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THERMOSTABLE PROTEASE PRODUCTION BY *Aneurinibacillus thermoaerophilus* MCW220, ISOLATED FROM A HOT WATER SPRING

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ABSTRACT

A thermophilic bacterium *Aneurinibacillus thermoaerophilus* MCW220 producing extracellular thermostable protease was isolated from Manikaran hot water spring, Himachal Pradesh (India). Till date there is no report of thermostable protease production by any *Aneurinibacillus thermoaerophilus.* The strain MCW220 was characterized morpho-biochemically followed by sequencing of its 16S rRNA gene. BLASTn search analysis of the sequence showed maximum identity with *A. thermoaerophilus* L420-91 and the G + C content was 57.2%. The *A. thermoaerophilus* MCW220 when quantitatively screened for protease activity showed maximum thermostable protease activity of 33.4 U L^{-1}. The optimum yield and maximum protease activity was achieved at 48 h incubation, with pH 7.0 at 60°C. The desired protein was precipitated and purified from the crude extract by using ammonium sulfate (80%) and Sephadex G-100 column. The procedure yielded 0.105 g protein with 2.35 fold purification with a yield 54.16%. The molecular mass of enzyme was found 45 kDa by SDS-PAGE. Purified protease showed maximum enzyme activity within the pH range of 9.0-9.5 at 50°C and possessed compatibility with various detergents which make it a potential candidate for use in detergent industry.

Keywords: *Aneurinibacillus thermoaerophilus,* hot water spring, SDS-PAGE, thermophile, thermostable protease, 16S rRNA

INTRODUCTION

Proteases, also known as proteinases or peptidyl-peptide hydrolases or proteolytic enzymes, are a large group of enzymes belonging to class hydrolases which catalyze hydrolytic reactions. The proteases are classified into several groups, depending whether they are active under acidic, neutral or alkaline conditions and on the characteristics of active site group of the enzyme i.e. metallo- (EC.3.4.24), aspartic- (EC.3.4.23), cysteine- or sulphydryl- (EC.3.4.22), or serine-type (EC.3.4.21). Proteases are single class of enzymes which occupy a great position so for as their application in both physiological and commercial fields are concerned. Advances in analytical techniques have demonstrated that proteases accomplish highly specific and selective proteins modification such as activation of zymogenic forms of enzymes by limited proteolysis, blood clotting and lysis of fibrin clots and processing and transport of secretory proteins across the membranes (Rao *et al.*, 1998).

The estimated worldwide sale of industrial enzymes is about $1 billion (Godfrey and West, 1996). Of the industrial enzymes, 75% are hydrolytic; and proteases represent one of the 3 largest

groups of industrial enzymes accounting for about 60% of world enzyme sale. Proteases are also used in various industrial products/processes such as detergents, pharmaceuticals, leather, meat tenderizers, protein hydrolyzates, food products and waste processing. *Bacillus* has most widely been exploited for alkaline proteases and several *Bacillus* species have commercially been used in bioremediation mixes or as probiotic agent in aquaculture (Sen *et al.*, 2009). Proteases find their use in soaking, dehairing and bating stages of preparing skins and hides. A formulation containing proteolytic enzymes from *B. subtilis*, *B. amyloliquefaciens* and *Streptomyces* sp. and a disulfide reducing agent (thioglycolate), that enhances hair degradation and helps in clearing pipes clogged with hair-containing deposits, is currently available in market (Sen *et al*., 2009).

Thermostable proteases are industrially attractive owing to the advantage that higher processing temperatures possible lead to faster reaction rates, enhance solubility of reactants and products, higher resistance and reduce contamination by mesophilic organisms. Therefore, worldwide intensive search of these organisms is on especially those thriving in under-explored habitats. A large number of thermostable enzymes from many bacterial genera have been reported which include *Chromohalobacter* sp. (Vidyasagar *et al.*, 2007), Streptomyces (Mizusawa *et al.*, 1969), *Geobacillus* sp. (Hawumba *et al.*, 2002; Chen *et al.*, 2004), *Bacillus* sp. (Kaur *et al.*, 2001; Kumar, 2002; Nascimento *et al.*, 2004; da Silvia *et al.*, 2007; Bajaj and Jamwal, 2013; Sharma *et al*., 2014; Pant *et al.*, 2015). However, *Bacillus* species have remained a major source of enzymes of industrial and commercial value. *Aneurinibacillus thermoaerophilus* has recently been identified as a thermo-stable lipase- and cellulose-producing bacterium (Rahman *et al.*, 2009; Masomian *et al.*, 2010; Acharya and Chaudhary, 2012). However, till now its potential in thermostable proteases production has not been reported. In present investigation, we report a new thermostable protease producing bacterium *Aneurinibacillus thermoaerophilus* MCW220 from Manikaran hot water spring, India.

MATERIALS AND METHODS

Isolation and screening of thermostable protease producing thermophilic bacteria

Samples in the form of water, soil, pebbles and rock matting from various sites of hot water spring, Manikaran, Himachal Pradesh (India) were collected in sterilized screw-capped vials and jars, brought to the laboratory and kept at 4^{o}C in refrigerator till further processing. For the isolation of thermostable protease producing thermophilic bacteria, the samples were serially diluted in sterile distilled water and enriched on skim milk broth, containing glucose (1 g), yeast extract (2.5 g), casein (5 g), skim milk (1 g) and incubated at 60ºC for 48-72 h. The cultures were sub-cultured after 48-72 h into freshly autoclaved 150 mL flasks containing 25 mL skim milk broth. Turbid cultures were streaked on plates containing solidified skim milk agar medium (2% agar) and incubated in covered water bath incubator for 24 h in autoclavable polythene bags to prevent drying. After 24 h, morphologically distinct colonies were sub-cultured on respective agar plates to get pure isolated colonies. A clear zone of casein hydrolysis indicated presence of thermostable protease producing organisms. The isolate with high zone of clearance was purified by sub-culturing on fresh plates. The isolate was preserved on skim milk agar medium at 4ºC sub-cultured periodically.

Characterization of bacterial isolate

Selected thermophilic bacterial isolate was studied for various morphological characteristics *viz.*, colour, Gram reaction, shape, spore formation and motility (Kristjansson *et al.*, 1986) as well as it was characterized for biochemical characters *viz.*, catalase, urease, oxidase, indole, citrate, MR-VP and sugar fermentation (Aneja, 2008; Harrigan, 1998). All biochemical test reagents were procured from Himedia, Mumbai (India).

Genomic DNA extraction

Bacterial culture was inoculated into 20 mL skim milk broth and incubated at 60^{o}C overnight. The culture was then centrifuged at 14,000 rpm for 5 min., cell pellet washed twice with distilled water and used for DNA isolation using Genomic DNA Extraction Mini-Kit (Real Genomics) according to the manufacturer's instructions.

PCR amplification of 16S rRNA gene

The PCR amplification of 16S rRNA gene from purified genomic DNA was carried out in 0.2 mL PCR tubes with 20 µL reaction volume by using universal primers *viz.*, B27F 5`-AGAGTTTGATC CTGGCTCAG-3' and U1492R 5`-GGTTACCTTGTTACGACTT-3'. All the amplifications were performed using thermal cycler (MultiGene PCR system, Labnet) and with a temperature profile standardized for 16S rRNA gene amplification.

Sequencing analysis

The PCR product obtained through amplification with universal primers targeting rRNA gene was sequenced, using same upstream and downstream primers, by a commercial sequencing facility (Xcleris Lab). For identification, the sequence of bacterial isolate was blasted using online NCBI BLAST program [*http://www.ncbi.nib.gov/blast*] (Altschul *et al.,* 1997). Phylogenetic analysis was used for comparative genomics to establish evolutionary relationships. The analysis began with aligning of sequences using tools like Clustal W, and after alignment phylogenetic tree was constructed with MEGA version 6.0 using Neighbor-joining method. The partial 16S rRNA gene sequence of isolate was submitted to GenBank database under Accession No. KF938887.

Optimization of culture conditions

The thermophilic bacterium was grown in different media, pH, incubation temperature and incubation time to assess its optimum growth requirement. The culture media evaluated for optimization of thermostable protease producing bacterial isolate were nutrient agar (NA) containing 1% skim milk, skimmed milk medium (Sneath *et al*., 1986) and minimal synthetic medium with skim milk (Zilda *et al*., 2012). The pH range was optimized at 60^{o}C for 24 h using standardized medium adjusted to pH 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0, separately. The temperatures investigated were 40, 50, 60 and 70^{o}C; whereas incubation times assessed for growth were 24, 48, 72, 96 and 120 h. In all cases optical density was monitored at 540 nm on a double beam UV/VIS scanning spectrophotometer.

Production of thermostable protease enzyme

Fresh inoculum @ 1% was inoculated into skim milk broth (pH 7.0) for thermostable protease production and incubated at 60°C for 24 h at 150 rpm**.** The cells were separated by centrifugation at 10,000 x g, 4^{o}C for 10 min. The culture supernatant, obtained by centrifugation, was used as crude extracellular enzyme and protein contents estimated by standard method (Lowry *et al.,* 1951).

Protease assay

Protease assay of enzyme with casein as substrate was determined by the modified method of Kunitz (Kunitz, 1947). The enzyme solution (200 µL) was added to 800 µL substrate solution (0.5% casein in 0.1 M tris-HCl buffer) and the mixture incubated at 60°C for 15 min. The reaction was stopped by the addition of 1 mL 10% trichloroacetic acid (TCA) and the sample incubated at room temperature for 15 min. It was followed by centrifugation at 12, 000 x *g* for 10 min and measurement of absorbance of supernatant at 280 nm. A control was also run simultaneously in which TCA was added prior to the addition of enzyme solution.

Ammonium sulphate precipitation

The precipitation of thermostable protease enzyme was performed at 4^{o}C using 0.1 M tris-HCl buffer, pH 7.0 and the technique employed was ammonium sulfate precipitation method (Scopes,

1982). The cell free extract was subjected to various concentration of ammonium sulfate (0-80%). Ammonium sulfate was added with continuous stirring and stored at 4°C for overnight. The precipitated proteins were recovered by centrifugation at 20,000 g for 20 min. and pellet dissolved in 0.1 M tris-HCl buffer (pH 7.0). The dialysis was carried out for 24 h against 3 successive changes of dialysis buffer (tris-HCl buffer) in dialysis bag.

Sephadex G-100 column chromatography

The protein pellet obtained after saturation with 0-80% ammonium sulfate and dissolved in 0.1 M tris-HCl buffer was passed through a Sephadex G-100 column (1.5 x 27 cm) which had previously been equilibrated with 0.1 M tris-HCl buffer. The column was eluted @ 0.5 mL min^{-1} using the same buffer. Fractions with high protease activity were pooled and used to estimate molecular weight of extracellular protease, elecrophoretically.

SDS polyacrylamide gel electrophoresis

The molecular mass of partially purified protease from bacterial isolate MCW220 was determined by SDS-PAGE (Laemmli, 1970) using 12.5% acrylamide gel. Gels were stained with Coomassie brilliant blue G-250 and destained with 10% methanol and 10% acetic acid. The standard protein was used as protein molecular weight marker for SDS-PAGE for the estimation of molecular size of polypeptides of protease.

Characterization of purified protease

The optimum pH of purified protease was determined by conducting enzyme reaction in the selected pH range (7.0 to 12.0; tris HCl buffer) for 30 min. at 50°C. For determining optimum temperature for protease enzyme activity, the reaction was carried out for 30 min. at 30 to 90°C using tris HCl buffer set to pH 9.0. The bacterial protease was pre-incubated for 1 h at 50° C with the selected detergent (1% w/v prepared in tris buffer, pH 9.0) that included a few commercially available detergents such as Surf Excel, Rin, Tide and Fena. The residual activity of protease was determined thereof by the standard assay method (Kunitz, 1947).

Statistical analysis

The data pertaining to the optimization of bacterial growth and enzyme activity was subjected to multiple regression analysis to study the effect of independent variables (time, pH and temperature) on dependent variables (growth and enzyme activity). Various models were tried to find out the relationship between these variables. R^2 was used for evaluating the performance of model.

RESULTS AND DISCUSSION

For isolation of extracellular thermostable protease producing thermophilic bacteria, the samples were collected from Manikaran hot water spring Himachal Pradesh (India) and isolations performed using skim milk agar media. Skim milk medium proved best medium for the isolation of thermostable protease producing bacterium *Aneurinibacillus thermoaerophilus* isolate MCW220. The bacterial isolate was distinguished by the formation of a zone of clearance on casein medium due to proteolytic activity. By visual observation, bigger clear zone forming one thermophilic bacterial isolate was selected (Fig. 1). Since the fat content of whole milk inhibits the growth of bacteria, skim milk was used throughout the study. The widest zone of 12 mm dia. was produced by bacterial isolate MCW220. Habib *et al.* (2012) and Azlina and Norazila (2013) have also used skim milk medium for the successful isolation of thermostable protease producing bacteria. However, nutrient with skim milk medium and minimal synthetic medium supplemented with skim milk have also been used for this purpose (Zilda *et al.*, 2012).

Characterization of thermostable extracellular protease producing bacteria

Morpho-biochemical characterization of thermostable extracellular protease-producing isolate MCW220 revealed that the isolate grew on skim milk agar medium when incubated for 24 h at 60°C. The colony was white and rhizoid shaped with curled edge. The texture was rough with flat elevation. The microscopic studies showed the isolate MCW220 to be rod shaped Gram positive bacterium. The isolate produced free oxygen as gas bubbles in catalase reaction and showed oxidase positive reaction. The isolate showed positive results in methyl red and negative for Voges- Proskauer, indole formation and urease tests. The isolate grew on citrate agar and fermented sugars such as glucose, sucrose and lactose.

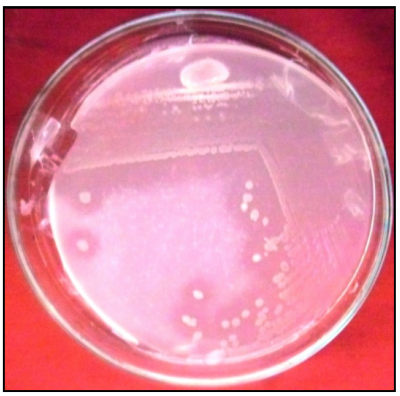

Fig. 1: Zone of clearance produced by MCW220 bacterial isolate on skimmed milk medium

Identification of bacterium and 16S rRNA gene analysis

The PCR amplification and sequencing of 16S rRNA gene of strain MCW220 revealed that the product of PCR amplification was approximately 1500 bp and the G+C content was 57.2%. The 16S rRNA gene sequence of this strain when compared with 16S rRNA gene sequences of other isolates revealed that isolate MCW220 belonged to the genera *Aneurinibacillus* (*http//www.ncbi.nlm. gov/BLAST/*). Multiple sequence alignment of MCW220 nucleotide sequence possessed 99% homology with *Aneurinibacillus thermoaerophilus* strain L420-91, 16S ribosomal RNA, partial sequence (NR_029303.1). Finally the phylogenetic relationship of this isolate with other sequences retrieved from NCBI database using MEGA 6.0 software generated a Neighbor-Joining tree which was divided into many branches and 16S rRNA gene of MCW220 isolate was supported by a bootstrap value of 100 (total of 100 replicates) (Fig. 2). The data of 16S rRNA gene sequence has been submitted to GenBank (Accession No. KF938887) and the strain designated as *Aneurinibacillus thermoaerophilus* MCW 220.

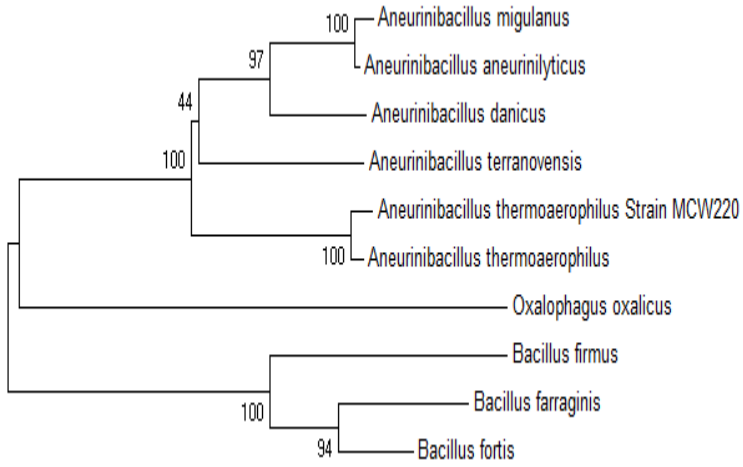

Fig. 2: Phylogenetic tree of *Aneurinibacillus thermoaerophilus* strain MCW220 16S rDNA. The GenBank accession numbers of the analyzed sequences are shown and a total of 100 bootstrap replicates were performed, and the bootstrap values are indicated at the branching points.

Optimization of the growth conditions of the selected bacteria

The various culture conditions such as different media, incubation time, pH of medium and incubation temperature were optimized for maximum growth and thermostable protease activity of *Aneurinibacillus thermoaerophilus* MCW220 strain. Skim milk medium was found to be best for both the growth and thermostable protease enzyme activity as maximum growth O.D. of 0.69 at wavelength of 540 nm and thermostable protease activity of 30.6 U L^{-1} was observed by using this medium (Fig. 3). Similar reports of skim milk medium for thermostable protease production have been communicated by Habib *et al.* (2012) and Azlina and Norazila (2013).

Maximum growth (1.25 growth O.D. at 540 nm) was observed after 48 h incubation while

maximum thermostable protease activity (30.5 U L^{-1}) was observed after 24 h incubation (Fig. 4). In the reports on thermostable alkaline protease production by *Bacillus* sp. an incubation period of 24 to 36 h has been reported to be optimum for enzyme production (Bharadwaj *et al*., 2014; Pant *et al.*, 2015). Maximum growth (2.1 growth O.D. at 540 nm) and thermostable protease activity of 29.4 U L^{-1} was observed at pH 7.0 (Fig. 5). Different *Bacillus* sp. reportedly produce alkaline protease in alkaline range e.g. *Bacillus subtilis* at pH 7.4 (Pant *et al.*, 2015), *B. aryabhattai* K3 at pH 8.0 (Sharma *et al*., 2014).

Incubation temperature of 60°C was found optimum for growth and maximum thermostable protease activity. Maximum growth OD of 0.69 at 540 nm and maximum thermostable protease enzyme activity of 31.1 U L^{-1} was observed at 60°C temperature (Fig. 6). The optimum temperature for different bacterial proteases has been reported to be 50-75°C (Chu *et al.*, 2007; Pant *et al*., 2015). The selected optimal parameters were used for production of thermostable protease enzyme.

Table 1 shows multiple regression data on optimization, in which quadratic model was fitted good for the effect of time on bacterial growth, effect of temperature on bacterial growth and effect of temperature on enzyme activity. Likewise, cubic model was fitted good for the effect of pH on bacterial growth, effect of time on enzyme activity and effect of pH on enzyme activity. It was observed that incubation time, pH and temperature significantly affected the bacterial growth and laccase activity as R^2 values were greater than 0.6 in every case.

Protease assay

Extracellular thermostable protease activity of crude enzyme of bacterial isolate was determined quantitatively by

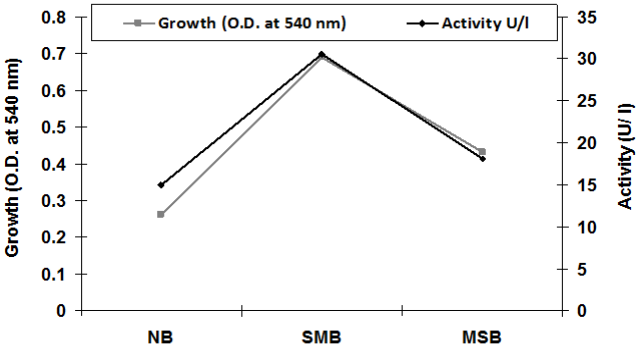

Fig. 3: Effect of media on growth and thermostable protease activity of *A. thermoaerophilus* MCW220

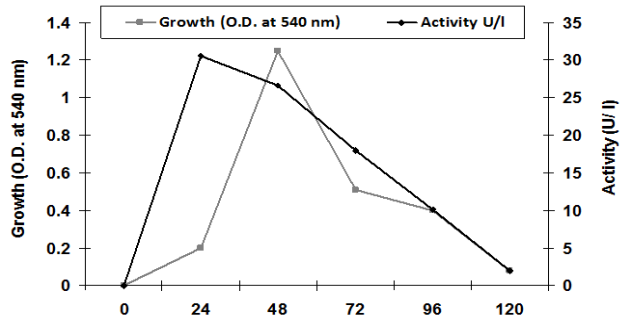

Fig. 4: Effect of incubation time (h) on growth and thermostable protease activity of *A. thermoaerophilus* MCW 220

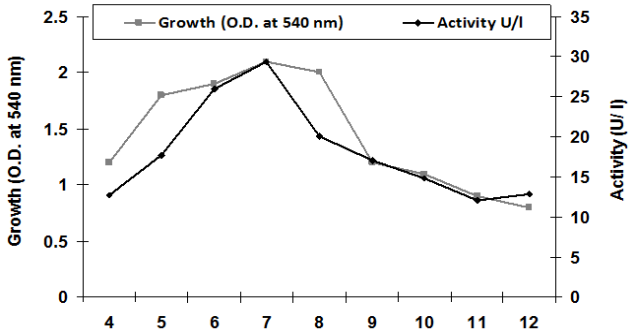

Fig. 5: Effect of pH on growth and thermostable protease activity of *A. thermoaerophilus* MCW220

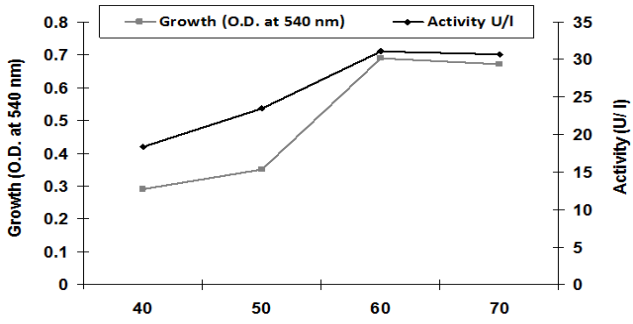

Fig. 6: Effect of temperature (°C) on growth and thermostable protease activity of *A. thermoaerophilus*

Table 1: Regression analysis of various variables

Independent variables	Dependent variables			
	Growth (O.D. at 540 nm)	R^2	Activity (U L^{-1})	R^2
Time (X)	$-0.043 + 0.027X + 0.001X^2$		$1.164 + 1.696 X - 0.029X^2 + 0.001X^3$	
	(0.015) (0.011) (0.0004)	0.603	(0.45) (0.330) (0.007) (0.0004)	0.957
pH (Y)	$-7.203 + 3.582Y - 0.437Y^2 + 0.016Y^3$		$-116.682 + 54.614Y - 6.66Y^2 + 0.251Y^3$	
	(1.953) (0.820) (0.108) (0.004)	0.930	(32.262) (13.538) (1.777) (0.074)	0.871
Temperature (Z)	$-0.894 + 0.037Z + 0.0001Z^2$		$-39.340 + 1.988Z - 0.014Z^2$	
	(0.210) (0.0079) (0.00003)	0.844	(12.221) (0.620) (0.004)	0.952

modified protease assay (Kunitz, 1947). Casein is a milk protein which acts as a high source of amino acids for the growth of bacteria to high cell densities and proteases/thermoproteases degrade this substrate into peptides followed by amino acids. Casein degradation initiated by extracellular serine protease such as subtilisins in several *Bacillus* species also has previously been reported (Mazar *et al.,* 2012; Thakur and Tiwari, 2013). However, azocasein has also been reported to be used for determining thermostable protease activity (Wilson and Remigio, 2012; Azlina and Norazila, 2013). Thermostable protease activity was determined after 24 and 48 h incubation for the test bacterial isolate. MCW220 bacterial isolate showed maximum extracellular thermostable protease activity of 33.4 U L^{-1} after 24 h incubation and maximum extracellular activity of 29.2 U L^{-1} after 48 h incubation.

Production and partial purification of thermostable protease enzyme

Thermostable protease production was carried out for extracellular preparation of the enzyme using all the standardized and selected optimum conditions of medium, temperature, time and pH. 1% primary inoculum size was used to inoculate skim milk broth at pH: 7.0, at 60^{o}C for 24 h. Thermostable protease enzyme activity as well as protein content of extracellular crude extract was calculated and it was found that extracellular protein content was 0.00304 mg mL^{-1} with an extracellular thermostable protease activity of 0.0080 U/ml and specific activity of 2.63 U mg^{-1}. Crude extract was further used to precipitate thermostable protease enzyme using ammonium sulphate (0-80%) saturation and it was found out that maximum thermostable protease enzyme activity of 1.43 U mL^{-1} was detected at 0-80% level of saturation. Bajaj and Jamwal, 2013 partially purified thermostable alkaline protease from *Bacillus pumilus* D-6 by ammonium sulphate precipitation at 40-60% level of saturation. Sharma *et al.* (2014) reported approximately 6.6 fold purification of protease from *Bacillus aryabhattai* K3 by ammonium sulphate precipitation (80%) with a recovery of 54.62% enzyme.This precipitated protein was dissolved in 0.1 M tris-HCl buffer (pH 7.0). The enzyme preparation at this stage was treated as ammonium sulphate fraction (ASF) (Table 2). Thermostable protease was precipitated by ammonium sulphate precipitation to 1.3-fold purification with 67.5% recovery from *Aneurinibacillus thermoaerophilus* MCW220 strain, which then applied to Sephadex G-100 column chromatography. It further purified protease to 2.35-fold with 54.16% protein recovery from *A. thermoaerophilus* MCW220 strain obtained from this study (Table 2). Moharam *et al.* (2003) purified protease from two strains of *Bacillus sephaericus* by using ammonium sulphate fractionation and Sephadex G-100 column chromatography while,

Table 2: Partial purification of thermostable protease from *Aneurinibacillus thermoaerophilus* MCW220 strain

Steps	Total enzyme activity (U)	Total soluble protein (mg)	Specific activity (U mg^{-1} protein)	Fold purification	Yield (%)
Crude extract	1.20	0.456	2.63	1.00	100.00
Ammonium sulfate precipitation, dialyzed	0.81	0.235	3.44	1.30	67.50
Gel filtration chromatography	0.65	0.105	6.19	2.35	54.16

Nilegaonkar *et al.* (2002) recorded 10 folds increase in specific activity of protease when purified using Sephadex G-100 column chromatography. SDS-PAGE analysis of protease exhibited a single band with molecular mass estimated to be 45 kDa (Fig. 7). Previously, a variety of molecular mass for proteases from other *Bacillus* species had been reported: 30.9 kDa (Huang *et al.*, 2006), 34 kDa (Kunitate *et al.*, 1989), 38 kDa (Kim *et al.*, 2001), 49 kDa (Akel and Atoum, 2003) and 75 kDa (Jung *et al.*, 2007).

Characterization of purified protease

The purified enzyme was assayed at various pH of buffer (pH 7-10; Fig. 8). The enzyme showed maximum activity at pH 9.0 and 9.5. Previously, an alkaline protease of *Bacillus* sp. with similar properties has been reported (Bharadwaj *et al*., 2014). The enzyme reaction of purified protease was carried out at 30-90^{o}C and maximum enzyme activity was observed at 50^{o}C (Fig. 9). Alkaline proteases of *Bacillus* sp. with similar temperature optima have been reported (Bharadwaj *et al*., 2014). Hence, protease from *Aneurinibacillus thermoaerophilus* MCW220 can be considered as thermostable enzyme.

Stability of thermostable protease in the presence of detergent(s)

For the possible commercial exploitation of an alkaline protease in detergent industry, the purified alkaline protease of *A. thermoaerophilus* MCW220 was tested for its compatibility/stability with each of the four commercial detergents of common use (Table 3). The protease incubated with the detergent (either in water/buffer adjusted to pH 9.0; 0.5%, w/v) revealed that Fena and Rin showed maximum compatibility with the bacterial purified protease. Bharadwaj *et al*. (2014) reported high activity alkaline protease from *Bacillus* sp. showing maximum compatibility in the presence of

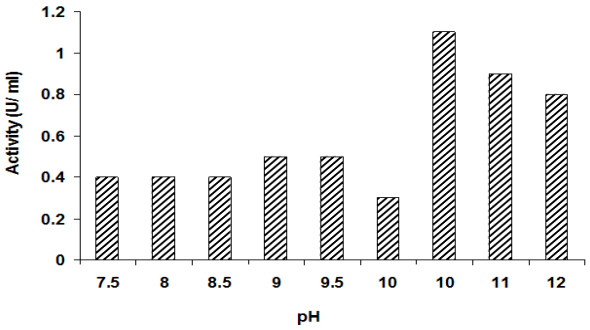

Fig. 8: Effect of pH of the buffer on the purified bacterial protease

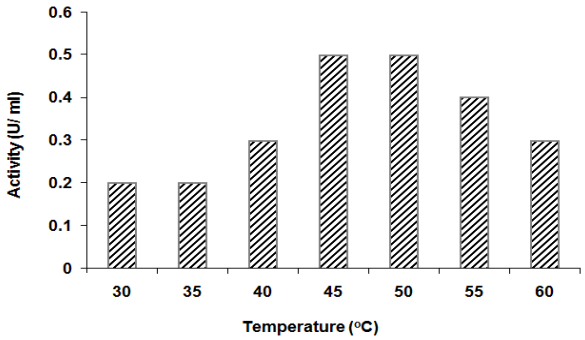

Fig. 9: Effect of temperature on the activity of the purified protease

Fig. 7: SDS- polyacrylamide gel electrophoresis of protease; Lane 1: Protein molecular weight marker; **Lane 2:** Purified protein eluted from gel filtration chromatography

Table 3: Compatibility of alkaline protease of *Aneurinibacillus thermoaerophilus* MCW220 with commercial detergents

Residual protease activity (in presence of detergent used at 0.5% w/v)				
Surf Excel	Rin	Tide	Fena	No detergent (control)
0.3	0.4	0.3	0.4	0.5

commercial detergents. The results showed that this enzyme is compatible with various detergents which make it a potential candidate for use in detergent industry.

Conclusion: In the present study, a new thermostable protease producing thermophilic bacterium *Aneurinibacillus thermoaerophilus* MCW220 has been isolated from Manikaran thermal spring, India. The bacterium was identified on the basis of morpho-biochemical and molecular characterization. This is the first report of thermostable protease production by *A. thermo-aerophilus*. The sequence has been submitted to NCBI database under Accession No. KF938887. Although the final yield of thermostable protease was low, yet the study demonstrates the capability of thermostable protease production by this bacterium. Further investigations are required enhance enzyme yield by immobilization or cloning and expression of the protease gene.

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REFERENCES

Acharya, S. and Chaudhary, A. 2012. Alkaline cellulose produced by a newly isolated thermophilic *Aneurinibacillus thermoaerophilus* WBS2 from hot spring, India. *African Journal of Microbiology Research,* **6**: 5453-5458.

Akel, H. and Atoum, M. 2003. Genetic polymorphism by RAPD-PCR and phenotypic characterization of isolated thermotolerant *Bacillus* strains from hot water spring sources. *Microbiologica,* **26**: 249-256.

Altschul, S.F., Thomas, L.M., Alejandro, A.S., Jinghui, Z., Zhang, Z., Webb, M. and David, J.L. 1997. Gaped BLAST and PSIBLAST: New generation of protein database search program. *Nucleic Acids Research*, **25**: 3389-3402.

Aneja, K.R. 2008. *Experiments in Microbiology, Plant Pathology and Biotechnology* (4th edn.). New Age International, New Delhi, India.

Azlina, I.N. and Norazila, Y. 2013. Thermostable alkaline serine protease from thermophilic *Bacillus* species. *International Research Journal of Biological Sciences,* **2**: 29-33.

Bajaj, B.K. and Jamwal, G. 2013. Thermostable alkaline protease production from *Bacillus pumilus* D-6 by using agro-residues as substrates. *Advances in Enzyme Research*, **1**(2): 30-36.

Bharadwaj, A., Puri, N., Chauhan, P.S., Cheema, B.S. and Gupta, N. 2014. Studies on alkaline-thermostable protease from an alkalophilic bacterium: Production, characterization and applications. *International Journal of Environmental Sciences,* **5**: 353-371.

Chen, X.G., Stabnikova, O., Tay, J.H., Wang, J.Y. and Tay, S.J.L. 2004. Thermoactive extracellular proteases of *Geobacillus caldoproteolyticus,* sp. nov., from sewage sludge. *Extremophiles,* **8**: 489-498.

Chu, W.H. 2007. Optimization of extracellular alkaline protease production from species of *Bacillus*. *Journal of Industrial Microbiology and Biotechnology*, **34**: 241-245.

da Silvia, C.R., Delatorre, A.B. and Martins, M.L.L. 2007. Effect of the culture conditions on the production of an extracellular protease by thermophilic *Bacillus* sp and some properties of the enzymatic activity. *Brazilian Journal of Microbiology*, **38**: 253-258.

Godfrey, T. and West, S. 1996. Introduction to industrial enzymology pp. 1-8. **In**: *Industrial Enzymology* (2nd edn.). Macmillan Press, London, UK.

Habib, S.M.A., Fakhruddin, A.N.M., Begum, S. and Ahmes, M.M. 2012. Isolation and screening of thermostable extracellular alkaline protease producing bacteria from tannery effluents. *Journal of Scientific Research*, **4**: 515-522.

Harrigan, W.F. 1998. Biochemical tests for identification of microorganisms. pp. 100-118. **In**: *Laboratory Methods in Food Microbiology* (3rd edn.). Academic Press, San Diego, California, USA.

Hawumba, J.F., Theron, J. and Brozel, V.S. 2002. Thermophilic protease producing *Geobacillus* from Buranga hot springs in Western Uganda. *Current Microbiology*, **45**: 144-150.

Huang, G., Ying, T., Huo, P. and Jiang, Y.Z. 2006. Purification and characterization of a protease from thermophilic *Bacillus* strain HS08. *African Journal of Biotechnology*, **5**: 2433-2438.

Jung, S.C., Paik, H., Kim, M.S., Baik, K.S., Lee, W., Seong, C.N. and Choi, S.K. 2007. InhA-like protease secreted by *Bacillus* sp. S17110 inhabited in turban shell. *Journal of Microbiology*, **45**: 402-408.

Kaur, S., Vohra, R.M., Kapoor, M., Beg, Q.K. and Hoondal, G.S. 2001. Enhanced production and characterization of a highly thermostable alkaline protease form *Bacillus* sp. P-2. *World Journal of Microbiology and Biotechnology*, **17**: 125-129.

Kim, S.S., Kim, Y.J. and Rhee, I. 2001. Purification and characterization of a novel extracellular protease from *Bacillus cereus* KCTC 3674. *Archives of Microbiology*, **175**: 458-461.

Kristjansson, J.K., Hreggvidson and Alfredsson, G.A. 1986. Isolation of halotolerant *Thermus* spp. from submarine hot springs in Iceland. *Applied and Environmental Microbiology*, **52**: 1313-1316.

Kumar, C.G. 2002. Purification and characterization of a thermostable alkaline protease from alkalophilic *Bacillus pumilus*. *Letters in Applied Microbiology*, **34**: 13-17.

Kunitate, A., Okamoto, M. and Ohmori, I. 1989. Purification and characterization of a thermostable serine proteases from *Bacillus thuringiensis*. *Agricultural and Biological Chemistry*, **53**: 3251-3256.

Kunitz, M. 1947. Crystalline soybean trypsin inhibitor II. *Journal of General Physiology*, **30**: 291-310.

Laemmli, U.K. 1970. Cleavage of structural protein during the assembly of the head of bacteriophage T4. *Nature*, **5:** 227-280.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. 1951. Protein measurements with folin phenol reagent. *Journal of Biological Chemistry*, **193**: 265-275.

Masomian, M., Rahman, R.N.Z.R.A., Salleh, A.B. and Basri, M.A. 2010. Unique thermostable and organic solvent tolerant lipase from newly isolated *Aneurinibacillus thermoaerophilus* strain HZ: Physical factor studies. *World Journal of Microbiology and Biotechnology*, **26**: 1693-1701.

Mazar, F.M., Mohammadi, H.S., Rad, M.E., Gregorian, A. and Omidinia, E. 2012. Isolation, purification and characterization of a thermophilic alkaline protease from *Bacillus subtilis* BP-36. *Journal of Sciences, Islamic Republic of Iran*, **23**: 7-13.

Mizusawa, K., Ichishima, E. and Yoshida, F. 1969. Production of thermostable alkaline proteases by thermophilic *Streptomyces*. *Applied Microbiology*, **17**: 366-371.

Moharam, M.E., EI-Bendary, M.A. and Foda, M.S. 2003. Purification and biochemical characterization of an alkaline serine protease of two highly mosquitocidal strains of *Bacillus sphaericus* and possible applications. *New Egyptian Journal of Microbiology*, **4**: 36-48.

Nascimento, W.C.A. and Martin, M.L.L. 2004. Production and properties of an extracellular protease from thermophilic *Bacillus* sp. *Brazilian Journal of Microbiology*, **35**: 91-96.

Nilegaonkar, S.S., Kanekar, P.P., Sarnaik, S.S. and Kelkar, A.S. 2002. Production, isolation and characterization of extracellular protease of an alkaliphilic strain of *Arthrobacter ramosus*, MCM B-351 isolated from the alkaline lake of Lonar, India. *World Journal of Microbiology and Biotechnology*, **18**: 785-789.

Pant, G., Prakash, A., Pavani, J.V.P., Bera, S., Deviram, G.V.N.S., Kumar, A., Panchpuri, M. and Prasuna, R.G. 2015. Production, optimization and partial purification of protease from *Bacillus subtilis*. *Journal of Taibah University for Science*, **9**: 50–55.

Rahman, R.N.Z.R.A., Masomian, M., Salleh, A.B. and Basri, M.A. 2009. New thermostable lipase by *Aneurinibacillus thermoaerophilus* strain HZ: Nutritional studies. *Annals of Microbiology*, **59**: 133-139.

Rao, M.B., Tanksale, A.M., Ghatge, M.S. and Deshpande, V.V. 1998. Molecular and biotechnological aspects of microbial proteases. *Microbiology and Molecular Biology Reviews*, **62**: 597-635.

Scopes, R.K. 1982. Ammonium sulfate precipitation table. pp. 125-131. **In**: *Protein Purification: Principal and Practice*. Springer Verlag, New York, USA.

Sen, S., Mandal, B. and Veeranki, V.D. 2009. Alkaline protease: A tool to clean environment. pp. 72-73. **In**: *Spring Meeting & 5th Global Congress on Process Safety*. American Institute of Chemical Engineers (AIChE), New York, USA.

Sharma, K.M., Kumar, R., Vats, S. and Gupta, A. 2014. Production, partial purification and characterization of alkaline protease from *Bacillus aryabhattai* K3. *International Journal of Advances in Pharmacy, Biology and Chemistry*, **3**: 290-298.

Sneath, P.H.A., Mair, N., Sharpe, E.M. and Holt, J.G. 1986. *Bergey's Manual of Systematic Bacteriology* (9th edn.). Williams and Wilkins, Baltimore, USA.

Thakur, S. and Tiwari, E. 2013. Isolation and characterization of thermostable protease producing bacteria from soap industry effluent. I*OSR Journal of Pharmacy and Biological Sciences*, **6**: 50-52.

Vidyasagar, M.S., Prakash, S.K., Jayalakshmi, K. and Sreeramulu. 2007. Optimization of culture conditions for the production of halothermophilic protease from halophilic bacterium *Chromohalobacter* sp. TVSP101. *World Journal of Microbiology and Biotechnology*, **23**: 655-662.

Wilson, P. and Remigio, Z. 2012. Production and characterization of protease enzyme produced by a novel moderate thermophilic bacterium (EP1001) isolated from an alkaline hot spring, Zimbabwe. *African Journal of Microbiology Research*, **27**: 5542-5551.

Zilda, D.S., Harmayani, E., Widada, J., Asmara, W., Irianto, H.E., Patantis, G. and Fawzya, Y.N. 2012. Screening of thermostable protease producing microorganisms isolated from Indonesian hot spring. *Squalen*, **7**: 105-114.