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Isolation, Production, Partial Purification, and Biochemical Characterization of a Thermophilic Alkaline Protease from Tuwa Hot Spring with Industrial Applications

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Abstract

Thermophilic bacteria were isolated from the Tuwa hot spring region of Gujarat, India, to evaluate extracellular thermostable alkaline protease production. Total twenty-five isolates were screened on skim milk agar, and five potent strains were selected for submerged fermentation. The 16S rRNA gene sequence of *Bacillus licheniformis* MT_1 has been deposited in the NCBI GenBank database under accession number PZ221934.1. Among them, *Bacillus licheniformis* MT_1 showed the highest enzyme yield and was selected for further study. Partial purification using ammonium sulfate precipitation (20–70% saturation) resulted in 1.08-fold purification, 90.82% yield, and specific activity of 65.00 U/mg. SDS-PAGE analysis revealed a predominant protein band corresponding to the expected molecular weight of the partially purified protease. The enzyme exhibited maximum activity at pH 9.0 and 80°C, confirming its alkaline and thermophilic nature. It retained over 92% activity after prolonged exposure to elevated temperatures and remained stable across alkaline pH conditions. Substrate specificity studies showed highest activity with bovine serum albumin, followed by gelatin and casein. Kinetic analysis using the Lineweaver–Burk plot gave a Km of 0.25 g% and V_{max} of 5000 U/mL. Enzyme activity was enhanced by Mg^{2+} and Mn^{2+} ions and retained partial activity in the presence of organic solvents, surfactants, NaCl, and urea, with tolerance varying by agent and concentration. This protease shows strong potential for detergent and industrial applications.

INTRODUCTION

The microbial enzymes have become important biocatalysts in different processes in every industry due to their high specificity, efficiency and environmental friendliness[1]. Proteases from microorganisms are one of the most economic significant categories of enzymes found in microorganisms, accounting over 60% of the world enzyme market. Food processing, medicines, waste management, leather processing, and most importantly the detergent industry all make extensive use of proteases. Protein peptide bond hydrolysis is catalyzed by them. The continuous demand for robust enzymes that can function in challenging industrial environments has drawn a lot of interest to proteases produced by thermophilic bacteria[2].

Industrial proteases are still largely employed by the detergent industry, which employs them to increase stain removal performance while minimizing the requirement for harsh chemicals and high energy input[3]. Enzyme-based detergents significantly contribute to ecologically friendly and sustainable cleaning solutions by facilitating effective washing at lower temperatures, conserving water, and reducing chemical pollution. Thermostable proteases improve product performance and consumer satisfaction by continuing to work throughout hot wash cycles and prolonged storage[4].

Determining the appropriateness of enzymes for industrial formulations, especially for detergent uses, requires this type of knowledge[5]. Although thermoproteases are recognized to have economic potential, their effective usage needs rigorous purification and characterization[6]. Purification is essential for removing interfering chemicals, enhancing enzyme stability, and facilitating precise evaluation of biochemical characteristics[7]. Characterization studies provide important information on the molecular weight, optimal pH and temperature, kinetic parameters, substrate selectivity, and stability in the presence of metal ions, inhibitors, surfactants, and oxidizing agents[8,9]. However, despite extensive studies on thermophilic proteases, limited information is available on enzymes derived from the Tuwa hot spring ecosystem, particularly with respect to their combined thermostability, alkaline tolerance, and compatibility with industrial additives such as detergents and organic solvents[10,11].

The Tuwa hot spring, located in Gujarat, India, represents a unique geothermal ecosystem characterized by elevated temperatures and alkaline conditions that support diverse thermophilic microbial communities. Previous investigations of this ecosystem have primarily focused on microbial diversity and metagenomic characterization, revealing the presence of several thermophilic and protease-producing bacterial taxa with potential biotechnological applications[12]. However, despite these findings, only limited studies have isolated individual thermophilic bacteria from this habitat and comprehensively

characterized their extracellular enzymes for industrial applications. Consequently, the Tuwa hot spring remains an important but relatively underexplored source of novel thermostable enzymes.

Despite the reported microbial diversity of the Tuwa hot spring ecosystem and extensive studies on alkaline proteases from various *Bacillus* species, comprehensive purification and biochemical characterization of thermostable alkaline proteases isolated from Tuwa thermophilic bacteria remain scarce. Information regarding their combined thermostability, alkaline tolerance, detergent compatibility, and industrial applicability is still limited. Therefore, further investigation of thermophilic isolates from this unique geothermal habitat may facilitate the discovery of enzymes with enhanced industrial potential.

MATERIALS AND METHOD

Media and Chemicals

Nutrient agar, nutrient broth, skim milk agar, casein, ammonium sulfate, glycine, Tris-HCl, bovine serum albumin (BSA), gelatin, SDS, Tween 20, Tween 80, and other analytical-grade chemicals used in this study were procured from HiMedia Laboratories Pvt. Ltd. (Mumbai, India). All chemicals and reagents used were of analytical grade.

Sample Collection and Cultivation of Thermophilic Bacteria

The samples were collected using sterile 1-liter glass bottles from 3 different hot Kunds of Tuwa, Panchmahal, Gujarat. Samples were processed within 24 h of collection. For isolation of thermophilic bacteria, serially diluted samples were spread on nutrient agar and skim milk agar plates and incubated at 55°C for 24–48 h. Distinct bacterial colonies showing growth under thermophilic conditions were purified by repeated streaking on nutrient agar plates. Pure cultures were maintained on nutrient agar slants at 4°C for further analysis. For isolation, selected isolates were cultivated in nutrient broth under submerged fermentation conditions at 55°C with shaking at 120 rpm.

Isolation

Total twenty-five bacterial isolates were isolated and tested for the production of proteases. Skim milk agar plates were used for qualitative screening, and conventional enzymatic tests were used to measure protease activity quantitatively. Based on enzyme production efficiency, five powerful protease-producing isolates were chosen and treated to submerged fermentation (SmF). The most productive isolate, *Bacillus licheniformis* MT_1, was chosen for large-scale enzyme production following the optimization of culture and process parameters. This strain produced a thermoprotease, which was later purified and characterized.

Molecular Identification of the Isolate

Genomic DNA of the selected protease-producing bacterial isolate (MT_1) was extracted from a pure culture using a commercial genomic DNA purification kit according to the manufacturer's instructions. The quality and integrity of the isolated DNA were confirmed by agarose gel electrophoresis, which showed a distinct high-molecular-weight DNA band.

The nearly full-length 16S rRNA gene was amplified by polymerase chain reaction (PCR) using universal bacterial primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-CGGTTACCTTGTTACGACTT-3'). PCR amplification was performed with an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 90 s, and a final extension at 72°C for 10 min.

The amplified PCR product was analyzed by agarose gel electrophoresis, which confirmed the presence of a single amplified fragment of approximately 1.5 kb corresponding to the expected size of the bacterial 16S rRNA gene. The PCR product was

purified and subjected to Sanger sequencing using forward and reverse primers to obtain high-quality nucleotide sequence data. Multiple sequence alignment and phylogenetic analysis were performed using the Neighbor-Joining method with bootstrap analysis in MEGA software to further confirm the taxonomic position of the isolate.

Protease assay and total protein estimation

Enzyme production and activity were determined following the procedure originally described by Anson (1938) with modifications introduced by Hagihara (1958) (Hagihara *et al.*, 1958). The reaction mixture consisted of 0.5 mL of enzyme extract and 2.0 mL of 0.6% (w/v) casein prepared in 5 mM glycine-NaOH buffer (pH 9.0). The mixture was incubated at 55°C for 30 minutes. The reaction was subsequently stopped by adding 10% (w/v) trichloroacetic acid (TCA), followed by centrifugation at 10,000 rpm for 10 minutes to separate the precipitated proteins. Protease activity was quantified using a tyrosine calibration curve. One unit (U) of alkaline protease activity was defined as the amount of enzyme required to release 1 µg of tyrosine per minute under the specified assay conditions.

Total protein content was measured using the Folin-Lowry method (Lowry *et al*., 1951), with tyrosine serving as the standard reference. A reagent blank without protein was used for baseline correction, and no interference from other components was detected. During purification steps, protein concentration was determined spectrophotometrically by recording absorbance at 750 nm.

Statistical Analysis

The present study was designed as a preliminary biochemical characterization of the partially purified protease. Therefore, descriptive experimental observations are presented, and no inferential statistical analyses were performed. Future investigations will include biological replicates and appropriate statistical analyses to validate the findings.

Partial Purification and Biochemical Characterization

Ammonium Sulfate Precipitation

Following the incubation time, biomass was removed from the culture media by centrifuging it at 10,000 rpm for 10 minutes at 4°C. The cell-free supernatant was then collected. Ammonium sulphate precipitation was used to partially purify the crude enzyme solution. Stepwise saturation ranges of 0–20%, 20–40%, and 40–60% were reached by progressively adding solid ammonium sulphate (enzyme grade) while stirring constantly. During precipitation, the mixture was kept at 4°C. Centrifugation at 10,000 rpm for 15 minutes at 4°C was used to recover the precipitated proteins. The final protein pellets were gathered and put to use in additional purifying procedures.

All purification procedures were carried out under refrigerated conditions using a Remi refrigerated centrifuge maintained at 4°C. Ammonium sulfate precipitation was performed by gradual addition of solid ammonium sulfate under continuous stirring in an ice bath to maintain low temperature and preserve enzyme stability. Partial purification of the enzyme by ammonium sulfate precipitation and dialysis was performed following standard protein purification procedures described previously (Gupta *et al.*, 2005).

Dialysis

A minimum amount of 3.0 mL of 0.2 M glycine-NaOH buffer (pH 9.0) was used to resuspend the protein precipitates after ammonium sulphate fractionation. A dialysis bag was filled to three-fourths of its capacity with the resuspended enzyme solution, and both ends were tightly shut. Dialysis was performed for 24 hours at 4°C against 0.2 M glycine-NaOH buffer while gently swirling with a magnetic stirrer. To guarantee that ammonium sulphate was completely removed, the dialysis buffer was changed out for new buffer every two hours. The caseinolytic activity of this partial purified protease was

determined by Hagihara (1958) method. Dialysis was carried out using a HiMedia dialysis membrane tubing (Dialysis Membrane-50) having an average flat width of 24.26 mm, average diameter of 14.3 mm, and approximate capacity of 1.61 mL/cm.

Determination of Molecular Weight

Using SDS-PAGE technique, the molecular weight of the *Bacillus licheniformis* MT_1 protease was checked. After mixing the enzyme sample with SDS loading buffer and heat-denaturing it, it was electrophoresed on a 10–12% polyacrylamide gel using standard protein markers. Following Coomassie Brilliant Blue R-250 staining, the relative mobility of the protease was compared to the molecular weight standards to determine its molecular weight. SDS-PAGE analysis was performed according to the method described by Laemmli (1970).

Effect of Reaction Time and Substrate Specificity

Through incubating the enzyme-substrate reaction mixture under ideal test conditions for various time periods spanning from 0 to 110 minutes, the impact of reaction time on protease activity was ascertained. Protease activity was evaluated using the standard protease assay technique after the process was stopped at predefined time intervals. Units per milliliter were used to calculate and express the remaining enzyme activity.

Using several protein substrates at a final concentration of 1% (w/v), the protease's substrate specificity was assessed. Casein, gelatin, and bovine serum albumin (BSA) were among the substrates examined. Protease activity was measured using the standard assay technique, and the enzyme test was performed at the ideal pH and temperature for each substrate. Units per milliliter were used to express the enzyme activity achieved with various substrates.

Effect of pH and pH Stability

Enzyme experiments were performed using suitable buffer systems at various pH values ranging from 6.5 to 11.0 in order to assess the impact of pH on protease activity. The following buffers were used: citrate (pH 6.5-7.0), phosphate (pH 7.5), Tris-HCl (pH 8.0–9.0), and glycine-NaOH (pH 9.5–11.0). The protease assay technique was used to measure enzyme activity under standard assay conditions; the activity at optimal pH was regarded as 100%. The enzyme was pre-incubated under controlled conditions at various pH levels in the same buffer systems for pH stability investigations[32]. Under normal test conditions, residual protease activity was evaluated after aliquots were removed at regular intervals (0–130 min). The residual activity was represented as a percentage of the original activity, with the enzyme activity at 0 minutes acting as the control and being taken as 100%.

Effect of Temperature and Thermal Stability

The effect of temperature on protease activity was evaluated over a range of 20°C to 80°C. Enzyme activity was measured using the standard protease assay after incubating the reaction mixture, containing enzyme and substrate, at the specified temperatures under optimal assay conditions. The maximum activity observed was considered as 100%, and relative activities at other temperatures were calculated accordingly.

Thermal stability of the protease was examined by pre-incubating the enzyme at its optimum temperature in the absence of substrate for varying time intervals (0-100 minutes). At specific time points, samples were withdrawn, immediately cooled, and the remaining enzyme activity was determined under standard assay conditions. Residual activity was expressed as a percentage of the initial activity, with the activity at 0 minutes taken as 100%.

Effect of Metal Ions

The partly purified enzyme was incubated with various metal salts at a final concentration of 5 mM in order to assess the impact of different metal ions on protease activity. AgCl, $AgSO_4$, $BaCl_2$, $Ca_3(PO_4)_2$, $FeCl_2$, H_3BO_3, KCl, KI, $MgSO_4$, $MnSO_4$, molybdic acid, NaCl, and $ZnSO_4$ were among the metal ions that were evaluated. The control was an enzyme solution devoid

of any metal ions. The standard protease assay was used to measure residual protease activity after the reaction mixtures were incubated under ideal assay conditions. When metal ions were absent, enzyme activity was regarded as 100%.

Effect of Organic Solvents

Protease was incubated with the various solvents with an end concentration of 20% (v/v) to determine the stability of the enzyme in the respective solvents. There was a control that was kept solvent-free. The rest of the protease activity was ascertained when the solvent mixtures of the enzyme were subjected to the normal test conditions. The assumption of the control group is that the control group activity was 100%.

Effect of NaCl Concentration

The influence of salinity on protease activity was examined by incubating the enzyme with varying NaCl concentrations ranging from 0.5% to 25% (w/v). A reaction mixture without NaCl served as the control. After incubation under optimal assay conditions, protease activity was measured using the standard assay method. The activity observed in the control was considered as 100%, and the relative activity at different salt concentrations was calculated accordingly.

Effect of Surfactants

The effect of surfactants on protease stability was evaluated by incubating the partially purified enzyme with Tween 20, Tween 80, and SDS at a final concentration of 1% (v/v). After incubation under standard assay conditions, residual protease activity was determined and compared with a control sample without surfactant.

Effect of Urea

The impact of urea on protease stability was investigated by subjecting the enzyme to varying urea concentrations (0.05 M, 0.1 M, and 0.5 M). The control was an enzyme solution devoid of urea. Enzyme activity was assumed to be 100% when urea was not present.

Determination of Km and V_{max} Using Lineweaver–Burk Plot

Casein was used as the substrate to measure the purified protease's kinetic characteristics, such as the Michaelis-Menten constant (Km) and maximal reaction velocity (V_{max}). Under ideal pH and temperature conditions, protease activity was measured at different substrate concentrations ranging from 0.2% to 1.2% (w/v). The standard protease test technique was used to measure protease activity in order to calculate the reaction rate. For every substrate concentration, the first reaction velocities were computed and reported in milliliters per minute. A Lineweaver–Burk double reciprocal plot was created by plotting the reciprocal values of substrate concentration (1/[S]) and reaction velocity (1/V). The slope and intercept of the linear regression line derived from the plot were used to get the Km and V_{max} values.

Detergent compatibility and washing performance

The compatibility of the protease with commercially available detergents (such as Tide, Rin, Ariel, and Wheel) was evaluated by pre-incubating the enzyme with individual detergent solutions. Detergent stocks were prepared at 1.0% (w/v), and 0.3 mL of each detergent solution was mixed with 1.0 mL of enzyme solution, resulting in a final detergent concentration of 7.2 mg/mL. After incubation, the residual enzyme activity was determined using the standard assay method and compared with a control sample that contained no detergent, which was considered as 100% activity.

The potential application of the protease as a detergent additive was further assessed through a washing performance test using white cotton fabric pieces (10 × 10 cm) stained with dal fry. Four experimental setups were prepared: (i) stained cloth washed with 300 mL of water (control), (ii) stained cloth treated with 5.0 mL of crude enzyme in 300 mL of water, (iii) stained cloth treated with a combination of 5.0 mL crude enzyme and 5.0 mL of 1.0% detergent in 300 mL of water, and (iv) stained cloth treated with 5.0 mL of 1.0% detergent in 300 mL of water. All samples were incubated at room temperature for 30 minutes. Following incubation, the cloth pieces were removed, rinsed thoroughly with water, and air-dried.

The stability of the enzyme in the presence of commercial detergents was determined by pre-incubating the enzyme with detergent solutions for 1 h at room temperature, followed by measurement of residual protease activity under standard assay conditions. Washing performance was evaluated qualitatively by visual comparison of stain removal among the different treatment groups. No quantitative image analysis or percentage stain removal measurements were performed in the present study.

Enzymatic Hydrolysis of Gelatin from X-ray Films

In order to sterilize used X-ray films, they were first cleaned with distilled water to get rid of any surface impurities and then wiped with cotton that had been saturated with ethanol. The cleaned films were cut into uniform pieces (2 × 2 cm) after being dried for 30 minutes at 55°C in a hot air oven. In sterile Petri dishes, each film piece was incubated in 20 milliliters of glycine-NaOH buffer (pH 9.0) that contained 1% (v/v) pure protease. The film was completely submerged in the reaction mixture for the 60-minute incubation period at 65°C. To track the elimination of gelatin, observations were made every 20 minutes. To assess non-enzymatic effects, a control experiment was conducted using the same parameters but a buffer devoid of enzyme.

Enzymatic Dehairing of Goat Skin

Goat skin was selected and washed with water to remove salt and other debris. It was cut into small pieces (5cm×5cm) and treated with purified enzyme and 7% sodium sulfide and lime for 24h under shaking at 120 rpm. Pieces of goat skin that were considered negative controls were treated only with distilled water. The pH and temperature for all treatments were adjusted to 9.0 and 55°C, respectively. After the treatment, the process of depilation was evaluated by examining the features such as color, and general appearance of the leather.

RESULTS AND DISCUSSION

Sample Collection Site

Samples were collected from the Tuwa hot spring located in Panchmahal district, Gujarat, India (22.8°N, 73.7°E). The hot spring is characterized by elevated temperature and alkaline conditions favourable for thermophilic microorganisms.

Isolation

A total of twenty-five thermophilic bacterial isolates were obtained from the samples of the Tuwa hot spring. All the isolates were then screened on skim milk agar plates to check their protease production. Among them, five isolates showed significant zones of clearance, indicating proteolytic activity. The diameter of the hydrolysis zones varied among the isolates, suggesting differences in enzyme production capacity. Among the selected isolates, *Bacillus licheniformis* MT_1 exhibited the highest protease activity and was therefore selected for further studies including enzyme production, purification, and characterization. However, the processes of isolation and screening remain major limiting steps in the discovery of novel bioactive compounds from microbial sources (Godfrey, 1985).

Molecular identification

Phylogenetic analysis further confirmed the taxonomic position of the isolate (Fig. 1). The obtained sequences were assembled to generate a consensus sequence and compared with sequences available in the NCBI GenBank database using the BLAST algorithm. BLAST analysis revealed 99% similarity with *Bacillus licheniformis* strains, confirming the identity of isolate MT_1 as *Bacillus licheniformis*. The 16S rRNA gene sequence of isolate MT_1 was deposited in the NCBI GenBank database under accession number PZ221934.1.

Protease assay and total protein estimation

Protease activity was determined using casein as a substrate following the modified Anson method. With a protein content of 6,636.79 mg and a total activity of 398,500 U, the crude enzyme preparation had a specific activity of 60.04 U/mg. After ammonium sulfate fractionation, non-enzymatic proteins were removed, as evidenced by the total enzyme activity of 361,892 U and the decreased protein content of 5,567.57 mg. With a high yield of 90.82% and a 1.08-fold purification, this step raised the specific activity to 65.00 U/mg.

Dialysis and Molecular weight determination

The protease enzyme produced by *Bacillus licheniformis* MT_1 isolated from the Tuwa water sample was successfully submitted to partial purification and molecular characterization, and the findings are presented in the SDS-PAGE profile and purification table (Fig. 2). Crude enzyme extraction and ammonium sulfate precipitation (20–70%) were the steps in the purification process that worked well to improve enzyme purity while maintaining significant catalytic activity described in Table 1.

Table 1
Partial Purification of Protease

Partial purification of protease						
Purification steps	**Total Enzyme (mL)**	**Total activity (U)**	**Protein (mg)**	**Specific activity (U/mg)**	**Fold purity**	**Yield (%)**
Crude enzyme	1450	398500	6636.79	60.04	1	100
Ammonium sulphate Concentrate (20–70%)	45	361892	5567.57	65.00	1.08	90.82%

SDS-PAGE analysis showed a predominant protein band corresponding to the partially purified protease, indicating a substantial improvement in protein purity following ammonium sulphate precipitation. The estimated molecular weight of the enzyme was approximately 29 kDa, which is within the reported range for extracellular proteases from *Bacillus licheniformis*. Similar extracellular serine proteases from *Bacillus* species have been extensively reported for detergent and industrial applications.

The comparison in Table 2 indicates that the protease obtained from *Bacillus licheniformis* MT_1 possesses a favourable combination of high thermostability, alkaline stability, and detergent compatibility compared with previously reported Bacillus proteases. The enzyme also demonstrated effective performance in stain removal and gelatine hydrolysis, highlighting its promising industrial applicability.

Table 2
Comparison of biochemical and industrial properties of alkaline protease from *Bacillus licheniformis* MT_1with previously reported protease

Organism/Strain	Molecular Weight (kDa)	Optimum pH	Optimum Temperature (°C)	Important Characteristics	Industrial/Application Potential	Reference
Bacillus licheniformis MT_1 (Present study)	~ 29	9	80	Thermostable, alkaliphilic, detergent-compatible, stable in surfactants, metal ions, and organic solvents	Laundry detergent additive, gelatin hydrolysis, eco-friendly silver recovery from X-ray films	Present study
Bacillus licheniformis ER-15	58	10	70	Keratinolytic alkaline protease with good thermal stability	Feather degradation and dehairing applications	Tiwary and Gupta (2010)
Bacillus mojavensis	28	9.5	60	Oxidation-stable and thiol-dependent serine alkaline protease	Detergent formulation applications	Beg and Gupta (2003)
Haloalkaliphilic *Bacillus* sp.	30	10	60	Stable under alkaline and saline conditions	Industrial biocatalyst and detergent additive	Gupta *et al.* (2005)
Bacillus sp. AH-101	27	10	75	Extremely thermostable alkaline protease	High-temperature industrial processing	Takami *et al.* (1989)
Bacillus pumilus	31	9	60	Urea- and detergent-stable serine protease	Laundry detergent formulations	Han and Damodaran (1998)

Effect of Reaction Time and Substrate Specificity

Over the course of 0-110 minutes, the impact of reaction time on the activity of the isolated protease from the Tuwa hot spring was assessed. Enzyme activity rose quickly and peaked at 20 minutes (22 U/mL), as shown in Fig. 3A, suggesting effective substrate hydrolysis during the reaction's early stages. The activity then stayed almost constant for 100 minutes (~ 16.5 U/mL), indicating substrate saturation and steady-state kinetics in line with the Michaelis-Menten model. Enzyme instability or product inhibition might be the cause of a modest drop-in activity after extended incubation.

Differential hydrolytic activity toward different protein substrates was found by substrate specificity studies (Fig. 3B). Bovine serum albumin (BSA) had the highest residual activity (68.23 U/mL) among the substrates examined, followed by gelatin (45.3 U/mL); casein (25 U/mL) had the lowest activity. The enzyme's selective catalytic efficiency and potential for industrial applications are highlighted by this preference, which indicates a stronger affinity for globular proteins. Bezawada *et al.* (2011) reported that proteases have shown the highest activity toward casein.

Effect of pH and pH Stability

Over a pH range of 6.5–11.0, the impact of pH on protease activity was investigated. The alkaline nature of the protease was demonstrated by the gradual rise in enzyme activity with pH, which peaked at pH 9.0 (6500 U/mL), as shown in Fig. 4A. Beyond this optimum, a significant drop in activity was observed at higher pH values (pH 10–11), indicating decreased catalytic effectiveness in strongly alkaline environments. Alkaline proteases have an ideal pH of 9.0, which supports the enzyme's potential for industrial use. The enzyme maintained over 98% of its initial activity for 50 minutes, as seen in Fig. 4B, after which it gradually decreased. This stability profile validates the enzyme's resilience in alkaline environments and shows a moderate pH tolerance. A similar optimum pH of 9.0 has also been reported for a protease obtained from *B. cereus* MCM B-326 (Tiwary & Gupta, 2010). The commercially available subtilisin-type of proteases meet these requirements with a pH range of 8.0 to 11.0 and temperature range of 50°C to 70°C (Beg and Gupta., 2002). The level of pH where alkaline protease works best is usually found within the pH 9–11 (Maal *et al.*, 2009; Singh *et al.*, 2001 and Joshi, 2007).

Effect of Temperature and Thermal Stability

Over a temperature range of 20–80°C, the impact of temperature on protease activity was assessed. The thermophilic character of the protease isolated from the Tuwa hot spring was confirmed by the steady rise in enzyme activity with increasing temperature, as shown in Fig. 5A, which peaked at 80°C (7000 U/mL). A progressive decrease in activity was noted beyond this peak, most likely as a result of the enzyme becoming thermally denatured at higher temperatures. The structural modifications that allow for catalytic performance in extremely hot environments are reflected in the high optimal temperature.

The enzyme demonstrated exceptional heat stability by maintaining more than 92% of its initial activity for up to 100 minutes, as seen in Fig. 5B. The enzyme's potential for industrial applications requiring strong and heat-resistant biocatalysts is highlighted by its stability at high temperatures. The optimum temperature of protease ranges from 50°C to 100°C (Takami *et al.*, 1989; Banerjee *et al.*, 1999). Thermostability of the enzymes: *Bacillus sp., Streptomyces sp.* and *Thermus sp*. alkaline proteases were relatively stable at high temperatures and addition of $CaCl_2$ only improved the thermostability of the enzymes (Gupta *et al.*, 2005).

Effect of Metal Ions

To comprehend their modulatory role, the impact of different metal ions (5 mM) on protease activity was assessed. Most alkaline proteases are significantly stabilized by the addition of Ca^{2+}. $MgSO_4$ and $MnSO_4$ considerably increased protease activity, as seen in Table 3, suggesting their potential role as cofactors that support catalysis or stabilize enzyme structure. Ag_2SO_4 and $BaCl_2$ also showed a moderate boost. $FeCl_2$, on the other hand, produced a discernible inhibition, indicating modification of the enzyme's structure or interaction with the active site. The presence of NaCl, KCl, and KI did not affect the enzyme's near-control activity. These findings suggest that protease activity is affected by ions, a feature frequently seen in thermophilic enzymes. Beg *et al.* (2003) revealed that Mn^{2+} ions increased the protease activity up to 36% in *B. mojavensis*, but the activity was inhibited by Cu^{2+} whereas in contrast our enzyme was stimulated in presence of Mn^{2+}. Adinarayana *et al.* (2003) also reported that the protease from *B. subtilis* PE-11 was strongly activated by metal ions such as Mg^{2+}, Ca^{2+} and Mn^{2+}. In our investigation, the stimulation effect was found to be with Ag^{+2}, Ba^{+2}, Mg^{+2}, Mo. Acid and Mn^{+2} and the inhibition effects were with Ag^{+}, Ca^{+2} and Fe^{+2}.

Effect of Organic Solvents

To evaluate the protease's solvent tolerance, its stability in the presence of several organic solvents (20%) was investigated. Ethanol and isopropanol maintained comparatively high residual activity, as seen in Table 3, indicating that the enzyme preserves structural integrity in polar solvents. Additionally, xylene had little negative impacts on activity. Acetone, on the other hand, produced significant inhibition, demonstrating its destabilizing effect on the structure of the enzyme. Hexane and toluene showed moderate decreases in activity, indicating the impact of solvent polarity on enzyme stability. The protease's solvent tolerance indicates that it may find use in commercial bioprocesses that use organic medium. Depending

on the type of solvent system, activity and stability of enzymes are affected due to change in the hydration layer around the protein which results in higher compaction, thereby stabilizing the protein. Protease of A. fumigatus and A. flavus have been reported to be tolerant to organic solvents (Yadav *et al.*, 2011).

Table 3
Effect of metal ions, organic solvents, NaCl concentration, surfactants, and urea on the activity of purified protease from Tuwa hot spring

Parameter Category	Agent / Condition	Concentration	Residual Activity (%)
Metal ions	Control	5 mM	100
	AgCl	5 mM	96.95
	Ag_2SO_4	5 mM	128.90
	$BaCl_2$	5 mM	143.00
	$Ca_3(PO_4)_2$	5 mM	89.20
	$FeCl_2$	5 mM	80.44
	H_3BO_3	5 mM	135.20
	KCl	5 mM	100.70
	KI	5 mM	103.25
	$MgSO_4$	5 mM	146.80
	$MnSO_4$	5 mM	174.76
	Mo acid	5 mM	143.34
	NaCl	5 mM	105.53
	$ZnSO_4$	5 mM	102.80
Organic solvents	Control	20%	100
	Acetone	20%	29.48
	Ethanol	20%	88.88
	Hexane	20%	64.96
	Isopropanol	20%	79.80
	Toluene	20%	70.72
	Xylene	20%	94.52
NaCl concentration	Control	—	100
	NaCl	0.5%	58.92
	NaCl	10%	57.44
	NaCl	15%	55.30
	NaCl	20%	54.48
	NaCl	25%	53.00
Surfactants	Control	1%	100
	Tween 20	1%	64.14
	Tween 80	1%	54.14
	SDS	1%	27.73
Urea	Control	—	100

Parameter Category	Agent / Condition	Concentration	Residual Activity (%)
	Urea	0.05 M	79.48
	Urea	0.1 M	73.36
	Urea	0.5 M	66.00

Effect of NaCl Concentration

The enzyme was incubated with different doses of NaCl to determine how salinity affected protease activity. Even at the lowest tested concentration (0.5% w/v), NaCl reduced enzyme activity to below 60% of the control, as seen in Table 3, indicating that the enzyme is sensitive to NaCl across the entire concentration range examined. Residual activity declined further and gradually as the NaCl concentration increased from 0.5% to 25%, suggesting a progressive, concentration-dependent inhibitory effect rather than a stabilizing one. Nonetheless, the enzyme retained more than half of its activity even at the highest salt concentration tested (25%), indicating a degree of tolerance that may support its use in moderately saline or marine-related industrial applications.

Effect of Surfactants

To assess the stability of the enzyme in detergent-rich environments, the impact of surfactants (1%) on protease activity was examined. Non-ionic surfactants like Tween 20 and Tween 80 reduced protease activity to a lesser extent than the anionic surfactant SDS, as shown in Table 3. This suggests that the enzyme retains partial structural integrity in the presence of non-ionic surfactants. SDS, an anionic surfactant, on the other hand, caused a much more pronounced decrease in activity, most likely as a result of secondary structure disruption and protein denaturation. These results indicate that the protease is comparatively more compatible with non-ionic surfactant systems than with anionic ones, supporting its prospective use in detergent and cleaning formulations that rely on non-ionic surfactants. Rachael *et al.* (2015) reported that *B. licheniformis* 3C5 protease had preserved more than 80% of its initial activity after 1h incubation in the presence of various organic solvents.

Effect of Urea

The effect of urea on protease activity was studied to assess enzyme stability under denaturing conditions. As shown in Table 3, low concentrations of urea retained substantial residual activity, indicating partial resistance to denaturation. However, increasing urea concentration led to a progressive decline in enzyme activity, suggesting gradual unfolding of the protein structure. Despite this reduction, the enzyme retained measurable activity even at higher urea levels, demonstrating moderate structural robustness. This resistance to chemical denaturation further supports the stability of the protease and its suitability for industrial applications involving harsh processing conditions. Urea stable serine protease from *Bacillus pumilus* and *Conidiobolus macrosporus* are reported by Han and Damodaran (1998) and Tanksale *et al.* (2001), respectively.

Determination of K_m and V_{max} Using Lineweaver–Burk Plot

Using different casein concentrations as the substrate, the purified protease's kinetic properties were ascertained. As the casein concentration increased, the substrate saturation curve demonstrated a steady rise in protease activity, plateauing above 0.6% (w/v), signifying substrate saturation and adherence to Michaelis-Menten kinetics (Fig. 6A). The Lineweaver–Burk technique was used to plot the reciprocal values of reaction velocity (1/V) versus reciprocal substrate concentration (1/S) in order to determine kinetic constants (Fig. 6B). The enzyme's typical Michaelis-Menten behavior was validated by the resultant linear plot. The apparent K_m and V_{max} values were computed using the plot's slope and intercept. The higher V_{max} implies effective catalytic activity, whereas the comparatively low K_m value shows a strong affinity of the protease for

casein. These kinetic characteristics indicate the thermophilic protease's applicability for high catalytic efficiency applications in biotechnological and industrial settings. High substrate affinity and effective catalysis were demonstrated by the protease's low K_m value of 0.25 g% and V_{max} of 5000 U/mL. V_{max} is higher as compared to 89.28 U/mL for protease from *Burkholderia stabilis* (Park *et al.*, 2020).

Detergent compatibility and washing performance

The practical application of the alkaline protease isolated from the Tuwa hot spring as a laundry detergent ingredient was assessed by evaluating its detergent compatibility and washing performance using pieces of stained cotton material. Different treatment techniques, such as water alone, detergent alone, protease alone, and a combination of detergent and protease, were applied to cloth samples stained with dal fry. After 30 minutes at room temperature, all treatments were rinsed and dried (Fig. 7). Commercial detergents were heat-treated at 100°C for 15 min prior to use in order to inactivate endogenous detergent proteases that could interfere with enzyme activity measurements.

Visual comparison of the treated fabric pieces indicated noticeable differences among the washing treatments. The control and water-treated samples exhibited minimal stain removal, whereas treatment with protease alone showed visible reduction of proteinaceous stains. The combination of detergent and protease produced the most apparent improvement in stain removal compared with the other treatments Protease treatment alone produced a discernible elimination of proteinaceous stains, indicating the enzymatic degradation of stain components, whereas detergent alone demonstrated a modest decrease in stain. In comparison to other treatments, the combination of detergent and alkaline protease had the best stain removal efficacy, resulting in a noticeably cleaner fabric. The improved performance is explained by the protease and detergent components working in concert to break down protein-based stains enzymatically so that surfactants may more easily remove them. These qualitative observations suggest that the protease is compatible with detergent formulations and has potential as a detergent additive. However, quantitative evaluation of stain removal efficiency will be necessary to confirm these findings in future studies (Kanmani *et al.*, 2011).

Enzymatic hydrolysis of Gelatin from X-ray Films

Alkaline protease treatment of X-ray films produced effective hydrolysis of the gelatin layer in 60 minutes at 65°C in an alkaline environment (Fig. 8). As the incubation period increased, the gelatin layer gradually disappeared, exposing the translucent polyester basis completely. Effective bioprocessing of the film was demonstrated by the enzymatic breakdown of gelatin, which made it easier for embedded silver particles to separate. The specificity of protease-mediated hydrolysis was confirmed by the fact that control films that were treated without enzyme maintained their black gelatin covering. The protease's thermophilic and alkaline characteristics are highlighted by its capacity to operate well at high temperatures and alkaline pH levels. By lowering chemical risks and environmental effect, this enzymatic technology provides a sustainable and environmentally acceptable substitute for traditional chemical methods for recovering silver from old X-ray films.

Dehairing Activity of Protease

The dehairing efficiency of the partially purified protease was evaluated using goat skin, and the results are presented in Fig. 9. The result from glycine-NaOH buffer (20mM, pH 9.0) considered as control, where no dehairing was observed that indicate dehairing was not done by alkaline treatment. While the chemical based dehairing process was reduced the quality of the leather and damage the upper layer of the skin. However, the complete dehairing of the skin treated with protease was achieved after 18 h, which was good in texture and quality as compared to chemical process (Fig. 9).

LIMITATIONS AND FUTURE PERSPECTIVES

Although SDS-PAGE analysis confirmed the apparent molecular weight and relative purity of the partially purified enzyme preparation, no additional electrophoretic activity-based characterization was performed in the present study. Future

investigations involving advanced proteomic approaches may provide further insight into enzyme identity, catalytic properties, and structural classification. The purification strategy employed in this study was limited to ammonium sulfate precipitation followed by dialysis, resulting in a partially purified enzyme preparation. Although this approach was adequate for preliminary biochemical characterization, further purification using chromatographic techniques such as ion-exchange and gel-filtration chromatography will be necessary to obtain highly purified protease for detailed structural, kinetic, and mechanistic studies. Another limitation of the present study is that quantitative statistical analysis based on independent biological replicates was not performed. Future studies should include replicate experiments and appropriate statistical analyses to improve the robustness and reproducibility of the findings.

CONCLUSION

Bacillus licheniformis MT_1, a thermophilic alkaline protease-producing bacterium, was successfully isolated and described in this study from the Tuwa hot spring in Gujarat. Ammonium sulphate precipitation was used to partly purify the enzyme, resulting in improved specific activity and excellent recovery. SDS-PAGE analysis confirmed the apparent molecular weight of the partially purified protease and indicated improved purity following ammonium sulphate precipitation. The protease showed high thermophilic and alkaliphilic properties, with optimum activity at pH 9.0 and 80°C. It demonstrated exceptional structural resilience by maintaining significant stability throughout a broad range of pH, temperature, salinity, and denaturing conditions. A low Km and a noticeable V_{max} were found by kinetic study, indicating excellent substrate affinity and catalytic efficiency. The enzyme demonstrated compatibility with commercial detergents, organic solvents, metal ions, and surfactants. Its efficiency in enzymatic gelatin hydrolysis and detergent-assisted stain removal from used X-ray films for environmentally friendly silver recovery was validated by application tests. The alkaline protease from *Bacillus licheniformis* MT_1 demonstrated effective enzymatic dehairing of goat skin, highlighting its potential as an environmentally friendly biocatalyst for sustainable leather processing applications. All things considered, the enzyme has great industrial promise for use in detergents and environmentally friendly bioprocessing.

Declarations

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The authors used AI tools solely for language refinement. No AI was used in data collection, analysis, or interpretation.

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AUTHOR CONTRIBUTIONS

All authors contributed to the study and design.

Trivedi Mansi: Conceptualization, Methodology, Writing, Review and Editing, Formal Analysis, Investigation, Writing original Draft, Validation

Patel Jigisha and Arya Prashant: Resources, Methodology

Patadia Hemantkumar: Conceptualization, Review and Editing, Validation

All authors read and approved final manuscript.

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COMPLIANCE WITH ETHICAL STANDARDS

This article does not contain any studies with human participants performed by any of the authors.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this published article.

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Figures

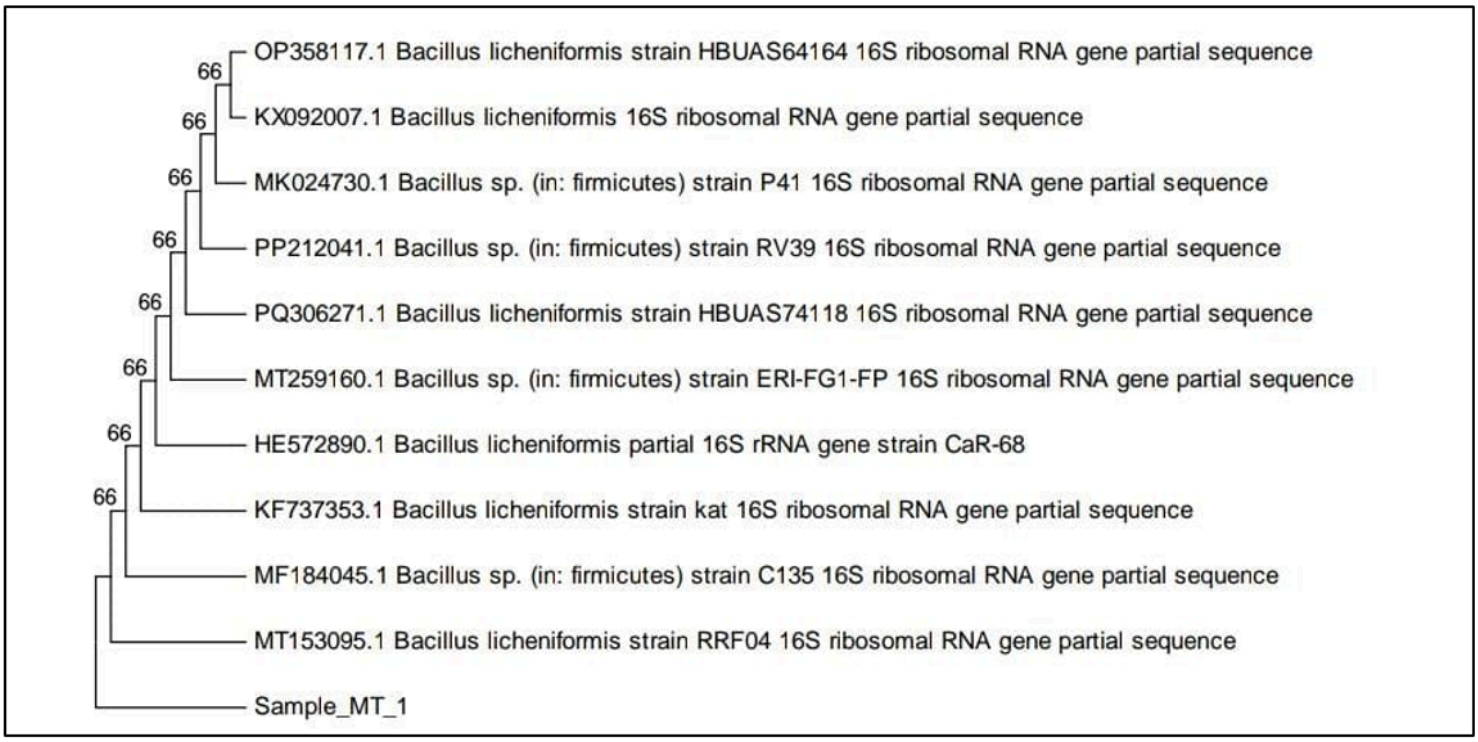


Figure 1

Phylogenetic analysis of *Bacillus licheniformis* MT_1 based on 16S rRNA gene sequences

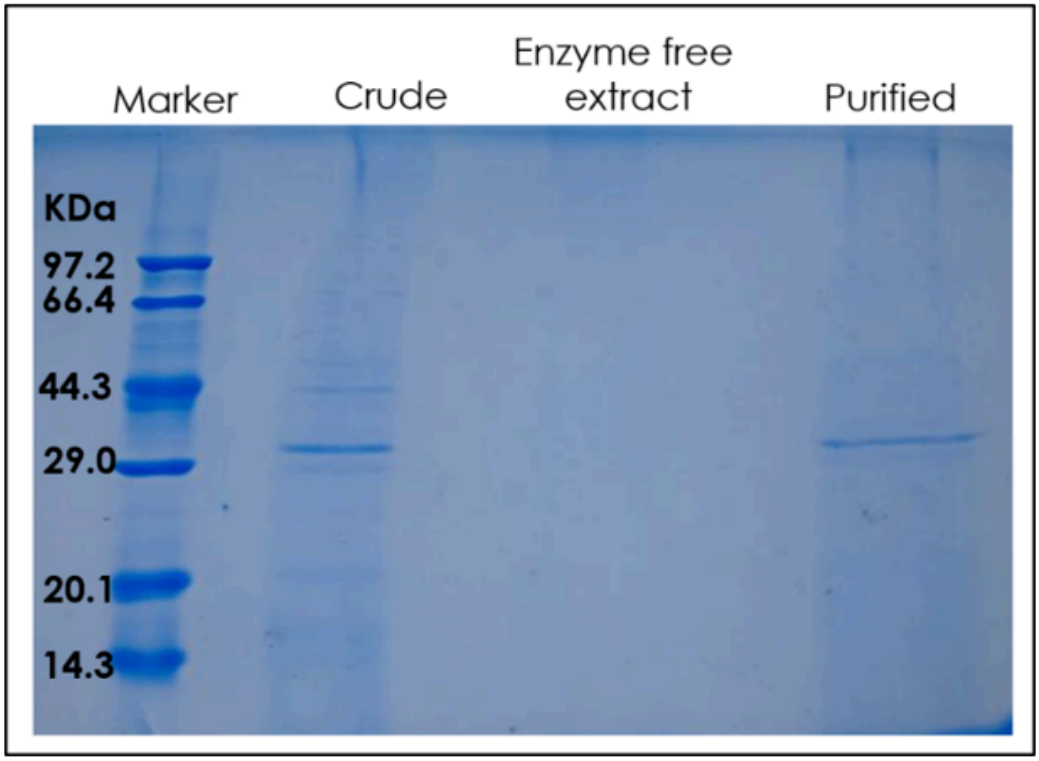


Figure 2

Determination of Molecular weight by SDS-PAGE

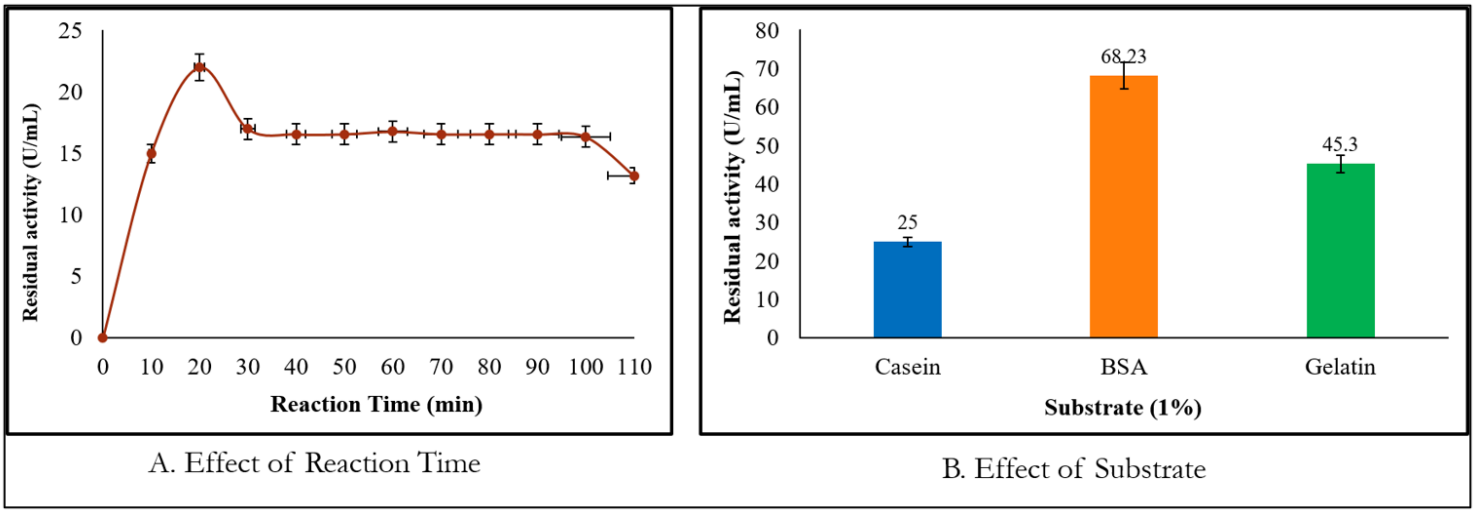


Figure 3

Effect of Reaction Time and Substrate Specificity on Protease activity

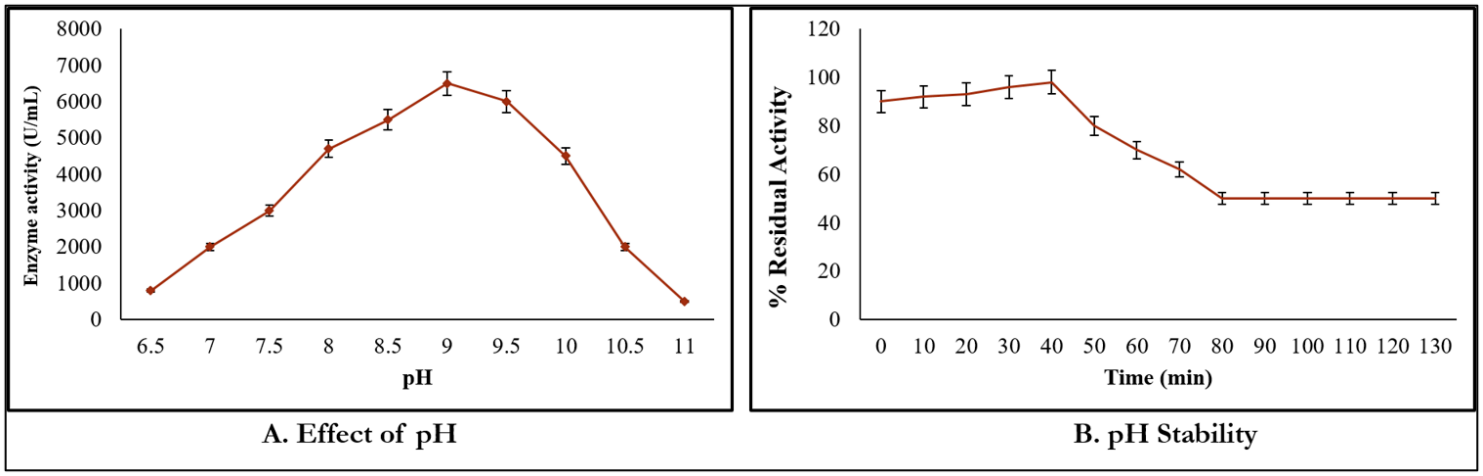


Figure 4

Effect of pH and pH Stability

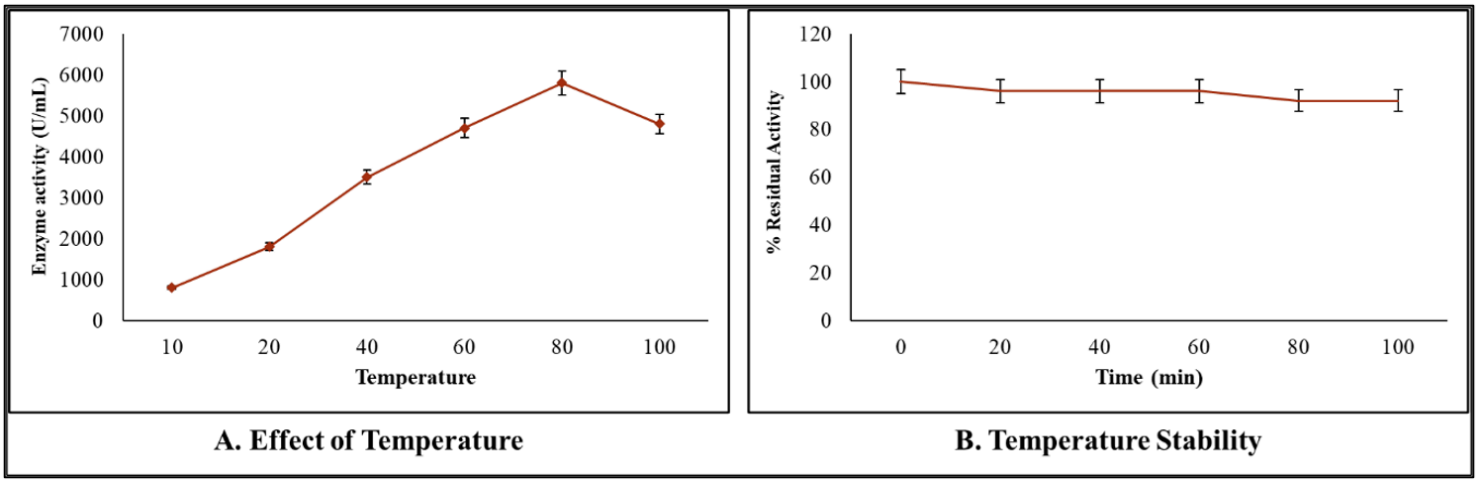


Figure 5

Effect of temperature and temperature stability

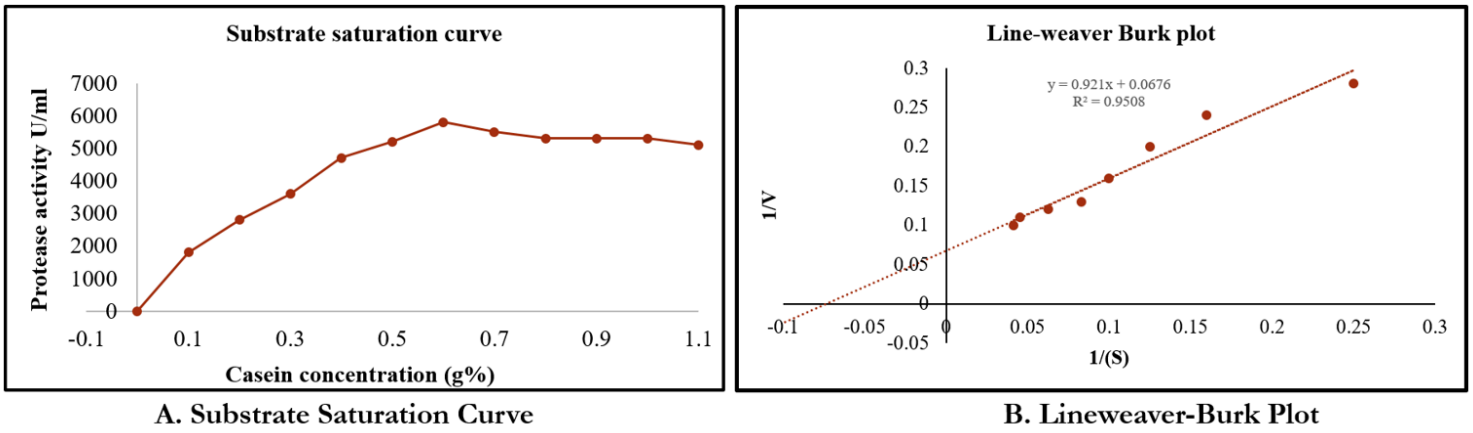


Figure 6

Determination of Km and V_{max} of purified protease using Lineweaver–Burk plot

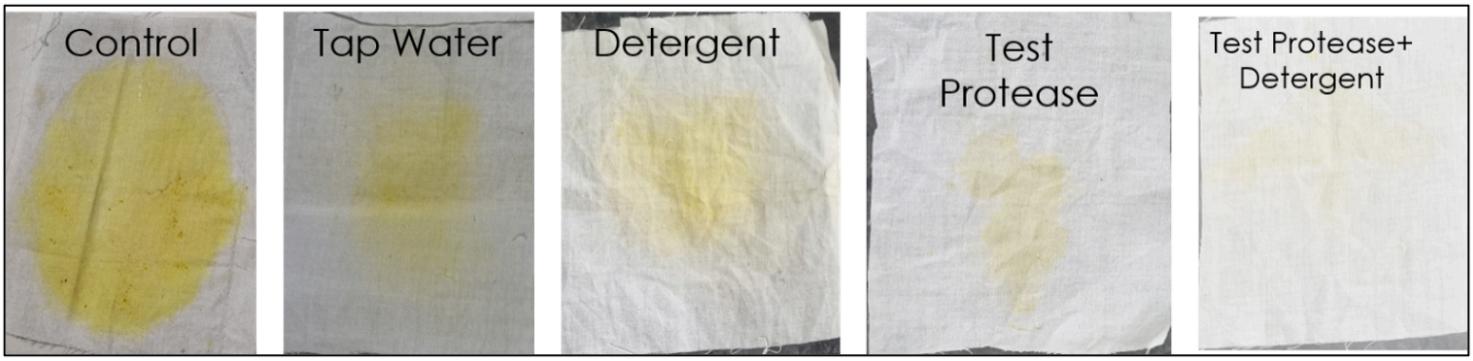


Figure 7

Detergent compatibility and washing performance

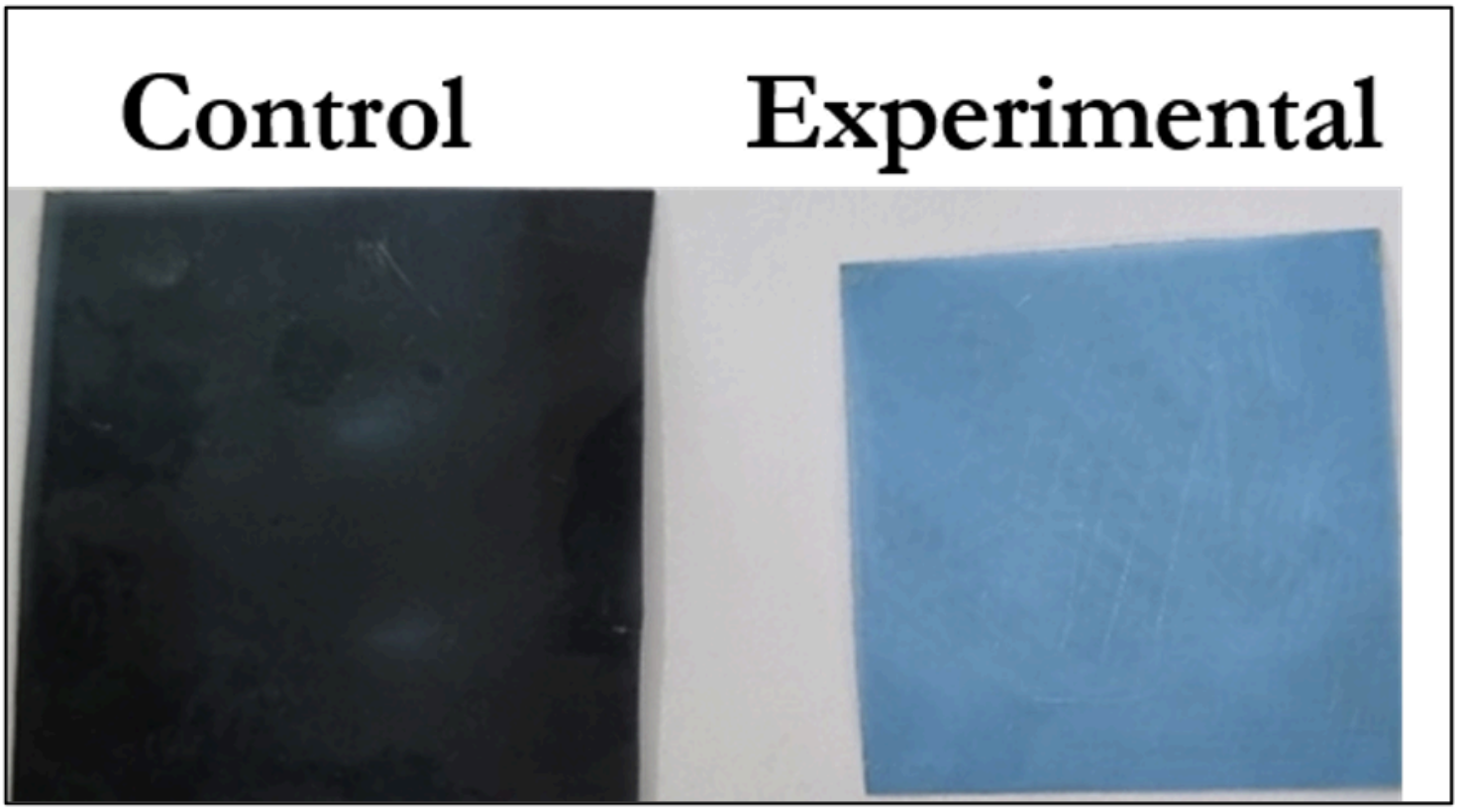


Figure 8

Enzymatic Hydrolysis of Gelatin from X-ray Films



Figure 9

Dehairing of Goat skin