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EXPLORATION OF A HOT SPRING FOR THERMOSTABLE PROTEASE PRODUCERS

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ABSTRACT

Unkeshwar hot spring of Maharashtra was explored for the isolation of industrially important efficient thermostable protease producers. These isolates were identified based on their morphological characters, microscopic features, enzymatic profile and biochemical characteristics as *Bacillus sphaericus* APP21, *Staphylococcus auricularis* APP32, *Microbacterium* sp. APP41, and *Kurthia* sp. APP42 from Unkeshwar hot spring. Thermostable protease production was carried out using casein-yeast extract medium at 50 ^{o}C. Maximum specific activity of partially purified thermostable protease was recorded from *Bacillus sphaericus* APP21 (447.75 U/mg). Molecular weight of partially purified proteases from these isolates has been determined in the range of 14-29 KDa. These proteases have showed excellent thermostability in the range of temperature 45 to 65 ^{o}C. These proteases were catalytically active in the range of pH 7 to 9 and belonged to the class serine protease. Km and Vmax values were found in the range 5.26-8.34 mg/mL and 446-1380 U/mg respectively. The divalent cations Mg^{2+} and Mn^{2+} enhanced the catalytic efficiency of these proteases. These proteases showed excellent catalytic efficiency in presence of selected solvents, surfactants and detergents. These properties make them excellent candidates to use in detergent formulation, food processing, and in many biotechnologically important industries where proteolysis is needed at an elevated range of temperature.

Keywords: Thermostable protease; Thermostability; Unkeshwar hot spring; SDS-PAGE; Km; Vmax

INTRODUCTION

A hot spring is produced by the emergence of geothermal heated groundwater from the Earth's crust. Some popular hot springs are Yellowstone national park, (U.S.A.), Suryakund (Bihar, India), Sohna hot spring (Sohna, Delhi), Atri hot spring (Khordha, Odisha), Manikaran hot spring (Himachal Pradesh, India), Cimanggu hot spring (West Java, Indonesia), hypersaline and heliothermal Ekho Lake (East Antarctica),Garampani hot spring (Assam), Unapdev and Sunapdev hot spring (Maharashtra, India), and Bakreswar hot spring (West Bengal, India) (**Ulfah *et al.*, 2011; Labrenz *et al.*, 1998**).

Proteases from thermophilic sources have been reported to have optimum catalytic activities at the high temperatures. Therefore thermostable proteases are receiving considerable attention for their usefulness in various enzymatic industrial processes. Proteases have found applications in laundry detergents as additives, leather finishing, silk industry, feeds modification, food processing, brewing, pharmaceuticals, diagnostic reagents, peptide synthesis, and silver recovery from X ray/photographic film. Some thermostable protease producers have been reported from the genera *Pyrococcus*, *Thermococcus*, *Staphylothermus*, *Desulfurococcus*, *Pyrobaculum* and *Sulfolobus*. In archaea, the hyperthermophilic *Desulfurococcus* strain was reported to produce the extremely thermostable serine protease (**Hanzawa *et al.*, 1996**). Thermophilic fungi from the genera *Achaetomium*, *Chaetomium*, *Penicillium*, *Rhizopus*, *Sporotrichum*, *Torula* and *Rhizomucor* have also been reported to produce novel thermostable proteases (**Emi *et al.*, 1976**). Among bacteria, the genus *Bacillus* is the dominant source of thermostable proteases.

Here we have selected the terrestrial Unkeshwar hot spring (19°51'21.3"N and 78°15'00.9"E) from Nanded district of Maharashtra state of, India and explored it for the isolation of various thermophiles. Further we have identified the industrially important thermostable protease producers. Production and characterization of thermostable proteases from the selected isolates were also assessed.

MATERIALS AND METHODS

Isolation and identification of thermophiles

Water samples from Unkeshwar hot spring were collected in March, August and December 2011 and March 2012. Temperature and pH of the water samples were recorded individually at the time of collection of samples (**Pathak *et al.*, 2014; Jadhav and Pathak, 2015**). Abiotic characterization of Unkeshwar hot spring water samples was performed and published (**Pathak and Rathod, 2014**). Composite water samples were spread on nutrient agar, tryptone-yeast extract agar, tryptone-yeast glucose agar, Vogel Johnson Agar, glucose sodium azide glycerol agar, thiosulphate agar, J agar, brain heart infusion agar, Gram negative agar and *Bacillus* agar plates and these plates were incubated at 50 ^{o}C to isolate different thermophiles (**Pathak and Rathod, 2015**).

Typical morphological characters viz. colony shape, size, margin, elevation, consistency, opacity and pigmentation of selected isolates were recorded. Microscopic characters viz. cell shape, cell size, sporulation and motility of selected isolates were observed (**Pathak and Rathod, 2015**). Optimization of physical parameters for maximum growth of selected isolates was thoroughly assessed (**Polkade *et al.*, 2015**). Sugar utilization pattern of selected isolates was determined by using dextrose, fructose, lactose, sucrose, mannitol, maltose, xylose, arabinose, galactose, glycerol, cellobiose, sorbitol, mellibiose, mannose, trehalose, ribose, salicin, rhamnose, inulin, adonitol, and raffinose (**Sneath *et al.*, 1986**). Catalase and oxidase tests were performed by using 3 % hydrogen peroxide and 1 % tetramethyl-p-phenylenediamine dihydrochloride respectively (**Polkade *et al.*, 2015**). Amylase, cellulase, gelatinase, pectinase and lipase production ability of selected isolates was evaluated by using starch, cellulose, gelatine, pectin and tributyrin substrates respectively. Antibiotic susceptibility pattern of selected isolates was evaluated using different types of antibiotic discs (HiMedia, Mumbai). The isolates were identified by comparing their morphological, microscopic and physiological characters, sugar utilization and antibiotic sensitivity pattern, and enzyme profile with the standard reference strains from the Bergey's manual of systematic bacteriology (**Joshi *et al.*, 2008; Sharma *et al.*, 2009; Pathak and Sardar, 2012; Pathak and Sardar, 2014; Sardar and Pathak, 2014; Jadhav and Pathak, 2015a; Pathak *et al.*, 2015; Pathak *et al.*, 2015a; Hingole and Pathak, 2013; Sharma *et al.*, 2015;**

Khairnar *et al*., 2012; Kolekar *et al*., 2013; Dutta *et al*., 2015; Pathak and Rathod, 2016).

Screening for thermostable protease production

Morphological distinct selected isolates were inoculated on casein agar plates and incubated at 50 ^{o}C for 24 h. After incubation, acidic $HgCl_2$ solution was flooded on the same plates to examine the protease production efficiency of the isolates. Protease production efficiency was calculated by taking ratio of size of zone to colony size. The thermostable protease producers were selected for further studies **(Pathak and Rathod, 2015)**.

Thermostable protease production

Protease production medium was composed of casein 1.0 %, yeast extract 0.3 %, peptone 0.5 %, KH_2PO_4 0.2 %, NaCl 0.3 %, $MgSO_4.7H_2O$ 0.005 %and distributed in different conical flasks. 5 % inoculum (11×10^5 cfu/mL) of each thermostable protease producer was inoculated individually in the flasks. All the flasks were incubated in orbital shaking incubator (CIS-BL 24, Remi Make, Mumbai) at 50 °C and 120 rpm agitation speed for 72 h to occur protease production (**Gupta and Khare, 2006; Liu *et al*., 2010**).

Extraction and partial purification of thermostable protease

After 72 h incubation, the culture broth from each flask was centrifuged at 10,000 rpm for 10 min at 4 ^{o}C in cooling centrifuge machine (Remi make, Vasai). Supernatants were collected individually and used as crude protease extracts. Solid ammonium sulphate was added slowly to the each crude protease extract at the rate of 60 % saturation and kept for overnight at 4 ^{o}C. The precipitates were collected by centrifuging at 10,000 rpm for 10 min and dissolved individually in 0.2 M phosphate buffer of pH 7.0. The dissolved form of precipitate was dialyzed against the same buffer at 4 ^{o}C for 24 h (**Pathak *et al*., 2014a; Rathod and Pathak, 2014; Rathod and Pathak, 2016a; Osho *et al*., 2015; Pathak and Rathod, 2013**).

Protease assay

Partially purified protease (1 ml) from each selected isolate was added individually to the test tubes containing 1 ml of 1 % casein solution prepared in 0.2 M phosphate buffer pH 7.0. These mixtures were then incubated at 50 °C for 10 min. 3 mL of 10 % trichloroacetic acid was added in each tube to terminate the reaction. The reaction in blank tube was stopped at zero hours. The supernatants were collected by centrifuging reaction mixtures at 9000 rpm for 10 min. The absorbance of each supernatant was measured as 280 nm. One unit activity of protease was defined as the amount of protease required to liberate 1 µmol min^{-1} ml^{-1} of tyrosine equivalent, under the assay conditions. Total protein content of each crude protease solution was determined by using BSA as standard (**Rathod and Pathak, 2014; Rathod & Pathak, 2016; Rathod and Pathak, 2017; Osho *et al*., 2015; Pathak and Rathod, 2013; Pathak et al., 2014b; Lowry *et al*., 1951**).

Molecular weight determination

Molecular weight of partially purified proteases from each selected isolate was determined by the sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE; 12%) as described by **Laemmli (1970)**.Protein broad range molecular weight markers (GeNei^{TM} Bioscience catalogue no. PMWB 623110475001730) were used to determine molecular weight of partially purified proteases from selected isolates (**Rathod and Pathak, 2014a**).

Effect of temperature and pH on catalytic efficiency of proteases

Effect of temperature on catalytic efficiency of partially purified proteases from selected isolates was determined at the temperature range from 35 to 85 ^{o}C. Catalytic efficiency of partially purified proteases from selected isolates was determined at pre-determined optimized temperatures and different pH values using appropriate buffers of pH 4 to 10 (**Rathod and Pathak, 2014; Osho *et al*., 2015; Pathak and Rathod, 2013; Rathod and Pathak, 2014a**).

Stability studies of proteases at pre-determined optimum temperature and pH values

Stability of partially purified proteases from selected isolates was examined by incubating the proteases at pre-determined optimum pH and temperature values for 0-6 hours without substrate and then measuring the residual (remaining) enzyme activity under standard assay conditions. The protease activity before pre-incubation at selected parameters was considered as 100 % and residual (remaining) activity was calculated (**Pathak *et al*., 2014a; Rathod and Pathak, 2014; Pathak and Rathod, 2013; Rathod and Pathak, 2014a**).

Effect of different casein concentration on protease activity and determination of Km and Vmax values

Partially purified proteases from the selected isolates were incubated with buffered casein solutions of different concentrations of casein ranging from 5 to 30 mg/mL. Protease assay was carried out at pre-determined optimum temperature and pH values of partially purified proteases for 10 min and protease activity per unit time was determined with each selected substrate concentration. The values of Km and Vmax of partially purified proteases from the selected isolates were determined after constructing Lineweaver-Burk plots (**Rathod and Pathak, 2014**).

Effect of selected metal ions on catalytic efficiency of partially purified protease

Effect of inducers and inhibitors on catalytic efficiency of partially purified proteases was determined using 1 % metal solution viz. $FeCl_3$, $MgCl_2$, $CuCl_2$, $CaCl_2$, $ZnCl_2$, $BaCl_2$, $HgCl_2$, $AgNO_3$, KCl, NaCl and $MnCl_2$. Protease activity in absence of metal ions was considered as 100 % and residual protease activities (%) were calculated (**Rathod and Pathak, 2014**).

Effect of activators and inhibitors on catalytic efficiency of partially purified protease

Effect of selected solvents (1 %) viz. Chloroform, Ethanol, Butanol, Hexane, Heptane, Tolune, Triton X-100, Acetonitrile, Benzene, Glycerol and Isopropanol and other additives viz. Disodium ethylene diamino tetra acetic acid (1 %), Hydrogen peroxide (1 %), Sodium dodecyl sulphate (1 %), 2-Mercaptoethanol (5 mM), Phenyl methyl sulphonyl fluoride (5 mM), Tween 20 (1 %) and Tween 80 (1 %) was also tested on catalytic efficiency of partially purified proteases from the selected isolates. Protease activity in absence of any activators and inhibitors was considered as 100 % and residual protease activities (%) were calculated (**Rathod and Pathak, 2014**).

Compatibility of partially purified proteases with detergents

Catalytic efficiency of partially purified proteases from the selected isolates with commercial detergents was assessed using Ariel® (Procter and Gamble, Suisse), Tide® (Procter and Gamble, Suisse), Rin® (Hindustan Lever Ltd India), Wheel® (Hindustan Lever Ltd India), Surf excel® (Hindustan Unilever Ltd India), Nirma® (Nirma Lever Ltd India), Ghadi® (Kanpur trading Co Pvt Ltd Kanpur, India), Sasa® (Sasa Detergent, Pune) and Vim® (Vim Co Ltd India) at final concentration 7 mg/mL in partially purified protease solution. The prepared detergent solutions were boiled for 30 min and cooled prior to use (**Rathod and Pathak, 2014**).

RESULTS AND DISCUSSION

Isolation, screening and identification of thermostable protease producers

The average values of temperature and pH of Unkeshwar hot spring water samples were recorded as 49.48 ± 7.07 ^{o}C and 7.92 ± 0.06 respectively. Abiotic characterization of Unkeshwar hot spring water samples was performed and published (**Pathak and Rathod, 2014**). Total 42 thermophiles were isolated from Unkeshwar hot spring water samples and maintained on nutrient agar slants. These isolates were designated as APP1 to APP42. Amongst these, 25 isolates have showed thermostable protease production. Protease production efficiency of these isolates are shown in Figure 1.

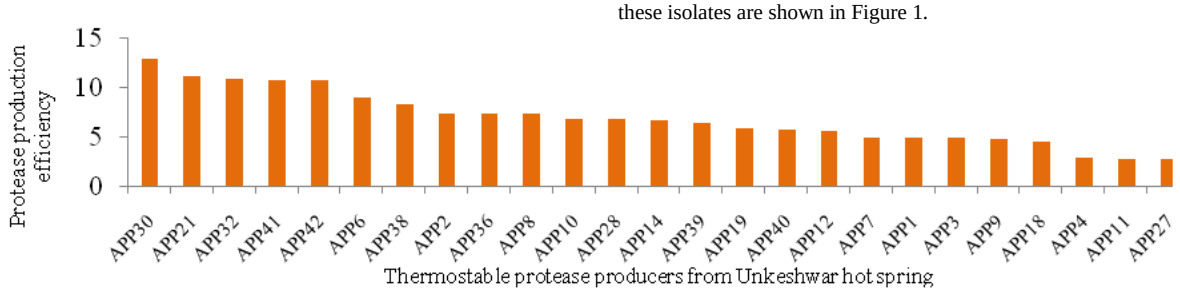

Figure 1 Protease production efficiency of bacterial isolates from Unkeshwar hot spring

Maximum thermostable protease production efficiency was showed by the isolate APP30 followed by the isolates APP21, APP32, APP41, APP42, APP6, APP38, APP2, APP36, APP8, APP10, APP28, APP14, APP39, APP19, APP40, APP12, APP7, APP1, APP3, APP9, APP18, APP4, APP11 and APP27.

Morphological and microscopic characters of APP21, APP32, APP41 and APP42 isolates have been given in Table 1. Sugar-utilization pattern and enzyme profile of these isolates have been given in Table 2 and Table 3 respectively. Optimum conditions required for growth and antibiotic susceptibility pattern of these thermostable protease producers have been given in Table 4 and Table 5 respectively. Antibiotic sensitivity pattern of isolates was used previously by many researchers as one of the taxonomic tools for microbial identification (**Lindberg et al., 1977; Sneath et al., 1986**).

Table 1 Morphological and microscopic characters of thermostable protease producers from Unkeshwar hot water spring, Maharashtra

Characters	*Bacillus sphaericus* APP21	*Staphylococcus auricularis* APP32	*Microbacterium* sp. APP41	*Kurthia* sp. APP42
Shape	Round	Round	Round	Circular
Size (mm)	1	1.5	2	2
Margin	Irregular	Entire	Cerate	Entire
Colour	Off white	white	Pale White	Creamy White
Elevation	Raised	Raised	Flat	Raised
Consistency	Sticky	Sticky	Sticky	Sticky
Opacity	Opaque	Opaque	Opaque	Opaque
Gram stain	Gram positive	Gram positive	Gram variable	Gram variable
Cell Size (µm)	L=1.0 B=0.5	D=1.0	L=1.0 B=0.5	L=1.0 B=0.5
Cell Shape	Coco-bacillary	Coccoid	Rod	Rod
Motility	Non motile	Non motile	Motile	Motile
Sporulation	+	-	-	-
Position of spore	Terminal	-	-	-

Table 2 Sugar utilization pattern of thermostable protease producers from Unkeshwar hot water spring, Maharashtra

Isolates→ Tests↓	*Bacillus sphaericus* APP21	*Staphylococcus auricularis* APP32	*Microbacterium* sp. APP41	*Kurthia* sp. APP42
Dextrose	-	w+	++	w+
Fructose	+	-	+	-
Lactose	-	-	-	-
Sucrose	-	-	+	-
Mannitol	-	-	-	-
Maltose	-	-	-	++
Xylose	-	-	+	-
Arabinose	-	-	-	-
Galactose	-	-	-	-
Glycerol	-	-	-	-
Cellobiose	-	-	-	-
Sorbitol	-	-	-	-
Mellibiose	-	-	-	-
Mannose	-	+	-	-
Trehalose	-	-	+	-
Ribose	-	-	+	+
Salicin	-	-	+	-
Rhamnose	-	-	-	-
Adonitol	-	+	-	-
Raffinose	-	-	-	-
Gas Production	-	-	-	-
Indole Test	-	-	-	-
MR Test	-	-	-	-
VP Test	-	-	-	-
Citrate Test	-	-	+	-

Table 3 Evaluation of enzyme profile of thermostable protease producers from Unkeshwar hot water spring, Maharashtra

Enzymes	*Bacillus sphaericus* APP21	*Staphylococcus auricularis* APP32	*Microbacterium* sp. APP41	*Kurthia* sp. APP42
Catalase	+	+	+	+
Oxidase	+	+	+	+
Caseinase	+	+ +	++	++
Amylase	-	-	+	-
Gelatinase	+	+	+	+
Cellulase	-	-	-	-
Urease	+	-	-	-
Lipase	-	-	+	-
Pectinase	-	-	-	-

Table 4 Optimum conditions required for growth of thermostable protease producers from Unkeshwar hot water spring, Maharashtra

Parameters	*Bacillus sphaericus* APP21	*Staphylococcus auricularis* APP32	*Microbacterium* sp. APP41	*Kurthia* sp. APP42
Temperature growth range (^oC)	25-65	25-45	25-55	30-50
Optimum temperature (^oC)	55	45	45	50
pH growth range	7-9	5-8	6-9	6-10
Optimum pH	8	7	8	7
Optimum growth period (h)	24	24	24	72

Table 5 Antibiotic susceptibility pattern of thermostable protease producers from Unkeshwar hot water spring

Isolates→ Tests↓	*Bacillus sphaericus* APP21	*Staphylococcus auricularis* APP32	*Microbacterium* sp. APP41	*Kurthia* sp. APP42
Amikacin (30µg)	2.5	R	3.5	3.3
Ampicillin (10µg)	4.0	3.5	3.5	2.4
Cefoperazone (75µg)	2.6	R	2.5	2.1
Cefoxitin (30µg)	1.0	2.5	2.5	2.5
Ceftazidime (30µg)	4.2	2	3.5	2.1
Ceftriaxon (30µg)	2.8	R	4.0	2.3
Chloramphenicol (25µg)	2.4	2.7	3.6	1.6
Chloramphenicol (30µg)	2.2	2.2	3.3	2.2
Ciprofloxacin (5µg)	2.1	3.4	3.2	2.6
Cotrimoxazole (30µg)	2.4	2.6	3.5	2.7
Gentamicin (10µg)	2.5	3.0	3.1	1.9
Netillin (30µg)	2.6	R	4.2	3.0
Ofloxacin (5 µg)	2.8	1.2	2.7	2.8
Penicillin (10U)	4.2	2.1	3.5	1.8
Penicillin G (1U)	2.5	R	3.0	R
Piperacillin (100 µg)	2.6	1.6	2.0	1.9
Streptomycin (10µg)	R	1.8	3.5	2.7
Sulphatriad (30µg)	3.5	2.9	3.0	2.2
Teicoplanin (30µg)	2.4	R	R	3.0
Tetracycline (30µg)	2.4	3.7	1.9	3.0
Tetracycline (25µg)	2.0	2.0	R	2.4
Vancomycin (30µg)	2.3	1.0	3.7	2.8

Size of zone of inhibition is given in centimeter, R=Resistant.

The isolates APP21, APP32, APP41 and APP42 were identified as *Bacillus sphaericus*, *Staphylococcus auricularis*, *Microbacterium* sp. and *Kurthia* sp. respectively based on their morphological and microscopic characters, sugar utilization pattern, enzyme profile and antibiotic susceptibility pattern (Table 1 to 5). In our previous studies, the isolates APP1, APP2, APP3 and APP4 were identified as *Bacillus alvei*, *Bacillus brevis*, *Bacillus sterothermophilus* and *Bacillus subtilis* respectively (**Pathak and Rathod, 2014**).APP6 was previously identified as *Bacillus firmus* (**Pathak *et al*., 2014c**). The isolates APP7, APP8, APP9, APP10, APP11, APP12 and APP14 were previously identified as *Bacillus licheniformis*, *Bacillus megaterium*, *Actinobacillus hominis*, *Lysinibacillus sphaericus*, *Paenibacillus alvei*, *Bacillus simplex* and *Pseudomonas fragii* respectively (**Pathak and Rathod, 2015**). The isolates APP18, APP27 and APP40 were previously identified as *Bacillus cereus*, *Bacillus stearothermophilus* and *Microbacterium* sp. respectively and communicated in a journal. The isolate APP19 was previously identified as *Bacillus atrophaeus* (**Pathak *et al*., 2014b**). The isolates APP28, APP30, APP38 and APP39 were previously identified as *Bacillus brevis*, *Pseudomonas oleovorans*, *Bacillus aminovorans* and *Bacillus firmus* and published in a journal (**Pathak and Rathod, 2015a**). The isolate APP36 was previously identified as *Bacillus subtilis* **(Pathak and Rathod, 2015b**).

Thermostable protease production

The data regarding total activity, total protein content, specific activity, purification fold and yield of partially purified proteases from selected isolates are given in Table 6. Maximum specific activity of partially purified thermostable protease was recorded from *Bacillus sphaericus* APP21 (447.75 U/mg) followed by *Staphylococcus auricularis* APP32, *Microbacterium* sp. APP41, *Kurthia* sp. APP42, *Bacillus atrophaeus* APP19, *Bacillus stearothermophilus* APP27, *Bacillus brevis*APP28, *Bacillus cereus* APP18, *Bacillus aminovorans* APP38, *Bacillus simplex* APP12, *Bacillus subtilis* APP36, *Paenibacillus alvei* APP11, *Bacillus subtilis* APP4, *Bacillus firmus* APP6, *Bacillus licheniformis* APP7, *Bacillus brevis* APP2, *Bacillus sterothermophilus* APP3, *Bacillus firmus* APP39, *Actinobacillus hominis* APP9, *Microbacterium* sp. APP40, *Pseudomonas fragii*APP14, *Bacillus megaterium* APP8, *Lysinibacillus sphaericus* APP10 and *Bacillus alvei* APP1.

Table 6 Thermostable protease production, partial purification and yield obtained from selected isolates from Unkeshwar hot spring, Maharashtra

	Partially purified protease				
Thermostable protease producers↓	Total activity (Units/mL)	Total protein (mg/mL)	Specific activity (U/mg protein)	Purification (fold)	Yield (%)
Bacillus sphaericus APP21	1298.5	2.9	447.75	2.6	76
Staphylococcus auricularis APP32	646.33	1.45	445.74	2.9	89
Microbacterium sp. APP41	875.76	2	437.88	2.2	72
Kurthia sp. APP42	512.67	1.6	320.41	2.5	71
Bacillus firmus APP6	756.345	7.5	100.85	2.23	28
Bacillus aminovorans APP38	923.45	6.4	144.29	2.14	52
Bacillus brevis APP2	656.75	7.3	89.97	2.5	37
Bacillus subtilis APP36	523.24	4.0	130.81	2.54	62
Bacillus megaterium APP8	240.45	5.8	41.46	2.0	75
Lysinibacillus sphaericus APP10	105.64	2.8	37.73	1.34	47

*Bacillus brevis*APP28	795.89	4.7	169.34	2.63	62
*Pseudomonas fragii*APP14	346.65	7.4	46.84	1.76	65
Bacillus firmus APP39	235.65	3.5	67.33	2.76	84
Bacillus atrophaeus APP19	498.67	1.6	311.66	2.8	71
Microbacterium sp.APP40	235.87	3.8	62.07	2.75	74
Bacillus simplex APP12	466.87	3.5	133.39	1.08	46
Bacillus licheniformis APP7	346.75	3.8	91.25	1.09	85
Bacillus alvei APP1	234.00	7.4	31.62	1.56	45
Bacillus sterothermophilus APP3	754.63	9.4	80.28	1.45	58
Actinobacillus hominis APP9	235.76	3.7	63.72	1.76	37
Bacillus cereus APP18	302.56	1.9	159.24	2.65	73
Bacillus subtilis APP4	664.53	6.2	107.18	1.67	56
Paenibacillus alvei APP11	563.20	4.5	125.16	1.65	27
Bacillus stearothermophilus APP27	357.8	2	178.9	2	68

Molecular weight determination

Molecular weight of partially purified thermostable proteases from *Bacillus sphaericus* APP21, *Staphylococcus auricularis* APP32, *Microbacterium* sp. APP41, and *Kurthia* sp. APP42 were determined as approximately 29 KDa, 14.3 KDa, 19 KDa and 20.1 KDa respectively (Figure 2). Anandharaj et al. (2016) isolated thermostable protease producer *Bacillus alkalitelluris* TWI3, from tannery waste and reported 42.6 kDa molecular weight of TWI3 protease by SDS-PAGE. Ansari et al. (2015) isolated a thermophilic bacterium *Aneurinibacillus thermoaerophilus* MCW220 from Manikaran hot spring, Himachal pradesh (India). MCW220 thermostable protease showed optimum pH 9 and temperature 50 °C. Molecular weight of MCW220 protease was 45 kDa, that is comparatively greater than the molecular weights of proteases from APP21, APP32, APP41 and APP42. Yildirim et al. (2017) reported 38.55 kDa molecular weight of thermostable protease from *Aeribacillus pallidus.*

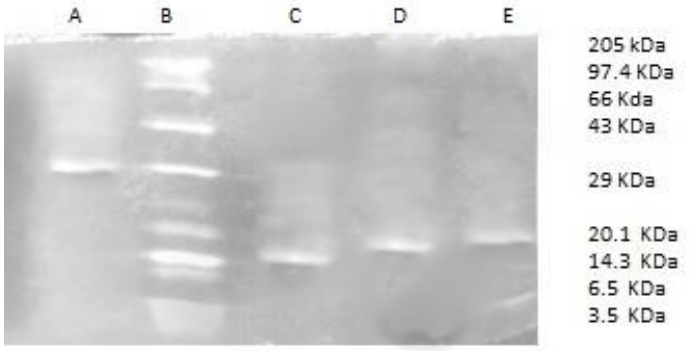

Figure 2 Determination of molecular weightsof partially purified thermostable proteases from selected isolates from Unkeshwar hot spring as revealed by 12 % SDS-PAGE

Lane A= Partially purified thermostable protease from *Bacillus sphaericus* APP21 (29 KDa)
Lane B= Protein broad range molecular weight markers(GeNei™ Bioscience catalogue no. PMWB 623110475001730)
Lane C= Partially purified thermostable protease from *Staphylococcus auricularis* APP32 (14.3 KDa)
Lane D=Partially purified thermostable protease from *Microbacterium* sp. APP41 (Approx. 19 KDa)
Lane E=Partially purified thermostable protease from *Kurthia* sp. APP42 (20.1 KDa)

Effect of temperature and pH on catalytic efficiency of proteases

Maximum catalytic efficiency of partially purified proteases from *Staphylococcus auricularis* APP32 and *Kurthia* sp. APP42 was found at temperature 55 °C. Maximum catalytic efficiency of partially purified proteases from *Bacillus sphaericus* APP21 was found at temperature 65 °C. Maximum catalytic efficiency of partially purified proteases from *Microbacterium* sp. APP41 was found at temperature 45 °C (Figure 3). Selim (2015) reported thermostable alkaline protease production from *Bacillus aryabhattai* J4. This protease exhibited optimum temperature 60 °C and showed stability at a broad pH range 6-10. Antranikian and Klingeberg (1991) published a patent on thermostable protease from *Thermococcus celer*. This protease showed optimum catalytic activity at temperature range 75-100 °C. Dammak et al. (2016) reported optimum temperature and pH of thermostable protease from *Halorubrum ezzemulense* strain ETR14 as 9 and 60 °C respectively.

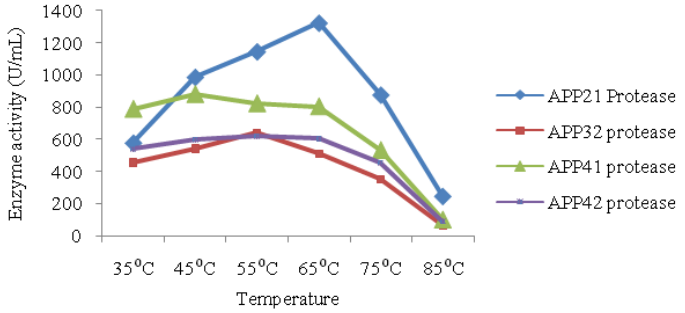

Figure 3 Effect of temperature on catalytic activity of protease

Partially purified proteases from APP21, APP32, APP41 and APP42 have showed remarkable catalytic activity at alkaline pH. Partially purified protease from *Staphylococcus auricularis* APP32 has showed maximum catalytic efficiency (1056 U/mL) at pH 8. Partially purified protease from *Bacillus sphaericus* APP21 has showed maximum catalytic efficiency (1543 U/mL) at pH 9. Maximum catalytic efficiency of partially purified proteases, 880 U/mL and 620 U/mL, respectively from *Microbacterium* sp. APP41 and *Kurthia* sp. APP42 was found at the pH value 7.0 (Figure 4).

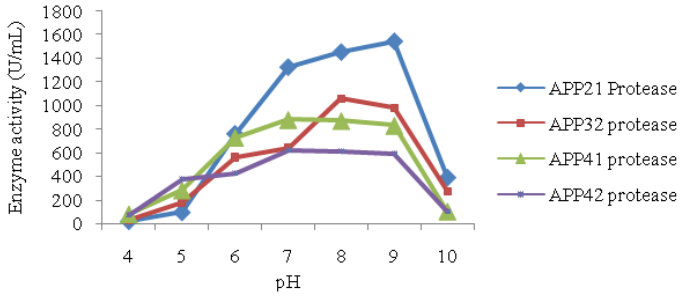

Figure 4 Effect of pH on catalytic activity of protease

Stability studies of proteases at pre-determined optimum temperature and pH values

Partially purified proteases from *Bacillus sphaericus* APP21, *Staphylococcus auricularis* APP32, *Microbacterium* sp. APP41,and *Kurthia* sp. APP42 exhibited greater than 50 % of their remaining activity even after 4 h of incubation period at pre-determined optimum temperature and pH values (Figure 5). Partially purified protease from *Bacillus sphaericus* APP21 exhibited 70.84, 63.71, 56.51, 50.55, 6.55 and 4.21 % of residual activity after 1, 2, 3, 4, 5 and 6 h of pre-incubation period at pH 9.0 and temperature 65 °C respectively. Partially purified protease from *Staphylococcus auricularis* APP32 exhibited 95.83, 92.99, 88.64, 77.75, 27.56 and 8.24 % of residual activity after 1, 2, 3, 4, 5 and 6 h of pre-incubation period at pH 8.0 and temperature 55 °C respectively. Partially purified proteases from *Microbacterium* sp. APP41 exhibited 94.77, 93.18, 91.14, 83.18, 65.23, and 23.07 % of residual activity after 1, 2, 3, 4, 5 and 6 h of pre-incubation period at pH 7.0 and temperature 45 °C respectively. Partially purified proteases from *Kurthia* sp. APP42 exhibited 98.71, 94.19, 70.48, 63.23, 16.45 and 7.26 % of residual activity after 1, 2, 3, 4, 5 and 6 h of pre-incubation period at pH 7.0 and temperature 55 °C respectively (Figure 5). Jordan and Lin (1993) published a patent on thermostable acid protease, thermopsin, from *Sulfolobus acidocaldarius*. This protease exhibited thermostable activity at pH range 1-11 and at temperatures between 0 to 100 °C.

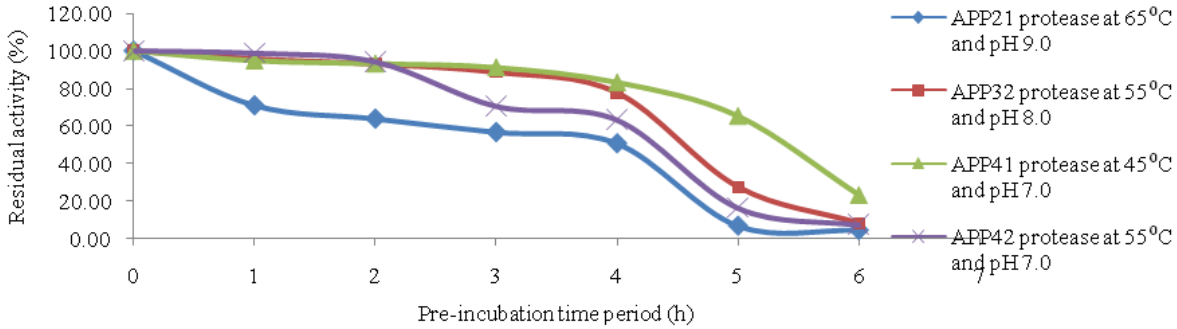

Figure 5 Stability of proteases at pre-determined optimum temperature and pH values

Effect of different casein concentration on protease activity and determination of Km and Vmax values

Partially purified proteases from *Bacillus sphaericus* APP21, *Staphylococcus auricularis* APP32, *Microbacterium* sp. APP41 and *Kurthia* sp. APP42 have showed maximum proteolytic activity at 10, 20, 15 and 25 mg/mL of casein concentrations respectively. The Lineweaver-Burk plots have been reflected in Figure 6 to Figure 9 and Km and Vmax values of these proteases have been given in Table 7. **Thebti et al., 2016** reported Km and Vmax values of thermostable protease from *Geobacillus toebii* strain LBT 77 as 1 mg/mL and 217.5 U/mL respectively.

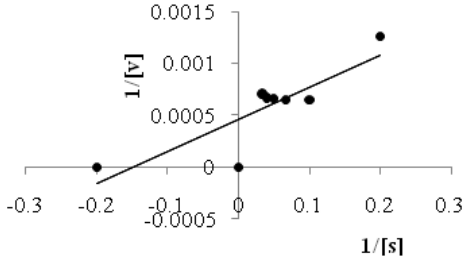

Figure 6 Lineweaver Burk plot for *Bacillus sphaericus* APP21 protease

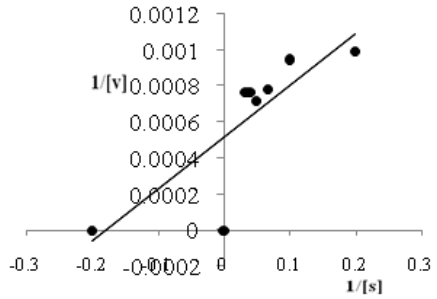

Figure 7 Lineweaver Burk plot for *Staphylococcus auricularis* APP32 protease

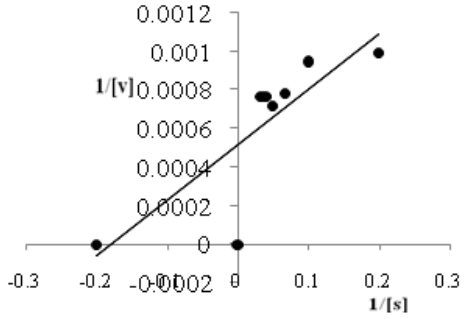

Figure 8 Lineweaver Burk plot for *Microbacterium* sp. APP41 protease

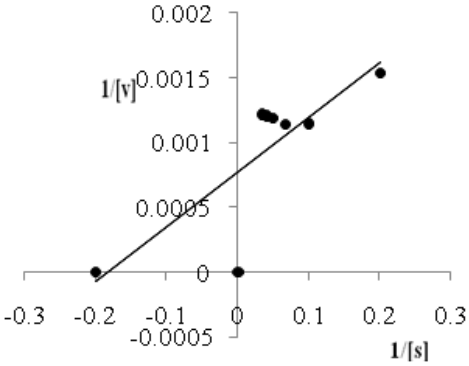

Figure 9 Lineweaver Burk plot for *Kurthia* sp. APP42 protease

Table 7 Optimum casein concentration for protease activity and Km and Vmax values of thermostable proteases from microbial isolates from Unkeshwar hot spring

Thermostable proteases from Unkeshwar hot spring isolates	Optimum casein concentration for protease activity (mg/mL)	Vmax (U/mg)	Km (mg/mL)
Bacillus sphaericus APP21	10	766.28	6.67
Staphylococcus auricularis APP32	20	1379.31	5.56
Microbacterium sp. APP41	15	735.29	5.26
Kurthia sp. APP42	25	446.42	8.34

Effect of inducers and inhibitors on catalytic efficiency of partially purified protease

Residual activities of partially purified proteases from *Bacillus sphaericus* APP21, *Staphylococcus auricularis* APP32, *Microbacterium* sp. APP41 and *Kurthia* sp. APP42 in presence of selected metal ions have been given in Table 8. Catalytic activity of partially purified protease from *Bacillus sphaericus* APP21was enhanced by 20, 5, 25 and 6 % in presence of Mg^{2+}, Ca^{2+}, Ba^{2+} and Na^{+} cations respectively. Catalytic activity of partially purified protease from *Staphylococcus auricularis* APP32 was enhanced by 30, 10, 11, 4 and 9 % in presence of Mg^{2+}, Ca^{2+}, Ba^{2+}, K^{+} and Mn^{2+} cations respectively. Catalytic activity of partially purified protease from *Microbacterium* sp. APP41 was enhanced by 25, 7, 4, 1 and 5 % in presence of Mg^{2+}, Ca^{2+}, Ba^{2+}, Na^{+} and Mn^{2+} cations respectively. Catalytic activity of partially purified protease from *Kurthia* sp. APP42 was enhanced by 33 and 7 % in presence of Mg^{2+} and Mn^{2+} cations respectively. **Thebti et al., 2016** reported that, catalytic activity of thermostable, haloalkaline, solvent stable, and detergent compatible serine protease from *Geobacillus toebii* Strain LBT 77 was enhanced in presence of Mg^{2+} and Ca^{2+} cations.

Table 8 Residual activities of partially purified proteases in presence of selected metal ions

Metal ions (1 %)	*Bacillus sphaericus* APP21 protease	*Staphylococcus auricularis* APP32 protease	*Microbacterium* sp. APP41 protease	*Kurthia* sp. APP42 protease
Fe^{3+}	88	90	81	70
Mg^{2+}	120	130	125	133
Cu^{2+}	80	82	86	70
Ca^{2+}	105	110	107	100
Zn^{2+}	92	98	91	100
Ba^{2+}	125	111	104	90
Hg^{2+}	80	88	90	92
Ag^{3+}	90	92	94	88
K^{+}	100	104	97	100
Na^{+}	106	100	101	98
Mn^{2+}	110	109	105	107
Control	100	100	100	100

Effect of Effect of inducers and inhibitors on catalytic efficiency of partially purified protease

Catalytic activity of partially purified proteases from *Bacillus sphaericus* APP21, *Staphylococcus auricularis* APP32, *Microbacterium* sp. APP41 and *Kurthia* sp. APP42 was completely inhibited in presence of 5 mM phenyl methyl sulphonyl fluoride, which indicates that these proteases belong to the class serine proteases. These proteases have showed remarkable catalytic efficiency in presence of selected solvents and surfactants. Residual activities of these partially purified proteases in presence of selected activators and inhibitors have been presented in Table 9.

Table 9 Residual activities of partially purified proteases in presence of selected activators and inhibitors

Solvents (1%)	*Bacillus sphaericus* APP21protease	*Staphylococcus auricularis* APP32 protease	*Microbacterium* sp. APP41 protease	*Kurthia* sp. APP42 protease
Chloroform	68	69	63	73
Ethanol	96	94	73	75
Butanol	86	84	83	86
Hexane	94	64	96	92
Heptane	86	88	89	93
Tolune	83	86	82	88
Triton X-100	98	94	93	95
Acetonitrile	75	78	85	75
Benzene	84	86	88	82
Glycerol	94	92	99	100
Isopropanol	91	92	83	84
Na2EDTA (1 %)	90	94	98	100
Hydrogen peroxide (1 %)	88	84	97	89
Sodium dodecyl sulphate (1 %)	98	99	97	100
2-Mercaptoethanol (5 mM)	87	90	92	91
PMSF (5 mM)	0	0	0	0
Tween 20 (1 %)	102	104	106	100
Tween 80 (1 %)	98	96	97	95
Control	100	100	100	100

Compatibility of partially purified proteases with detergents

Residual activities of partially purified proteases from *Bacillus sphaericus* APP21, *Staphylococcus auricularis* APP32, *Microbacterium* sp. APP41 and *Kurthia* sp. APP42 in presence of selected activators and inhibitors have been presented in Table 10. These proteases have showed remarkable catalytic efficiencies in presence of all selected detergents. Partially purified protease from *Bacillus sphaericus* APP21 has showed maximum 100 % compatibility with Ariel® and Nirma®. Partially purified protease from *Staphylococcus auricularis* APP32 has showed maximum 98 % compatibility with Vim®. Partially purified protease from *Microbacterium* sp. APP41 and *Kurthia* sp. APP42 have showed maximum 99 % compatibility with Nirma® and Surf excel® respectively.

Table 10 Residual activities of partially purified proteases in presence of selected commercial detergents

Detergents (7 mg/mL)	*Bacillus sphaericus* APP21protease	*Staphylococcus auricularis* APP32protease	*Microbacterium* sp. APP41protease	*Kurthia* sp. APP42protease
Ariel®	100	97	91	93
Tide®	97	93	94	94
Rin ®	98	91	95	92
Wheel®	94	80	93	94
Surf excel®	99	93	94	99
Nirma®	100	92	99	93
Ghadi®	93	95	92	94
Sasa®	95	92	91	92
Vim®	92	98	93	89
Control	100	100	100	100

CONCLUSIONS

Efficient thermostable protease producers were isolated from Unkeshwar hot spring of Maharashtra and identified as *Bacillus sphaericus* APP21, *Staphylococcus auricularis* APP32, *Microbacterium* sp. APP41 and *Kurthia* sp. APP42. Partially purified proteases from these isolates exhibited excellent thermostability above 45 ^{o}C temperature even after 4 h of pre-incubation period. These proteases were catalytically active at neutral to alkaline range of pH. These proteases have showed remarkable efficiency in presence of selected metal ions, solvents, surfactants and detergents. These proteases can be used in detergent formulation, food processing, peptide synthesis in presence of solvents and where proteolysis at elevated range of temperature is essential. The thermostable proteases producers from Unkeshwar hot spring can be exploited for production of many other thermostable enzymes beside protease

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