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Thermostable alkaline protease from *Bacillus* sp. and its potential applications

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Abstract: *A thermophilic strain producing thermostable protease and cellulase was isolated from hot water springs. The strain was identified as Bacillus licheniformis on the basis of 16S rRNA gene sequencing and biochemical properties. The protease was partially purified by ammonium sulphate precipitation. Thermostable alkaline protease showed highest activity and stability at pH (10-11) and at temperature 55°C. Enzyme activity increased in the presence of metal ions like Ca^{2+} and Cu^{2+}. The bacterial isolate produces a serine protease. The supplementation of the enzyme in detergents could significantly improve the cleansing performance towards the blood stains and suggest its possible use as a detergent additive. The enzyme also showed potential for application as a dehairing agent.*

Keywords: *Alkaline Protease; Bacillus licheniformis; Detergent; Dehairing agent; Thermostable*

I. INTRODUCTION

Proteases are one of the most commercially important groups of extracellular microbial enzymes. They have wide applications in the detergent, food, pharmaceutical, chemical and leather industries. Among the total industrial enzymes, proteases represent 60% of the total worldwide sales of enzymes and alkaline proteases account for 89% of the total protease sale [1]. Proteases are produced by microorganisms, plants and animals, but microbial community is preferred over the others for the large scale production of proteases. Microbial proteases, especially from *Bacillus* spp are the most widely exploited industrial enzymes. Among them, alkaline proteases are of particular interest due to their potential applications in the detergent industry as a cleaning additive [2].

Hot spring as one of the thermophile habitats is considered as a promising source for the direct isolation of thermostable enzymes. The microorganisms living in hot spring not only withstand elevated temperature but also the pH of environment and the presence of certain chemical compounds [3]. Thermostable proteases are advantageous in some applications, because higher processing temperatures can be employed, resulting in faster reaction rates, increase in the solubility of non gaseous reactants, products and reduced incidence of microbial contamination by mesophilic organisms [4].

Alkaline proteases have been given more attention because they can withstand high temperature, pH variation, surfactants and toxic metals. They can be easily grown in complex and synthetic media. Alkaline proteases are produced by a wide range of microorganisms including bacteria, molds, yeasts and also mammalian tissues. A large proportion of commercially available alkaline proteases are derived from *Bacillus* species because of their high pH and temperature stability. *Bacillus* species also have the ability to secrete large amount of alkaline protease from an industrial point of view [5].

Alkaline proteases are used as detergent and leather processing additives because of their unique abilities to digest proteinaceous stains such as blood, chocolate, milk, and keratin. Use of alkaline protease based detergents is preferred over the conventional synthetic ones. This is because of their better cleaning properties, higher performance, safer and healthier dirt removal conditions. They are active, stable and compatible at high temperature, high concentrations of salt, bleach, surfactant and alkaline environment [6].

Waste from the leather industry leads to environmental pollution. Alkaline proteases have dehairing properties and can be used in the leather processing industry. Conventional methods in leather processing involve the use of hydrogen sulphide and other chemicals which are pollutants. Thus for environmental reasons the enzymatic dehairing process has more advantages over the chemical dehairing process. Alkaline proteases swell hair roots and attack hair follicle proteins resulting in the easy removal of hair [1].

II. MATERIALS AND METHODS

2.1 Screening of water sample for Protease and Cellulase activity

The water sample was collected from Vajreshwari hot water spring (Palghar). Water sample was inoculated in 100ml Nutrient broth (pH-9) and enrichment was carried out for 72 hours at 55^{o}C under shaker conditions (120rpm). The enriched broth was appropriately diluted then plated on Milk-agar and Carboxymethyl cellulose agar (CMC). Incubation was carried out at 55°C for 24 hours. Colony characteristics were observed of the different colony types and then examined for halo around the colonies as the sign of protease producing ability of the organism on Milk agar and cellulase producing organism on CMC agar.

2.2 Bacterial Identification through Biochemical Tests and 16S rRNA Gene Sequences

Identification of the isolates was confirmed by biochemical and morphological tests as outlined by Bergey's Manual of Determinative Bacteriology and by 16S rRNA gene sequence analysis.

2.3 Fermentation for Protease Production

The bacterial isolate which showed proteolytic activity on milk agar plates under high alkaline conditions were selected for protease production. Protease production medium (pH-9) consisted of:
Glucose – 2 %(w/v),Peptone - 1 %(w/v),Yeast extract - 0.5% (w/v), Na_2HPO_4 - 0.4g/l, Na_2CO_3 - 0.8g/l, $ZnSO_4$ - 0.02g/l,$CaCl_2$ - 0.02g/l and $MgSO_4$ -0.02 g/l. 1 ml of culture (O.D - 0.1 at 620nm) was inoculated in the Protease production medium (PPM). Flasks were incubated at 55^{o}C for 72 hours under shaker conditions. After incubation the cell free supernatant was obtained by centrifuging the broth at 3000rpm for 20min. The supernatant obtained was considered as crude enzyme and was used for assaying the proteolytic activity.

2.4 Protease and Protein Assay

Enzyme assay mixture consisting of 1 ml of caesin (1% w/v in 0.1 M Glycine NaOH buffer) and 1 ml of purified enzyme was incubated at 55°C for 30 min. The reaction was stopped by adding 5 ml of TCA solution (5% w/v).Then the contents were centrifuged at 10,000g for 10 min and 1 ml of supernatant was taken out in separate test tube and the protein content was estimated by Folin Lowry. The absorbance of the solution was measured at 660 nm by spectrophotometer. The standard curve was obtained for series of known concentrations of Tyrosine 10-100ug/ml from the graph, the amount of protein liberated due to the action of enzyme protease in the supernatant was determined. One unit of protease activity was defined as the amount of enzyme required to liberate 1 g/ml tyrosine under the experimental conditions.

2.5 Partial Purification of Protease by Ammonium sulphate precipitation

The CFS sample of 100ml was divided into 5 parts of 20ml each. Different amount of ammonium suphate was added to each part to attain different levels of saturation i.e. 50%,60% ,70%, 80% and 90%.The addition of salt was carried out in an ice bath with constant stirring. The solution was kept overnight at 4^{o}C. The precipitate was collected by centrifugation at 3000 rpm for 10min. Each precipitate was dissolved in phosphate buffer and was dialyzed against phosphate buffer until it gave no precipitation with Nessler's reagent. The precipitated fractions were dialyzed and analyzed for protease activity and protein content. The fraction which showed the highest activity was used for further analysis

2.6 Effect of pH and Temperature on the Activity and Stability of Protease

For determining the effect of pH on protease activity, different buffers (50 mM) used in enzyme assay mixture were: phosphate buffer (pH 7 - 8), Tris-HCl buffer (pH- 9), and glycine NaOH buffer pH 10 – 11. For examining the pH stability of the protease the enzyme was pre-incubated with the buffer of appropriate pH for 30 min and then residual activity was assayed.

Thermostability of enzyme was determined by pre-incubating the enzyme at different temperatures ranging from 30°C to 100°C for 30 min (pH 9). Relative activity was determined by considering maximum activity as the standard reference.

2.7 Effect of Metal Ions and Inhibitors on the Activity and Stability of Protease

The effect of divalent ions viz. Mg^{2+}, Co^{2+}, Hg^{2+}, Zn^{2+}, Pb^{2+}, Ca^{2+} and Cu^{2+} on the activity of protease was studied by including each in the enzyme assay mixture at a final concentration of 10 mM and the activity was assayed.

The effect of various protease inhibitors (10mM and 50mM) such as serine inhibitors (phenylmethylsulphonyl fluoride [PMSF]), cysteine inhibitors (β-mercaptoethanol) and metallo inhibitors (ethylene diamine tetra acetic acid [EDTA]) were determined by preincubation with the enzyme solution for 30 minutes at 55°C before the addition of substrate. The established temperature and pH optimum conditions were

used to assay protease activity when testing the effects of divalent metal ions on the enzyme activity. Relative activity was determined by considering control as the standard reference.

All the analytical experiments were conducted in triplicates and results expressed are the mean of three different experiments.

2.8 Detergent application

Stain removal ability of the enzyme was assessed. A measured quantity of the enzyme was added with the solutions of the different commercial detergents at a detergent concentration of 7 mg/ml. Blood stained fabric pieces were rinsed in each tube for 30min dried and observed for stain removal. Detergent solutions were used as a positive control at the same concentration. After wash procedure, fabric pieces were rinsed with distilled water and dried. Blood-stained fabric, washed with only with distilled water was taken as negative control. Different fabric pieces were scanned before and after wash procedure to evaluate the efficiency of the removal of bloodstain.

2.9 Dehairing test

Pieces of goat skin with hair were incubated in the presence of protease at 25^{o}C, 37^{o}C and 55°C. Results were observed after 3 hrs and 6 hours. After incubating with the enzyme the hair on the skin pieces were pulled gently. The dehairing efficacy was assessed according to the depilated area of the skin at the end of the process [7].

III. RESULTS AND DISCUSSION

3.1 Screening of bacteria for protease and cellulase activity

Bacterial strain was isolated from a hot spring and screened for protease and cellulase producer at pH 9.0 at 55^{0}C. The bacterial strain showed clearance around the colony on milk agar plates indicating presence of

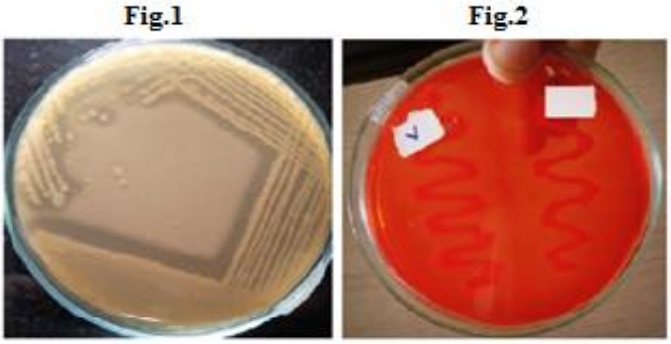


Fig.1Thermostable protease activity on Milk agar **Fig.2** Thermostable cellulase activity on CMC agar

protease activity (Fig.1). Bajaj, B.K, et al [8] has also reported the isolation of *Bacillus* strains from thermophilic environments. Clearance was also observed around the colony on CMC agar plate (Fig.2) indicating presence of cellulase activity. Similar results were also reported from *B. licheniformis* MVS1 (GU590781) and *Bacillus* sp. MVS3 [9].

3.2 Bacterial Identification through Biochemical Tests and 16S rRNA Gene Sequences

The isolated alkalophilic thermostable protease and cellulase producing strain was a spore forming Gram-positive rod .The strain showed growth over a wide range of pH and temperature. Identification of the isolates was confirmed by biochemical and morphological tests as outlined by Bergey's Manual of Determinative Bacteriology.

TABLE 1. Biochemical test for isolate as per Bergey's Manual of Systematic Bacteriology, Volume 3

BIOCHEMICALS	OBSERVATION
Catalase	+
Indole	-
Voges Proskauer	+
Methyl Red	-
Citrate utilization	+
Nitrate reduction	+
Acid from Glucose Arabinose Mannitol Xylose	 + + + +
Anaerobic growth	+
Caesin hydrolysis	+
Starch hydrolysis	+
Effect of NaCl 2% 5% 7% 10%	 + + + ND
Effect of temperature 4^0C 28^0C 37^0C 55^0C 65^0C	 - + + + -

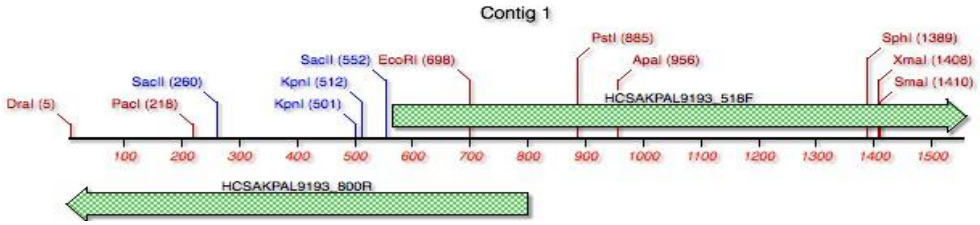


Fig .3.Assembled sequence of Forward and Reverse - Contig sequence

16S rRNA Analysis: The sequence of conserved sites by sequence analysis gave the following result:

GTTTTAAAGGGGGGTTTTTTTTAGGGTTTTTATATTAGTCAGGACGAACGCTGGCGGCGTGCCTAA
TACATGCAAGTCGAGCGGACCAGATGGGAGCTTGCTCCCTGATGTTAGCCGCCGACGGGTGAAGT
ACCCGTTGGTTACCTGCCTGGTAGGCTTGGATTACTTCGGGAAACCGGGGGTTATTCCCGAAGGCT
GGATGGACCGCCAGGTTTAATTAATAAAAGTGGGTTTCAGCTACCACTTACAGATGGACCCGCGG
CGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGT
GATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTC
CGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAAC
TCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATAGGGCGGTACCTTGACGGTACCTAACCAGAA
AGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTA
TTGGGCGTAAAGCGCGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGG
AGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTG
AAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTG
AGGCGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGA
GTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGCAGCAAACGCATTAAGCACTCCGCCTGGG
GAGTACGGTCGCAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGT
GGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAACCCTAGAGATA
GGGCTTCCCCTTCGGGGGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATG
TTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTA
AGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGA
CCTGGGCTACACACGTGCTACAATGGGCAGAACAAAGGGCAGCGAAGCCGCGAGGCTAAGCCAA
TCCCACAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAACTCGACTGCGTGAAGCTGGAATCGCTA
GTAATCGCGGATCAGCATGCGCGGGGGAAAAACCTTTCCCGGGGCCTTGTACACACCGCCCGTCA

CACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTTTTGGAGCCAGCCGCCGAAGGTG
GGACAATGATGGGGGATAGAAAAAGGGGAGGAAAGATATATAAAGGAACGGGGGGGGGG

The sequence was aligned for comparison with the existing databases by using BLAST. The biochemical tests and the 16S rRNA sequencing confirmed that the isolate belonged to the genus Bacillus. The identified organism was Bacillus licheniformis.

3.3 Enzyme production and partial purification of enzyme

Production of thermostable alkaline protease was carried by *Bacillus licheniformis* in protease production medium (PPM) (pH-9) for 72hrs at 55^0C. The crude extract obtained showed an enzyme activity of 189.4µM/min/ml. According to previous studies a maximum yield of 18.4µM/min/ml was reported from *B. licheniformis* LBBL-11 [10], 34277 µM/min/ml from *Bacillus* sp. strain B001 [11] and 3500 µM/min/ml from *B. licheniformis* NH1 [12]. The enzyme production varied according to the media composition and different organisms. Protease is produced by mostly all *Bacillus* spp. in 24 hours. The yield differs according to the different *Bacillus* species and their optimum conditions.

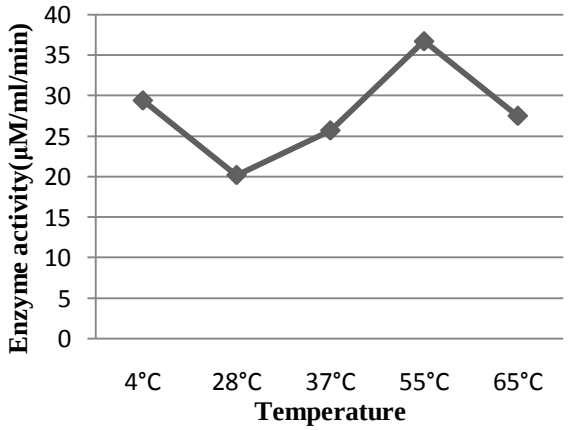
	Recovery		Specific activity $\mu Mmin^{-1}mcg^{-1}$	% Yield
	Protein content (mcg/ml)	Enzyme activity µM/ml/min		
Crude extract	103	189.4	1.8388	100
Ammonium sulphate precipitation 40-60%	80	147.1	1.8396	78

TABLE 2. Purification of protease from *B. licheniformis*

Ammonium sulphate precipitation of the enzyme at different saturation levels was carried out. Maximum activity was present in 40% - 60% salt precipitated fraction. The enzyme was purified and a specific activity of 1.8396$\mu Mmin^{-1}mcg^{-1}$ protein was obtained. A recovery of 42.39% was reported by Mani, P et al 2012[5].In the present study a yield of 78% was reported after purification.

3.4 Effect of Temperature and pH on Enzyme activity

The effect of different incubation temperature on the activity of protease was studied. The activity of the purified protease from *B. licheniformis* was determined at different temperatures ranging from 4°C-65°C. Results revealed that the optimum temperature at which protease exerted maximum enzyme activity (367µM/ml/min) was 55°C. Decrease in the enzyme activity was observed at 65°C. This indicates that enzyme has an optimum temperature for maximum enzyme activity and increase or decrease in the reaction temperature

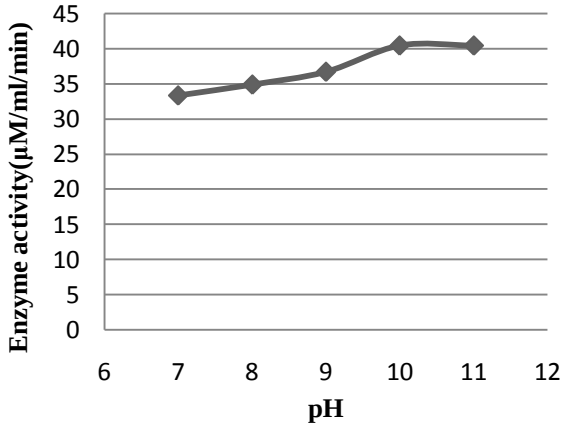


Graph .1 Effect of Temperature on Enzyme Activity

causes loss of activity. On the other hand, different optimum temperature for protease were reported by other investigators including 35°C from *B. licheniformis* MZK03 [13], 50°C from *B. licheniformis* S-40 [14], 70°C from *B. licheniformis* MP1 [2] and 55°C from *B. licheniformis* [5]. Lower optimum temperature has been reported for proteases from some *Bacillus* species [15].The protease was stable up to 55°C and 65°C even after 30 min of incubation. These contradict earlier reports of alkaline proteases from some bacteria strains which were completely denatured within 5 min of incubation at 55°C [16].Comparing these results, the alkaline protease from *B. licheniformis* is relatively more stable at high temperatures and exerts maximum proteolytic

activity at 55°C. Stability of protease at higher temperature might be an advantage for using this protease in industrial application such as laundry detergents formulations.

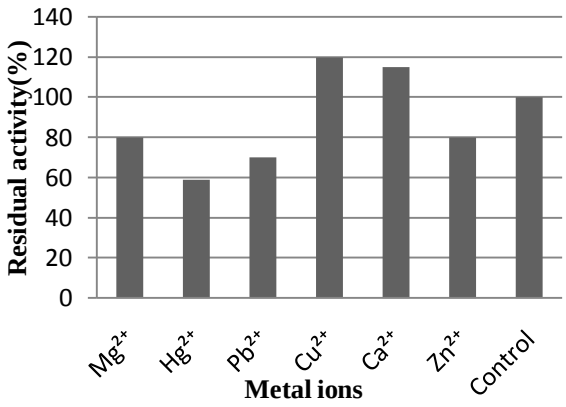
The effect of pH on the activity of protease was studied by conducting the assay at varying pH using appropriate buffers for 30mins. It was observed that the enzyme has ability to work under extremely alkaline pH. The enzyme showed activity over pH range of 7 - 9 and maximum activity was observed at higher pH 10-11(404µM/ml/min). The optimum pH range for the enzyme is 10-11, where it showed highest enzyme activity. However, a maximum proteolytic activity within pH range 8-10 was produced by *B.licheniformis* NH1 [17]. Other investigators recorded optimum proteolytic activity at different pH values such as pH 8.5 from *B. licheniformis* MZK03, pH 13 from *B. licheniformis* MIR29 and pH 9-10 from *B. licheniformis* LHSB-05 [18]. Large number of alkaline proteases isolated from *Bacillus* has a high optimal pH for their activity. However, alkaline protease from some *Bacillus* species have been reported to exhibit sharp decline in stability at pH 11 and 12, respectively. In the present study, however, the most significant level of growth and production of protease were supported by pH ranging from 10-11. These results indicate that the protease produced from *B. licheniformis* is a highly stable alkaline protease.



Graph .2 Effect of pH on Enzyme activity

3.5 Effect of Metal Ions and Inhibitors on the Enzyme Activity

Various metal ions were included in the enzyme assay reaction mixture at final concentration of 10mM. The enzyme activity was enhanced with an addition of Cu^{2+} and $Ca2^{+}$ resulting in the residual activity of 120% and 115% respectively. The increase in enzyme activity with $Ca^{2+}$and Cu^{2+}in this study suggests that these cations activated the protease and have the capacity to protect the enzyme from *B. licheniformis* against denaturation. This result correlates with the findings of Bajaj, B.K. et al [8] that Ca^{2+}, Co^{2+}, Zn^{2+} and Cu^{2+} increased the activity of protease from *B.pumilis* D6. Similar results were reported from *Bacillus* sp. SMIA-2 protease [19]. This suggests that the protease requires Ca^{2+} and Cu^{2+} for its maximum activity and stability.

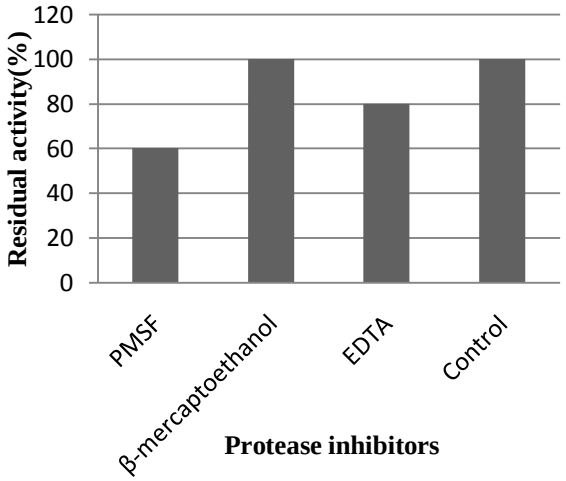


Graph.3 Effect of Metal ions on Enzyme activity

A universal feature among the subtilisin type proteases is the presence of calcium binding sites, which largely contributes to their stability against thermal denaturation and autolytic digestion some metal ions have been found to protect the protease against thermal denaturation and maintain active conformation of the enzyme at

high temperatures. Many enzymes also require the presence of metal atoms for full catalytic activity. The activity was reduced in the presence of Mg^{2+} and Zn^{2+}. Similar reduction in enzyme activity was observed from thermophilic bacterium (EP1001) [20] and *B.licheniformis* NH1 [17]**.** The toxic metal ions exert their toxicity by binding to a variety of organic ligands causing denaturation of proteins including enzymes. Pb^{2+} and Hg^{2+} caused an inhibitory effect on enzyme activity with residual activity of 70% and 59% respectively. Enzyme activity was not completely inhibited even in presence of inhibitors like Pb^{2+} and Hg^{2+} which are considered universal inhibitors of enzyme activity. Similar results were reported from *Bacillus pumilis* D6 [8] protease and from *B. licheniformis* MP1protease [2]. The above results thus indicate that the enzyme shows high stability even in presence of toxic metals and cannot be denatured easily.

PMSF is a known serine protease inhibitor and reacts with the serine residues of the active site in serine proteases. PMSF and EDTA showed a residual activity of 60% and 80% respectively. PMSF (10mM) inhibited enzyme activity up to 40%. Thus it can be suggested that the bacterial isolate may be producing a serine alkaline protease. Many of the *Bacillus* derived alkaline proteases belong to the class of serine proteases [21]. Serine alkaline proteases exhibit their optimum activity at pH range 9-11. These results are similar to what was reported for alkaline proteases [22] where 5 mM PMSF inhibited protease activity by up to 80%. EDTA, a metal chelating agent caused 20% inhibition at 10mM concentration, respectively.



Graph. 4 Effect of Inhibitors on Enzyme activity

The serine alkaline proteases are not generally inactivated by metal chelating agents like EDTA, but other examples alkaline proteases that are inhibited by EDTA have been reported [4]. These alkaline proteases probably require a metal ion for its activity and stability and hence in the present study protease was sensitive to presence of EDTA at 10mM concentration. Alkaline protease from a *B. subtilis* strain SH1 [23] was reported to be serine protease which is inhibited by EDTA. In addition, the stability of the enzyme in presence of EDTA is advantageous for its use as a detergent additive. The serine alkaline proteases are not generally inactivated by metal chelating agents like EDTA, but other examples alkaline proteases that are inhibited by EDTA have been reported. β-mercaptoethanol did not show significant inhibitory effects against the protease.

3.6 Detergent application

Blood stained fabric pieces were subjected to wash treatment at 55^{o}C for 30mins in distilled water solutions containing 7mg/ml of various detergent powders and supplemented with purified enzyme. Proteins are initially removed from the fabric surface either by components of the detergent matrix, or by water alone. When the enzyme was added with the detergent a complete removal of blood stain was observed. The supplementation of the enzyme preparation in detergents like Ariel, Rin and Tide significantly improved the cleansing performance towards the blood stain. Addition of the enzyme with the detergent Surf excel showed complete removal of the blood stain within 30mins. These results show the efficiency of *B. licheniformis* protease in proteinaceous stain removal efficiency. Several reports have reported the usefulness of alkaline proteases from *Bacillus liceniformis* KBDL4 [24] and *B. licheniformis* NH1 [17] in the facilitation of blood stains removal from

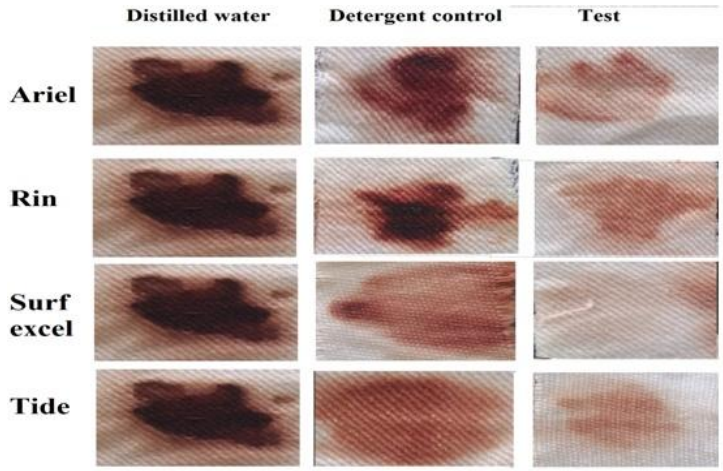


Fig.4 Blood stain removal using Bacterial protease Detergent
Control- Heat inactivated detergent Test- Heat inactivated detergent + purified enzyme

cotton cloth both in the presence and absence of detergents. The protease enzyme from *Bacillus licheniformis* MP1 [2] and *B. licheniformis* NH1 [12] was also efficient in removing bloodstains from fabric at 45^{o}C after keeping for 60mins. Protease from *B.licheniformis* N-2 [25] can also be used as a detergent additive and showed maximum activity at 40^{o}C. Proteases isolated from Bacillus sp. have excellent laundry detergent stability due to their ability to survive at alkaline pH and high temperature. Therefore, a preparation containing protease activity could be considered as a potential candidate for use as cleaning additive in detergents to facilitate the release of proteinaceous stains.

3.7 Dehairing of skin

Incubation of the enzyme for 6hrs with goat skin showed complete removal of hair when incubated at 55°C (pH-9). Incomplete and moderate removal of hair was observed after 3 hrs of incubation. No dehairing was observed at 28°C. The dehairing function during leather processing is generally carried out at a relatively high pH values ranging from 8-10. Hence proteases stable under alkaline conditions especially between the pH

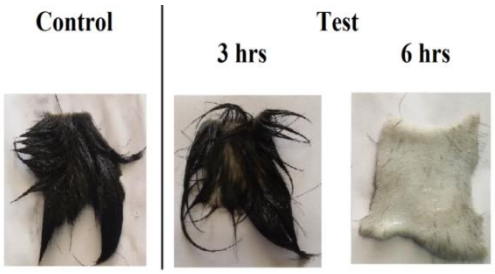


Fig.5 Dehairing of Goat skin

ranges 9-11 are used. Alkaline proteases from various Bacillus species are used as a dehairing agent because of their ability to survive at alkaline pH. Many proteases are not suitable for dehairing, since they have collagen degrading activity, which destroys the collagen structure of the hide. According to previous study *B. licheniformis* RP1 protease is advantageous to leather industry due to its poor collagenase activity [7]. Proteases from *B. circulans* [26] and *B. cereus* MCM B-326 [27] showed complete removal of hair from goat skin in 12 and 21 hours respectively. In the present study the protease isolated from *B.licheniformis* has an advantage over other bacterial proteases as it efficiently cause complete removal of goat hair in 6hrs indicating its potential application in leather industry for economizing the process. Enzymatic dehairing process is used as alternative to chemical methods as this process causes significant reduction of toxicity and also improves leather quality.

IV. CONCLUSION

It may be concluded that the organism isolated from hot spring showed the presence of thermostable protease and thermostable cellulase. Isolation of such multiple enzymes producing thermophilic organism can be of potential use in commercial industries as thermostable enzymes are less polluting and also aid in increasing the catalytic activity. The organism isolated was identified as *Bacillus licheniformis* by 16S rRNA

sequencing. The thermophilic organism was capable of surviving at diverse temperature and pH conditions.

Bacillus licheniformis produces high levels of an extracellular alkaline protease which has pH optima of 10-11 and temperature optima of 55 °C. This protease has a good stability at high alkaline pH value and broad heat stability thereby permitting its wide biotechnological application potentials to be exploited in many industries. Enzyme purification steps that incorporate not only ammonium sulphate precipitation but also other methods like passage through appropriate columns may increase yield. Strain improvement strategies may also prove to be beneficial. Optimal enzyme activity was studied by carrying out enzyme assays at various temperature and pH. The effect of metal ions on the enzyme activity helps us determine which metal ions act as inhibitors or aid in increasing the enzyme activity. The enzyme is an alkaline serine protease as indicated by the fact that it was inhibited by PMSF. Considering the high activity and stability at high alkaline pH and temperature the enzyme showed a potential application as a detergent additive. The enzyme also showed excellent dehairing property without causing any damage to the skin. The enzyme can be used as a dehairing agent in leather industry as it is non-polluting as compared to the other harmful chemicals used in dehairing process.

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