkar S, Kanekar P. 2014. Protease production and enzymatic soaking of salt-preserved buffalo hides for leather processing. IIOAB Letters **3**(1).

Zhu W, Cha D, Cheng G, Peng Q, Shen P. 2007. Purification and characterization of a thermostable protease from a newly isolated Geobacillus sp. YMTC 1049. Enzyme and Microbial Technology **40(6),** 1592-1597.