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A report on thermostable alkaline protease producing bacteria from a terrestrial thermal spring

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Five efficient protease producing moderate thermophiles were isolated from Unkeshwar (19°85' N and 78°25'E) thermal spring located in Nanded district of Maharashtra. The isolates were identified as *Bacillus firmus, Bacillus aminivorans, Bacillus sterothermophilus, Bacillus brevis* and *Pseudomonas oleovorans* using biochemical techniques. Being an efficient protease producer, *Pseudomonas oleovorans* was selected and further identified using 16s rRNA molecular analysis method as 'Uncultured bacterium clone APP30' and sequence was deposited in genebank nucleotide repository under accession number KC733812. The protease extracted and partially purified from APP30 showed remarkable caseinolytic activity at 55° C temperature and pH 9.0. The extracted protease also showed stability in presence of Chloroform, Ethanol, Butanol, Hexane, Toluene and Triton X-100 at 1 % (v/v) concentration and compatibility with different commercial detergents. Efficient destaining of blood stained cotton cloth piece was also recorded by partially purified protease. Enhanced proteolytic activity was observed in presence of $CaCl_2$, $MgSO_4$, KCl and NaCl at 1 % (w/v) concentration. Characterization of partially purified protease was carried out. Molecular weight of partially purified protease was determined as 28 kda.

[**Keywords:** Unkeshwar thermal spring, 16S rRNA gene sequencing, Thermostable alkaline protease, MEGA 6.06]

Introduction

Microbial proteases (peptidyl-peptide hydrolases, EC: 3.4.11-19), representing one among the three largest groups of industrial enzymes, account for 65 % of total worldwide enzyme sales[1]. Extensive use of proteases in detergent, laundry additives, leather finishing, silk industry, feeds modification, food processing, brewing, pharmaceuticals, diagnostic reagents, peptide synthesis, silver recovery from X-ray/photographic film, preparation of organic fertilizer and waste treatment was reported by many groups[2]. However, their applications in detergent formulations as a sole and key ingredient make up 89 % of total protease sales[3]. Majority of proteases used to date are obtained from mesophilic microorganisms and have restricted applications. Unlike these the proteases originated from thermophilic and hyperthermophilic sources have a number of commercial applications due to their inherent overall stability[4]. Thermostable proteases are gaining wide industrial and biotechnological interest in various industrial operations since the elevated processing temperature generally increases the rate of reaction by changing broth rheology[5].

In this context, we have explored an ancient thermal spring located at Unkeshwar in the Nanded district of Maharashtra state of India (19°85' N and 78°25'E) for isolation of thermostable protease producers (Figure 1). The spring is frequently visited by people of Maharashtra, Karnataka and Andhra Pradesh states of India. The spring water is believed to have curative properties especially against skin diseases. Various thermostable protease producers were isolated and characterized from this spring. Protease extracted from selected isolate was thoroughly assessed for optimization. Compatibility of same in various applications was also studied.

Materials and Methods

Water samples of Unkeshwar thermal spring were collected in pre-sterilized plastic containers and transported to laboratory within 24 h.

Fig.1—Unkeshwar terrestrial thermal spring, Nanded, Maharashtra, India

Samples were spread on six media namely nutrient agar, tryptone yeast glucose agar, tryptone yeast extract agar, Vogel Johnson Agar, glucose sodium azide glycerol agar and thiosulphate agar plates and incubated at 50° C

for 24 h[6]. Amongst these, the medium that showed highest diversity was used in consequent experiments. After incubation, isolated colonies were selected for further experiments. Selected five distinct isolates were inoculated on skimmed milk agar plates individually and incubated at 50° C for 48 h to confirm protease production[7]. Selected isolates were further identified using morphological and biochemical characters. Catalase, oxidase, indole, H_2S production and citrate utilization tests were carried out. Hydrolysis of gelatine, casein, starch, cellulose, urea, pectin and tween-80 was observed. Methyl red, Vogues-Proskauer, carbohydrate utilization tests were performed using standard procedures[8,9]. Appropriate positive and negative controls were used in all these tests. Antibiotic susceptibility was tested by disc diffusion method[10]. Effect of temperature on growth of organisms was determined by varying temperature in the range of 25 to 75° C with an increment of 10° C. Optimization of pH for growth was carried out by growing organisms in media having different pH in the range 4 to 10 with an increment of 1 unit. Optimum incubation period was determined by incubating cultures up to 96 h. Optimum NaCl concentration required for growth was determined by inoculating cultures in media having 0 to 5 % NaCl[11,12&13].

Being an efficient protease producer, APP30 was further used for protease production. Identification of the same was confirmed using 16s rRNA partial gene sequencing method[14]. In this method DNA was isolated from bacteria using cell lyses method and 16s rDNA was amplified by Thermocycler using the following pair of primers: (530F) GTGCCAGCAGCCGCGG and (1392R) ACGGGCGGTGTGTAC. The amplified 16s rDNA PCR product was sequenced using automated sequencer. Sequence similarity search was done for the 16s rDNA sequence using online search tool called BLAST (http://www.ncbi.nlm.nih.gov/blast). Isolate was identified using maximum aligned sequence through BLAST search. Phylogenetic tree was constructed from evolutionary distances using the MEGA 6.06 software[15,16,17].

Casein yeast extract medium was used for production of protease. Effect of temperature, pH and incubation period on production of protease was also assessed[18,19]. Fermented broth was centrifuged at 10,000 rpm for 10 min at 4° C. The cell free supernatants were collected. Solid ammonium sulphate (60 %) was added in supernatant. Enzyme precipitate were collected and dialyzed against 0.2 M phosphate buffer (pH 8.0). Dialyzed samples were used as partially purified enzyme. Estimation of partially purified protease at predetermined pH, temperature and substrate concentration was carried out by using Anson–Hagihara method[20,21]. Total protein content of partially purified protease was determined using BSA as standard.

Molecular weight of partially purified protease was determined after comparison its weight with the standard broad-range protein markers (Merck Biosciences, India) in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)[22,23]. Partially purified protease was characterized to optimize the parameters like temperature, pH and substrate concentration[22,23]. Effect of inducers and inhibitors on catalytic efficiency of partially purified protease was determined using 1 % metal solution viz. $FeSO_4$, $MgSO_4$, $CuCl_2$, $CuSO_4$, $CaCl_2$, $ZnSO_4$, $BaCl_2$, $HgCl_2$, $AgNO_3$, KCl, NaCl and $MnSO_4$[22,23]. Effect of selected solvents (1 %) viz. chloroform, ethanol, butanol, hexane, heptane, toluene, triton X-100, acetonitrile, benzene, glycerol and isopropanol and other additives viz. dithiothrietol (5 mM), Na_2-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 protease[22,23]. Compatibility of partially purified protease with commercial detergents was assessed using Ariel® (Procter and Gamble, Mumbai), Tide® (Procter and Gamble, Mumbai), Rin® (Hindustan Lever Ltd., India), Wheel® (Hindustan Unilever 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[22,23,24]. Blood stain removal capacity of partially purified protease was assessed. In this experiment, Hen blood was applied on white coloured cotton cloth pieces (5 × 5 cm) and blood stain was dried at 60° C and fixed with 1 % (v/v) formaldehyde[25]. The stained cloth pieces were immersed in distilled

water, detergent solution (Rin® 7 mg/mL (w/v) in tap water) and Rin® detergent fortified with partially purified protease (4300 Units) from APP30 strain for 30 minute at room temperature. After incubation each piece was rinsed with tap water for 2 minutes and then dried[25]. Decomposing efficiency of partially purified protease was evaluated using X-ray film. A piece of X-ray film of 2 × 5 cm size was incubated with 20 mL of partially purified protease (8600 Units) at 35° C and 50 rpm in orbital shaking incubator. This film was observed for decomposition of gelatinous coating after 8, 16 and 24 hours of incubation period[25].

Results and Discussion

The water samples from Unkeshwar thermal spring were neutral in pH, clean and odourless. The temperature of water sample was recorded 48° C. Inoculation of fresh samples has yielded remarkable growth of morphologically distinct colonies on nutrient agar. Amongst the six different media, nutrient agar yielded diverse morphotypes. CFU appeared on nutrient agar were 70. Out of these five isolates have developed zone of clearance indicating positive protease production. These isolates were designated as APP28, APP29, APP30, APP38 and APP39.

On the basis of morphological characters, microscopic features, sugars utilization patterns, enzyme profile, antibiotic sensitivity and

Table 1: Morphological characters of bacterial isolates from Unkeshwar thermal spring, India

Characters	*Bacillus brevis* (APP28)	*Bacillus sterothermophilus* (APP29)	*Pseudomonas oleovorans* (APP30)	*Bacillus aminivorans* (APP38)	*Bacillus firmus* (APP39)
Shape	Round	Irregular	Round	Rhizoid	Rhizoid
Size (mm)	2.2	2.9	3.1	3.4	2.7
Margin	Irregular	Filamentous	Entire	Fimbriate	Filamentous
Elevation	Raised	Semi-raised	Semi-Raised	Flat	Flat
Consistency	Semi-sticky	Brittle	Semi-sticky	Dry	Sticky
Density	Opaque	Opaque	Opaque	Opaque	Opaque
Surface	Rough	Rough	Smooth	Dull	Rough
Color	Dirty white	Dirty white	Apple white	Blue bell white	Pale white

Table 2: Microscopic features of bacterial isolates from Unkeshwar thermal spring, India

Characters	*Bacillus brevis* (APP28)	*Bacillus sterothermophilus* (APP29)	*Pseudomonas oleovorans* (APP30)	*Bacillus aminivorans* (APP38)	*Bacillus firmus* (APP39)
Cell shape	Rod	Rod	Thick Rod	Straight rod	Rod
Cell length (µm)	2.0	1.0	1.0	1.0	1.5
Cell width (µm)	0.5	0.5	0.5	0.5	0.5
Sporulation	+	+	-	+	+
Position of spore	T	T	NA	T	T
Motility	M	M	M	M	M
Gram stain	P	P	N	P	P
Flagellar arrangement	PF	PF	PF	PF	PF

Abbreviations: PF: Peritrichous flagella, **T**: Terminal, **M**: Motile, +: Spore former, -: Non spore former, **NA**: Not applicable, **P**: Positive, **N**: Negative

Table 3: Biochemical characteristics of bacterial isolates from Unkeshwar thermal spring, India

Tests	*Bacillus brevis* (APP28)	*Bacillus sterothermophilus* (APP29)	*Pseudomonas oleovorans* (APP30)	*Bacillus aminivorans* (APP38)	*Bacillus firmus* (APP39)
Dextrose	+	+	+	+	+
Fructose	+	+	+	+	+
Lactose	+	-	-	+	+
Sucrose	-	-	-	-	-
Mannitol	-	-	-	-	-
Maltose	+	-	-	-	-
Xylose	-	+	-	+	+
Arabinose	-	-	-	-	-
Galactose	-	+	-	+	-
Glycerol	-	-	-	+	-
Cellobiose	-	-	-	+	-
Sorbitol	-	-	-	-	-
Mellibiose	-	-	-	-	-
Mannose	+	+	+	+	-
Trehalose	+	-	-	+	-
Ribose	+	+	+	+	+
Salicin	-	-	-	+	+
Rhamnose	+	-	-	-	-
Inulin	-	-	-	-	-
Adonitol	-	-	-	-	-
Raffinose	-	-	-	-	-
Dulcitol	-	+	-	+	-
Gas and H_2S production	-	-	-	-	-
Indole test	-	-	-	-	-
MR test	-	-	-		-
VP test	-	-	-	-	-
Citrate test	+	+	-	+	-

Abbreviations: +: acid production or positive test, **-:** No acid production or negative test

physiological attributes, isolates were identified as *Bacillus brevis* (APP28), *Bacillus sterothermophilus* (APP29), *Pseudomonas oleovorans* (APP30), *Bacillus aminivorans* (APP38) and *Bacillus firmus* (APP39) (Table 1 - 6). All the isolates characterized and identified here are first time reported from Unkeshwar hot water spring. Out of these five, a fast growing and efficient thermostable protease producer APP30 strain was selected for further analysis. In our previous work, microbial diversity of Unkeshwar thermal spring was reported and efficient thermostable amylase, lipase, gelatinase and protease producers were isolated and characterized.

Table 4: Evaluation of enzymatic profile of bacterial isolates from Unkeshwar thermal spring, India

Enzymes	*Bacillus brevis* (APP28)	*Bacillus sterothermophilus* (APP29)	*Pseudomonas oleovorans* (APP30)	*Bacillus aminivorans* (APP38)	*Bacillus firmus* (APP39)
Catalase	+	+	+	+	+
Oxidase	+	+	-	-	-
protease	+	+	++	+	+
Amylase	+	+	-	+	+
Cellulase	-	-	-	-	-
Urease	w+	+	-	+	-
Lipase	+	-	+	-	-
Pectinase	w+	-	-	-	-

Abbreviations: w+**:** weakly positive**, +:** Positive test**,** **-:** Negative test

Table 5: Physiological attributes of bacterial isolates from Unkeshwar thermal spring, India

Parameters tested	*Bacillus brevis* (APP28)	*Bacillus sterothermophilus* (APP29)	*Pseudomonas oleovorans* (APP30)	*Bacillus aminivorans* (APP38)	*Bacillus firmus* (APP39)
Temperature growth range (^oC)	25-65	25-65	25-55	25-65	25-65
Optimum temperature (^oC)	45	45	35	45	45
pH growth range	4-8	5-8	5-9	6-8	5-8
Optimum pH	7	7	8	7	6
NaCl required for optimum growth	NR	NR	NR	NR	NR
NaCl tolerance (%)	0-4	0-4	0-4	0-3	0-2
Optimum growth period (h)	72	72	48	48	48

Abbreviation: NR: Not required

In our previous work, we have reported presence of diverse morphotypes viz. *Paenibacillus alvei* (APP1), *Bacillus brevis* (APP2), *Bacillus sterothermophilus* (APP3), *Bacillus subtilis* (APP4), *Bacillus farraginis* (APP5)[26], *Bacillus firmus*(APP6)[27], *Bacillus licheniformis* (APP7)[28], *Bacillus megaterium* (APP8)[28], *Actinobacillus hominis* (APP9)[28], *Lysinibacillus sphaericus* (APP10)[28], *Paenibacillus alvei* (APP11)[28], *Bacillus simplex* (APP12)[28], *Actinobacillus seminis* (APP13)[28], *Pseudomonas fragii* (APP14)[28], *Staphylococcus cohnii* (APP15)[28], *Streptococcus thermophilus* (APP16)[28], *Bacillus subtilis* UCTA8[29], *Fusarium* sp. CAT3[30], *Bacillus atrophaeus* (APP19)[31] and *Microbacterium* sp. (APP39)[32] in Unkeshwar thermal spring and these isolates were characterized[33].

Table 6: Evaluation of antibiotic sensitivity pattern of bacterial isolates from Unkeshwar thermal spring, India for their identification

Antibiotic discs	*Bacillus brevis* (APP28)	*Bacillus sterothermophilus* (APP29)	*P. oleovorans* (APP30)	*B. aminivorans* (APP38)	*Bacillus firmus* (APP39)
Amikacin (30 µg)	1.1	2.2	1.4	2.2	2.2
Amoxycillin (10 µg)	2.5	2.6	3.4	3.3	2.5
Ampicillin (10 µg)	2.9	R	R	1.3	R
Cefoperazone (75 µg)	2.7	1.9	3.2	3.2	2.2
Cefoxitin (30 µg)	2.4	3.2	1.5	2.6	3.8
Ceftazidime (30 µg)	1.5	2.4	3.3	2.6	1.9
Ceftriaxon (30 µg)	1.4	2.5	3.0	1.4	2.4
Chloramphenicol (25 µg)	2.3	2.3	R	1.5	3.2
Chloramphenicol (30 µg)	2.4	2.5	R	3.2	3.3
Ciprofloxacin (5 µg)	3.2	2.5	3.2	2.1	2.2
Cotrimoxazole (30 µg)	2.9	2.3	1.4	3.9	2.4
Gentamicin (10 µg)	2.5	3.5	2.4	3.6	2.5
Netillin (30 µg)	3.5	2.4	3.5	2.5	2.4
Ofloxacin (5 µg)	1.3	2.3	3.2	2.5	2.4
Penicillin (10 U)	2.3	R	2.4	2.1	1.5
Penicillin G (1 U)	1.2	1.5	1.3	1.5	1.4
Piperacillin (100 µg)	2.2	1.3	2.3	1.8	2.3
Streptomycin (10 µg)	2.4	2.3	2.5	2.3	3.3
Sulphatriad (30 µg)	1.4	1.3	1.1	1.2	1.3
Teicoplanin (30 µg)	2.3	2.3	2.6	2.1	1.9
Tetracycline (30 µg)	4.0	3.4	3.2	2.9	3.0
Tetracycline (25 µg)	3.5	2.8	3.1	2.3	2.1
Vancomycin (30 µg)	2.9	3.2	3.3	2.6	2.9

Abbreviations: R: Resistant; Size of zone is given in centimeters.

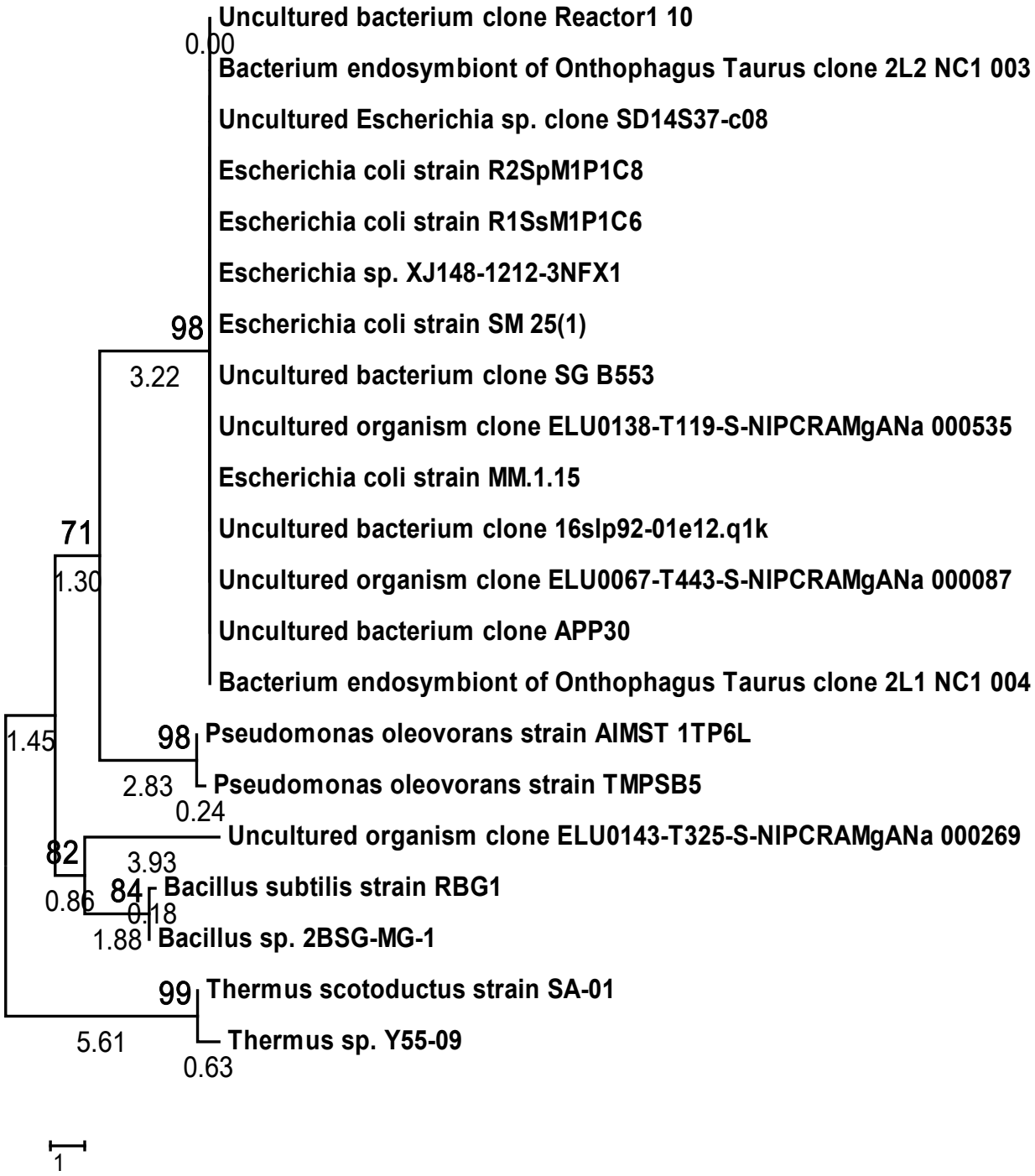

Fig.2— Phylogenetic tree (evolutionary relationship between selected taxa)

The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 21 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 236 positions in the final dataset. The optimal tree with the sum of branch length = 21.10745575 is shown. Evolutionary analyses were conducted in MEGA 6.06.

The PCR product was gel eluted, sequenced and the sequence data was submitted to NCBI Genebank under the accession number KC733812. The sequence data thus obtained was subjected to BLAST analysis. The sequence similarity analysis of 16s rDNA sequence showed its maximum identity of 99 % to 'Uncultured organism clone ELU0138-T119-S-NIPCRAMgANa_000465'.

The phylogenetic analysis of 16s rDNA sequence of the isolate along with the sequences retrieved from the NCBI was carried out with MEGA 6.06 using the neighbour-joining method with 1,000 bootstrap replicates. The result of phylogenetic analysis showed distinct clustering of the isolate and confirmed the results of the sequence similarity analysis (Figure 2).

APP30 produced 430.48 U/mL (dialysate) of protease at 50° C temperature and pH 8.0 after incubation of 72 h. The protein content of partially purified protease was determined as 725 µg/ml. The specific enzyme activity was calculated 593.76 U/mg. Optimum protease was produced at 50° C temperature, pH 8.0 and after 72 h incubation period.

SDS-PAGE analysis revealed that molecular weight of partially purified APP30 protease was 28 kda (Figure 3).

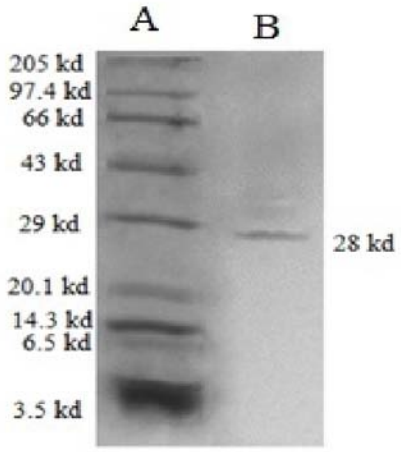

Fig.3— SDS–PAGE analysis of partially purified protease from APP30 strain; Lane A: Molecular weight markers (Merck Biosciences, India), Lane B: APP30 protease

be optimum for catalytic activity of protease. Partially purified protease showed remarkable stability from 45-55° C temperatures for 3 h. Catalytic efficiency of partially purified protease was reduced at 45, 55 and 65 ^{o}C after 6 h of incubation period. The partially purified protease retained 73.16, 44.11 and 18.61 % of its original activity after 3 h of incubation period at 45, 55 and 65 ^{o}C respectively. Partially purified protease lost 77.52 and 92.15 % of its initial activity at 45 and 55 ^{o}C temperatures respectively and completely lost its catalytic activity at 65 ^{o}C after 6 h of incubation period. An assay pH 9.0 was found to be optimum for catalytic activity of protease. Partially purified protease showed remarkable stability at pH 4 to 9 after 3 h of incubation period. Catalytic efficiency of partially purified protease was reduced after 6 h of incubation period at pH 4, 7, 9 and 11. The partially purified protease retained 90.62, 99.65, 49.82 and 28.31 % of its original activity after 3 h of incubation period at pH 4, 7, 9 and 11 respectively. Partially purified protease lost 77.34, 60.01, 83.31 and 92.07 % of its initial activity at pH 4, 7, 9 and 11 respectively after 6 h of incubation period. Optimum catalytic activity of partially purified protease was observed at 12 mg/mL of casein concentration. The calculated Km and Vmax values were 12.5 mg/mL and 6.79 µM/ mL/min respectively.

Fig.4— Blood stain washing performance analysis of partially protease from APP30 strain
A: Piece of cotton cloth washed with Rin® in tap water
B: Piece of cotton cloth washed with Rin® and APP30 protease in tap water

Fig.5— Degradation of gelatinous coating on X-ray film by APP30 thermostable alkaline protease
a: X-ray film before degradation of gelatineous coating
b: X-ray film after degradation of gelatineous coating

Catalytic activity of partially purified protease was enhanced in presence of Ca^{2+}, Mg^{2+}, K^{+} and Na^{+} by 21.62, 1.35, 1.08 and 0.67 % respectively. Residual activity of partially purified protease was determined in presence of Fe^{3+}, Cu^{2+}, Zn^{2+}, Ba^{2+}, Hg^{2+}, Ag^{3+}, K^{+} and Mn^{2+} as 40.54, 32.43, 36.48, 40.54, 40.54, 33.78, 99.99 and 28.37 % respectively. Residual activity determined in presence of chloroform, ethanol, butanol, hexane, heptane, toluene and triton X-100 was 60.81, 28.37, 28.64, 35.13, 56.75, 25.67 and 68.91 % respectively. Catalytic activity of partially purified protease was completely lost in presence of serine protease inhibitor-PMSF. Catalytic activity of partially purified protease was slightly enhanced in presence of surfactants tween-80 and tween-20 by 3.94 and 2.64 % respectively. Partially purified protease retained its original activity 25, 94.73, 78.94, 90.78 and 92.10 % in presence of DTT, Na_2-EDTA, H_2O_2, SDS and 2-mercaptoethanol respectively.

Relative enzyme activity of partially purified APP30 protease with various detergents was calculated as 48.32, 91.27, 13.42, 16.10, 57.71, 10.73, 53.69, 71.14 and 42.95 % with Ghadi®, Tide®, Surf excel®, Ariel®, Wheel®, Rin®, Nirma®, Sasa® and Vim® respectively at temperature 45° C and pH 8.0. Protease from APP30 in presence of Rin® had effectively removed blood stain from cotton cloth piece (Figure 4). Therefore protease from APP30 is recommended to be used as additive in laundry detergents. Protease from APP30 has completely decomposed the gelatinous coating on the piece of X-ray film after 24 h of incubation period (Figure 5).

Conclusions

The results obtained in present investigation mark the significance of efficient thermostable protease producer from Unkeshwar hot water spring, Maharashtra (India). Partially purified protease obtained here showed activity over a wide range of temperature 37 to 65° C. Maximum activity was recorded at 45° C; however 93.03, 93.67 and 53.16 % activity was recorded at 37, 55 and 65° C temperature respectively. Protease extracted here is catalytically active in alkaline pH and selected solvents, metal ions, detergents, surfactants, chelator, oxidizing and reducing agents. Thus APP30 protease may find the use in different industries and enzyme biotechnology field.

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