Pak. J. Biotechnol. 16 (1) 47-54 (2019) pISSN: 1812-1837 eISSN: 2312-7791 www.pjbt.org
http://doi.org/10.34016/pjbt.2019.16.1.8

PARTIAL PURIFICATION AND CHARACTERISATION OF COLD-ACTIVE METALLOPROTEASE BY *BACILLUS* SP. AP1 FROM APHARWAT PEAK, KASHMIR

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Article received 07.02.2019, Revised 20.03.2019, Accepted 24.03.2019

ABSTRACT

A gram-positive, rod shaped psychrotrophic and alkalotolerant bacterium, producing extracellular proteolytic enzyme was isolated from the peak of Apharwat, Kashmir. The strain was identified as *Bacillus* sp via 16S rDNA sequencing and was designated as *Bacillus* sp. AP1. Highest quantity of enzyme was secreted when strain was grown for 30 hours at 20ºC and pH 9.0. Glucose and skim milk were the best source of carbon and substrate respectively. The optimal activity of partially purified protease was recorded at pH 9.0, classifying the enzyme as alkaline protease. Similarly, the protease was found to be low temperature active with maximum enzyme activity at 20ºC. Strong inhibition of activity by EGTA and EDTA defines the enzyme as metalloprotease; among metal ions, Mn^{2+} enhanced enzyme activity. Finally, the washing test proved that enzyme could possibly be effective as an additive for cold washing purposes.

Keywords: *Bacillus* sp. AP1, Cold-active protease, Psychrotrophic, Alkalotolerant, Metallo protease.

INTRODUCTION

Mostly the environments which are exposed to cold are known to produce cold-loving microorganisms, termed as psychrotrophs and psychrophiles (Margesin 2009). Microbes tailor themselves to diverse places that direct advancement in their machinery at molecular level. To overcome harsh consequences of cold conditions, cold active microorganisms cultivate and nurture at comparable speed in contrast to narrowly associated species that growth in usual ecosystem (Furhan et al., 2019). Therefore, with the intent of survival, cold adapted microorganisms build up structural changes in their cellular and enzymatic processes slowly and carefully which helps them to reimburse for the harmful consequences of cold conditions. The cold-adapted microbes excrete enzymes that are cold-active in nature (Kuddus and Ramteke 2012).

The prospective of cold loving organisms their proteolytic enzymes have been evaluated at regular time intervals (Gerday et al., 2000; Cavicchioli et al., 2002; Collins et al., 2002). Cold-active alkalophilic proteases represent a vital kind of enzymes that show competence in their catalytic functions in cold conditions and represent an essential division of hydrolytic enzymes which are present within the entire forms of life. They are important for normal functioning, chemical transformations and biological processes (Gupta et al., 2002; Qureshi and Dahot 2009). Modern biotechnological industries are in need of polymers which can be capable of performing in the harsh conditions of cold and alkalinity. Cold active alkalophilic proteases are cost effective as they reduce the consumption of energy and can be interesting candidates for cold washing, bioremediation, food industry etc (Kasana 2010; Lashari et al., 2011; Chen et al., 2018). For that reason, more focus needs to be shifted on the study of proteolytic enzymes because of their effectiveness in industries. The purpose of this work intended to characterize the distinctive partially purified extracellular protease from psychrotrophic *Bacillus* sp. AP1.

MATERIALS AND METHODS

Chemicals: The entire chemicals used in the present work were of analytical grade and purchased from Hi-media Pvt. Ltd. (India) and SIGMA-ALDRICH (India).

Isolation of extracellular protease producing bacterial strain: For the isolation of cold-adapted bacterium, the glacier soil samples were collected from Apharwat Peak, positioned at a height of 4,390 meters (14,403 ft) above the sea level in Gulmarg Kashmir, India. The coordinates of the summit are 33°59′58″N 74°19′32″E, remains habitually layered by snow during the entire year. The soil sample in the quantity of 1g of was initially air dried, sieved and then serially diluted in sterile distilled water. Dilutions of 0.1ml were plated on alkaline skimmed milk agar media (pH 8-12) and incubated under cold conditions (0-30°C). Clear zone around colonies due to caesin hydrolysis indicated production of protease by microorganisms. For further study, one of the isolates making maximum clear zone diameter was chosen and designated as strain AP1. For additional purification, strain AP1 was repeatedly streaked on simi-

lar medium for identification and alkaline protease production.

Identification of strain AP1: Initially the strain AP1 was analyzed based on physical and microscopic appearance for colony size, shape, colour, motility, cell arrangement, spore forming, fluorescence, and growth on 5% NaCl. Various kinds of biochemical analysis were carried for categorizing the genera of the isolate as summarized in Bergey's identification manual book (Bergey et al. 1994).

For molecular identification of strain, CTAB Method was used for isolating the genomic DNA from strain AP1 (Chen and Ronald 1999). Automated sequencing was done according to the dideoxy chain-termination method with Applied Bio systems automated sequencer by Triyat Scientific services (India).

Phylogenetic analysis: Nucleotide sequence was investigated for sequence resemblance with the archived 16S rDNA sequences in the GenBank database using blastn (Altschul et al., 1997). By using CLUSTAL X numerous sequences were allied (Thompson et al., 1997). Phylogenetic analyses were performed accordant to the neighbor joining (NJ) method (Kumar et al., 2016) viz. MEGA version 7.0.

Optimizing protease production by strain AP1: The strain AP1 could grow in 250ml Erlenmeyer flasks with alkaline enzyme production media having the following composition; 5.0 g of Soymeal, 15.0 g of Peptone and 5.0g of NaCl, for 48 h at pH 8.0 using orbital shaker (80 rpm). The 5ml quantity of medium was withdrawn at regular intervals and readings were taken at 660 nm. The enzyme solution was subjected to centrifugation at 10,000 *g* for time interval of 15 minutes at 4ºC and collected supernatant was taken for determining enzyme activity.

Influence of time course on production of enzyme was studied by inoculating the test organism in nutrient broth medium was incubated at different time period ranging from 6 hours to 48 hours. After determining the optimum time source, different carbon sources (glucose, sucrose, lactose, maltose and fructose) and substrates (skim milk, caesin, BSA, gelatin and albumin) were tested for optimum production of protease.

For evaluating culture conditions, isolate was grown for one hour in the nutrient broth at varying pH (6-12) and temperatures (5-40ºC).

Enzyme Activity and protein content: The activity of enzyme was analyzed by the customized process illustrated earlier by Joshi et al., (2007). Solution including 4ml of casein (1% w/v) as substrate in 50 mM buffer (Tris-HCl, pH 9.0) along with 100µl of properly diluted enzyme sample was incubated at 20ºC for half an hour. The reaction was ended by the supplementation 5ml TCA (10 %) at 37ºC and 15 mins of incubation. Further the reaction mixture was again centrifuged for 15 min at 8000*g* and 20ºC. The spectrometric readings were taken at 660 nm. One Unit of protease activity was described as the quantity of enzyme necessary to release 1µg of tyrosine in 1.0 ml for 1 min in assaying conditions.

The protein content was checked by Bradford, (1976) method using BSA as standard.

Partial purification of enzyme: The strain AP1 cells were subjected to centrifugation at 8000*g* for 15 minutes, furthermore the culture broth having crude protease extract was allotted to $(NH_4)_2SO_4$ precipitation. The condensed protein mixture was detached by centrifuging at 10000*g* for 10min. Extra $(NH_4)_2SO_4$ was added to supernatant for producing saturation upto 90%. The precipitated protein fractions among 40 and 90% saturation were collected via centrifugation, the precipitate obtained was dissolved in buffer (0.05M Tris–HCl, pH 8.0). The buffered solution was dialyzed overnight at 4°C to eradicate ammonium sulphate residues. Dialyzed suspension was then subjected to centrifugation at 8000*g* for 15 minutes to eradicate inexplicable fussy material.

Molecular weight determination: The mol. wt. of partially purified enzyme was resolved using standard protein marker, Pre-stained Protein Standard (Life Technologies).

Effect of pH on enzyme activity/stability: To determine the maximum pH for activity, casein solution (1%, w/v) as a substrate was mixed with purified protease in various buffers: citrate buffer pH 6.0, sodium buffer pH 7.0, Tris–HCl buffer pH 8 and KCl/NaOH buffer pH 9.0-12.0; the solution were incubated at 20°C for 1 hour. Alkalistability was concluded by initially pre-incubating the protease with no substrate at varied range of pH 6.0-12.0 followed by one-hour incubation.

Effect of temperature on enzyme activity/ stability: To determine the highest temperature for activity, hydrolytic activity of purified enzyme was measured at various temperatures (5°C-45°C) and pH 9.0. Thermostability was calculated by pre-incubating the purified protease at temperature range of 5-45°C and pH 9.0 for 1 hour; then the residual protease activity was calculated under standard assaying conditions.

Effect of inhibitors/metal ions on protease activity: A variety of metal ions and inhibitors were supplemented in concentration of 0.5M and pre-incubated for 30 min along with appropriate amount of purified protease. The substrate was added

afterwards to measure the residual activity using standard assaying conditions. The control was deemed as maximum (100% of residual activity).

Statistical analysis: All the tests were carried out in three replications and results were applied in One-way ANOVA ranked with Duncan's several choice analyses using SPSS 16 Version software.

Washing test for blood stains removal: Function of *Bacillus* sp. AP1 derived cold active protease as enhancer in detergents was evaluated as per minor alteration in the method depicted by Chen et al., (2018). Tea stains were applied on white square pieces of cloth and subsequent sets were arranged and examined:

(a) 250ml Erlenmeyer flask containing 100ml of ddH_2O+200µl of commercial detergent (Ariel -200 µl/ml) + stained cloth.
(b) 250ml Erlenmeyer flask containing 100ml of ddH_2O + purified enzyme (200 µl) + stained cloth.
(c) 250ml Erlenmeyer flask containing 100ml of ddH_2O + purified enzyme (200 µl) + 200 µl of commercial detergent (Ariel -200 µl/ml) + stained cloth.
(d) 250ml Erlenmeyer flask containing 100ml of 100ml ddH_2O + purified enzyme (200µl) + 200µl of commercial detergent (Ariel -200µl/ml) + stained cloth.

The first three sets were incubated at 20°C and the last set was incubated at 40°C for 15 minutes. Furthermore, stained cloth pieces from each set were checked, washed and dried. Untreated piece of stained cloth was taken as control.

RESULTS AND DISCUSSION

Isolation and identification of bacterial strain: The bacterial isolate was attained from glacier soil sample collected from summit of Apharwat peak located in Kashmir region. The bacterial strain AP1 was selected based on maximum hydrolysis zone formation on alkaline skimmed milk agar plate, showing growth in cold and alkaline conditions (Figure 1). The strain was gram-positive, non-spore forming and rod-shaped bacteria showing characteristics like that of *Bacillus* species (Table 1). The rRNA gene sequence attained from strain AP1 illustrated highest similarity (99%) with the corresponding gene sequences of *Bacillus* sp. and was designated as *Bacillus* sp. AP1 (Figure 2). Previously cold-active protease producing *Bacillus* sp. has been reported from Nainital Lake (Joshi et al. 2007) and Gangotri glacier (Baghel et al., 2005).

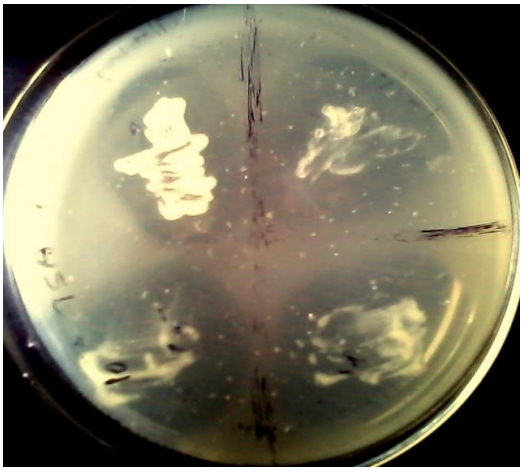

Figure 1: Zone of hydrolysis on skim milk agar

Table 1: Morphological and biochemical characters of strain AP1

Morphological Characteristics	
Cell Arrangement	Irregular
Size	Medium
Colour	Cream
Shape	Rod
Spore formation	-
Motility	+
Gram Staining	+
Biochemical Tests	
Growth on 1, 2,3,4 and 5% NaCl	+
Indole Test	-
MR test	-
VP test	+
Citrate Utilization	+
Catalase test	+
Nitrate reduction	+
H2S test	-
Oxidase test	+
Lysine decarboxylase	-
Arginine dihydrolase	-
Ornithine decarboxylase	-
Hydrolysis Tests	
Urea hydrolysis	-
Gelatin hydrolysis	+
ONPG hydrolysis	+
Acid Production	
Fructose	+
Galactose	-
Lactose	-
Maltose	-
Sucrose	+
Identified isolate	*Bacillus* sp.

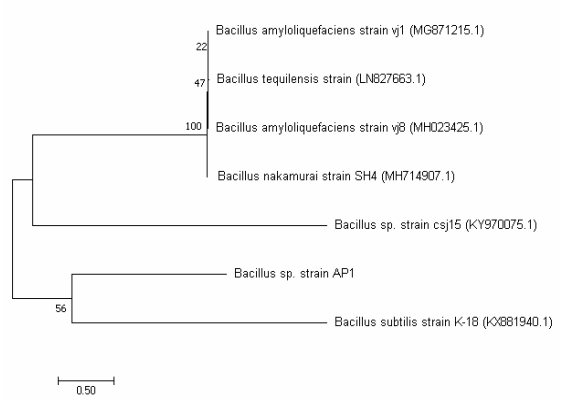

Figure 2: Molecular Phylogenetic analysis by Neighbor-Joining method

Optimizing conditions for crude protease production: It was been observed that production of protease inclined towards late log phase and highest enzyme production (45.4 U/ml) was recorded at 30 hours of incubation period at 20°C and pH 8.0 (Figure 3). However, ahead of 30 hours of time period, the production of enzyme gradually decreased. Among the various sources of carbon tested, glucose was able to yield maximum production, followed by sucrose (Figure 4). Among various substrates tested, skimmed milk gave the best results for protease production followed by caesin (Figure 5). Temperature and pH have significant influence on enzyme production of microorganisms. The *Bacillus* sp. AP1 produced protease in a broad pH (6.0–12.0) and temperature (5–40°C); however, optimum production was recorded at pH 9.0 (Figure 6) and 20°C (Figure 7). Based on pH and temperature optima, the *Bacillus* sp. AP1 can be classified as alkalotolerant (Babel 1985) and psychrotrophic (Morita 1975). Optimal temperature and pH were evaluated via distinct factor at once and afterwards that as standard for optimization of another parameter, increasing the production of protease to 75.0 U/ml.

The standardized conditions were applied for enzyme production to the media with glucose and skim milk being the source for carbon and substrate respectively. Further, the strain could grow in optimal temperature (20°C) and pH (9.0) conditions for 30 hours to obtain maximum protease production.

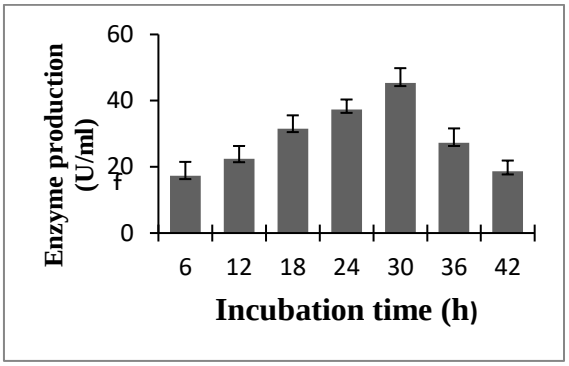

Figure 3: Effect of incubation on protease production

Figure 4: Effect of various carbon sources on protease production

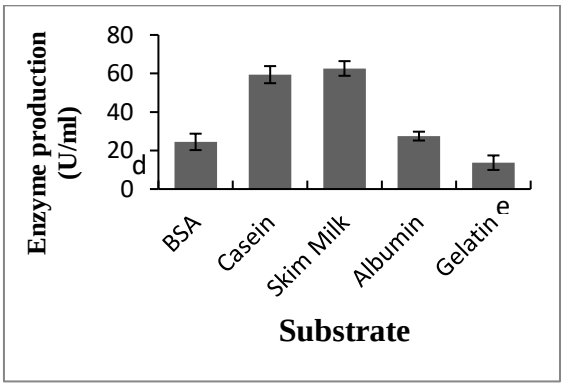

Figure 5: Effect of various substrates on protease production

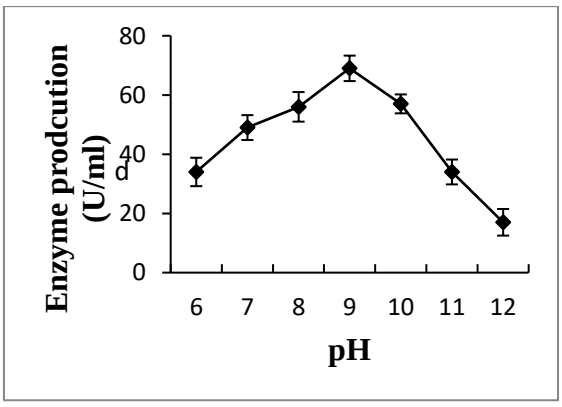

Figure 6: Effect of pH on protease production

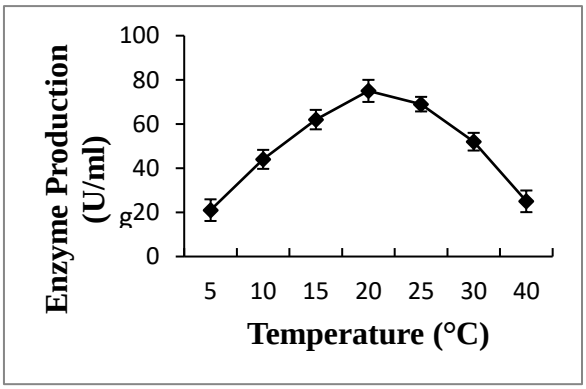

Figure 7: Effect of temperature on protease production

Partial purification of enzyme: The enzyme was partially purified by $(NH_4)_2SO_4$ precipitation at 60% dispersion stage under optimized conditions. Initially 2.77 folds in protein purification with yield of 88.90 % was obtained due to $(NH_4)_2SO_4$ saturation. The sample collected after overnight dialysis illustrated an enhancement of 3.82 folds in purification with final yield of 76 % and specific activity of 31.06 U/mg (Table 2). Enzyme purity was characterized by SDS-PAGE, which showed that our protein had a molecular weight of 62 kDa (Figure 8). A 66 kDa alkaline protease from *Bacillus caseinilyticus* has been previously reported by (Mothe and Sultanpuram 2016), but unlike our protease it was mesophilic. However, the molecular weight of our enzyme coincides with the cold active metalloprotease from *Flavobacterium psychrophilum* reported by Secades et al., (2003).

Table 2: Partial purification of protease from *Bacillus* sp. AP1

Purification method	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude enzyme	3200	392	8.12	1.00	100
Ammonium sulphate precipitation	2845	126	22.57	2.77	88.90
Dialysis	2432	78.3	31.06	3.82	76

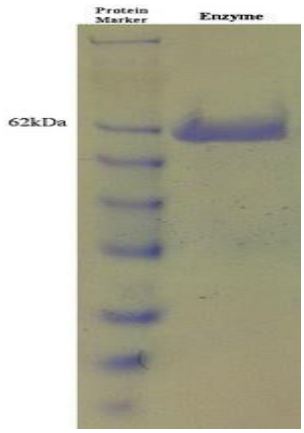

Figure 8: Mol. wt. of partially purified protease from *Bacillus* sp AP1

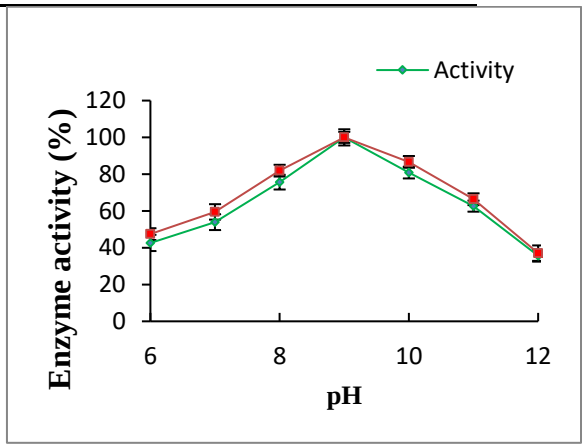

Figure 9: Effect of pH on activity and stability of purified protease from *Bacillus* sp. AP1

Effect of pH on activity and stability of purified protease: Partially purified protease illustrated activity within broad pH range of 6.0-12.0 and highest activity was observed at pH 9.0, proving the alkalophilic nature of enzyme. The *Bacillus* sp. AP1 purified protease was stable within alkaline pH range of 8.0-10.0 but showed considerable decline in stability below and above the stable range (Figure 9). Several *Baci-lli* derived cold-active proteases viz. from *Bacillus cereus* MTCC 6840 and *Bacillus cereus* SYP-A2-3 reported by Joshi et al., (2007) and Shi et al., (2005) respectively, showed maximum acti-vity at pH 9. The alkalophilic nature of the protease can prove beneficial for its application as an industrial enzyme.

Effect of temperature on activity and stability of purified protease: Hydrolytic activity of purified enzyme was measured at varying temperatures to determine the maximum activity which was found to be 20°C, indicative of cold-tolerant nature of enzyme. Significant stability was being observed within the temperature range of 5°C-20°Cat pH 9.0 and the enzyme was able to retain more than half the activity in between 20-30°C (Figure 10). Various cold-active prote-ases producing bacteria viz. *Bacillus cereus* (Joshi et al., 2007), *Curtobacterium luteum* (Kuddus and Ramteke 2008) and *Stenotropho- monas maltophilia* (Kuddus and Ramteke 2011) have been reported with highest activity at 20°C along with wide range of temperature stability.

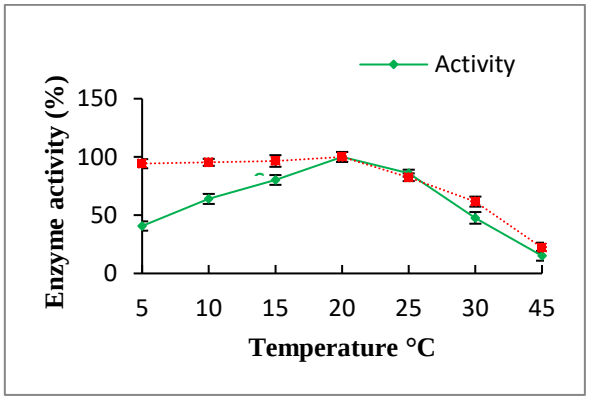

Figure 10: Effect of temperature on activity and stability of purified protease from *Bacillus* sp. AP1

Effect of inhibitors and metal ions: Partially purified enzyme by *Bacillus* sp. AP1 was significantly repressed by EGTA and EDTA; however, iodoacetate, PMSF and pepstatin didn't show any notable repressive effect on enzyme activity. Most of the metal ions tested showed considerable inhibition but Mn^{2+} enhanced the enzyme activity. EGTA and EDTA are well known inhibitors of metalloproteases as reported earlier by Secades et al., (2001) and Margesin et al., (2005). Iodoacetate, PMSF and pepstatin are common inhibitors of cysteine, serine and aspartic proteases respectively (Kuddus and Ramteke 2008), but none of them showed any considera-ble inhibitory effect unlike EDTA and EGTA. Our results and previous reporting proved that our enzyme from *Bacillus* sp. AP1 is a metallo-protease (Table 3).

Table 3: Effect of various inhibitors and metal ions on purified protease from *Bacillus* sp. AP1

Sl. No.	Inhibitors/ Metal ions	Residual activity (%) *
1.	Control	100
2.	EGTA	9
3.	EDTA	12
4.	PMSF	93
5.	Iodoacetate	88
6.	Pepstatin	84
7.	Ca^{2+}	43
8.	Cu^{2+}	31
9.	Mg^{2+}	26
10.	K^{+}	32
11.	Mn^{2+}	112
12.	Zn^{2+}	28

*Results based on three replications, and the standard error in all the tests was less than 5%

Washing test for blood stain removal: Tea stains were partly cleaned in case of set (a) and set (b) whereas tea stains were completely cleaned in the blend of purified protease + detergent after 15 minutes of incubation at 20°C (c). Rela-tively the activity was reduced at higher temp-erature of 40°C (d), proving that the protease can be significantly useful for cold washing purposes as an additive for detergents (Figure 11). Psychrotrophic enzymes are known to be greatly active at low temperatures unlike meso-philes, this ability attracts the modern day bio-tech industries (Gerday et al., 2000). The prot-ease from *Bacillus* sp. AP1 showed similar potential and can prove to be a vital candidate for cold washing purposes.

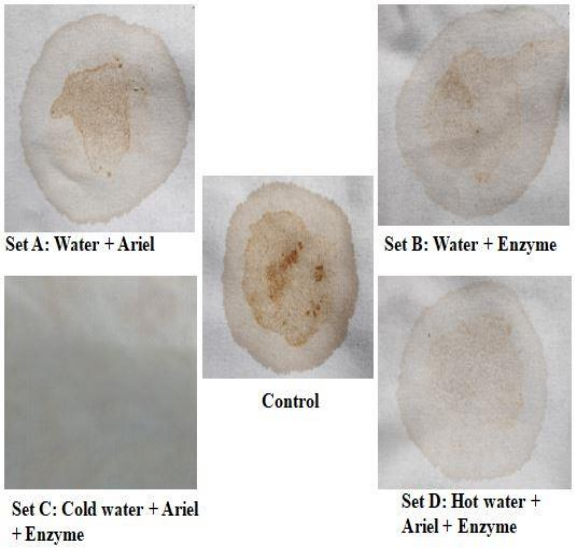

Figure 11: Washing test of purified protease from *Bacillus* sp. AP1 on tea stains from white cloth

ACKNOWLEDGMENTS: The authors are grateful to Arni University and SKAUST-Kashmir for supplying necessary research facility and support. We would also like to thank Moham-mad Naufal and Kaleem Qadri for their support throughout the gathering of samples.

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