

# Hide Dehairing and Laundry Detergent Compatibility Testing of Thermostable and Solvents Tolerant Alkaline Protease from Hot Spring Isolate *Bacillus cohnii*U3

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**Abstract:** In the present study, alkaline protease producing thermophilic *Bacillus cohnii*U3 strain was isolation from Unnai hot spring, India. The maximum production of protease (344 U/mL) was reported after 72 h of inoculation at 50°C in shake flask culture using the gelatin casein medium. The protease was found to remain active up to 96 h and production was growth dependent. The activity of parital purified protease was reported in abroad range of pH ranging from 4.0 to 11.0 with an optimum of 9.0 pH; Enzymes was indicated the highest activity at 50°C temperature. Salt independent catalysis and activity with a broad range of substrate concentration is the key feature of the protease. Thermos table nature, the stability in alkaline pH and stability in high salt concentration for 45 min were outstanding features of protease. The enzyme was stable in the presence of various organic solvents like Dimethyl Sulphoxide (DMSO), Methanol, Butanol, n-Hexane, Benzene at 25% (v/v) concentration. A good compatibility of the enzyme with most commercial detergents indicated its application in detergent industry. The remarkable dehairing in goat hide and destaining of blood spot in 2 h using 10 U/mL of protease assure that it could be a potential candidate for leather and detergent industries.

**Keywords:** Alkaline Protease, *Bacillus cohnii*, Hot Spring, Solvent Tolerant, Detergent Compatible, Hide Dehairing

## Introduction

With an increasing emphasis on environmental protection, the use of hydrolytic enzymes particularly from thermophiles, has gained significant attention in many industrial processes. Enzymes from thermophiles are of particular interest since the high temperature does not usually denature them and remain active at elevated temperature (Adams and Kelly, 1998). Furthermore, they are more resistant to chemical reagents and extreme pH in comparison to their mesophilic homologues (Hough and Denson, 1999). Their thermostability is associated with performing the enzymatic reaction at high temperature allows higher substrate concentrations, lower viscosity, the fewer risk of contamination and often higher reaction rates (Seatovic *et al.*, 2004). Therefore, thermophiles were the promising choice for massive enzyme production for industrial applications. Global requirements of thermostable biocatalysts are far greater than those of the

mesophilic biocatalysts suggesting the huge need of thermophilic enzymes (Haki and Rakshit, 2003).

Thermophilic bacteria have gained a significant attention to industrial scale. Microbiologists are more interested in thermophilic bacteria as a natural source of enzymes that are active and stable at elevated temperatures. Hot springs are the rich source for the thermophilic bacteria for the direct isolation of thermostable enzymes (Olajuyigbe and Kolawole, 2011). Recently, few hot spring isolated thermophilic *Bacillus* species are well studied for the alkaline protease production including, *Bacillus licheniformis* (Dudhagara *et al.*, 2014), *Bacillus subtilis* (Priya and Archana, 2008) and *Geobacillus sp.* (Wei *et al.*, 2007).

*Bacillus* species are the main producers of extracellular proteases (Pant *et al.*, 2015). Use of the *Bacillus* species for protease offers several advantageous like significant activity, stability, substrate specificity,

short period of fermentation, mere downstream purification and low cost production (Maurer, 2004; Anissa *et al.*, 2009; Hazem *et al.*, 2012).

Thermostability is the crucial properties of enzyme for industrial application. The application of thermostable enzymes offers higher reaction rates, increased half-life and operational stability, increased the resistance towards organic solvents and lower contamination risks during fermentation.

Proteases are the single class of enzymes which occupy a pivotal position with respect to their wide applications in commercial fields. Protease is widely employed as technical grade enzymes in various industrial processes. The largest consumption of protease is in laundry detergent and leather industries (Banerjee *et al.*, 1999). In detergent industries, alkaline proteases are employed as cleansing additives and preferably, proteases should be stable in detergents and at a broad range of pH and temperature (Gupta *et al.*, 2002). Enzymatic dehairing is being increasingly looked upon as a reliable alternative to the conventional lime-sulphide process, to avoid the problems created by the sulphide pollution (Paliwal *et al.*, 1994).

In the present study, we had isolate and identify the proteolytic thermophilic *Bacillus cohnii*U3 from Unai hot spring, Gujarat state, India. The study also probed enzyme's suitability through characterization for the biotechnological purpose, especially for the detergent and leather industries.

## Materials and Methods

### *Isolation and Identification of Bacteria*

Isolation of strain was carried out by the enrichment of water collected from Unnai hot spring (20°85'33"N, 73°33'42"E), India into Gelatin Casein (GC) broth containing g/L: gelatin, 5.0; casein enzymatic hydrolysate, 1.0; peptone, 5.0; yeast extract, 1.5; meat extract, 1.5; NaCl, 5.0; pH 7.5 followed by incubation at 50°C for 48 h under shake flask conditions. After 48 h, a loop full of culture was streaked on same solid medium and incubated at 50°C. Protease production was detected by plate assay. Strain U3 was identified by 16s rRNA gene sequencing. The gene sequence was submitted to NCBI nucleotide database with accession number GU979027.1.

### *Protease Production and Partial Purification*

The inoculum was developed by inoculating a single colony of isolate into 25 mL of sterile GC medium pH 7.5 and incubated at 50°C on a shaker for 24 h. A 10% inoculum ( $A_{660\text{nm}}$ : 1.0) was added to GC broth (pH 7.5) and incubated for 96 h at 50°C under shaking condition (120 rpm). Growth was measured colorimetrically at 660 nm and culture was harvested by centrifugation at 8000

rpm for 10 min at 5°C. The cell-free extract was used as crude preparation to measure protease activity. Partial purification of the protease was done using 70% saturation of anhydrous Ammonium sulphate followed by dialysis. The partial purified protease was used for further experiments throughout the study.

### *Protease Assay*

Protease activity was measured by Anson-Hagihara's method using the 0.6% casein as a substrate (Hagihara, 1958). One unit of protease activity was defined as the amount of enzyme liberating one  $\mu\text{g}$  of tyrosine/min under standard assay conditions. Enzyme units were measured using tyrosine (0 to 100  $\mu\text{g}$ ) as standard.

### *Growth Profile of Protease Secretion*

The kinetics of growth with enzyme secretion in the GC broth was performed to arrest the maximum enzymes secretion time. Enzymes activity and growth were measured up to 96 h at every 6 h regular interval.

### *Effect of pH, Temperature, Salt and Substrate on Protease Activity*

In order to investigate the influence of pH on protease activity, 0.5 mL of protease was added to 3.0 mL of 0.6% casein prepared in 20 mM Acetate buffer (pH 4.0 and 5.0), 20 mM Phosphate buffer (pH 6.0, 7.0 and 8.0) and 20 mM Borax-NaOH buffer (pH 9.0, 10.0 and 11.0). The enzyme activity (U/mL) was measured as per the standard enzymes assay mentioned as earlier. The effect of temperature on protease activity was examined at various temperatures by incubating enzyme reaction mixtures for 10 min at different temperatures including 25, 30, 35, 40, 45, 50, 55 and 60°C afterword enzyme activity was measured. To study the effect of salt, 0 to 1%, w/v of NaCl was added into enzyme reactions and protease assay was performed. The substrate effect on protease activity was determined by taking the 0.2 to 2% w/v of casein concentrations that was prepared in phosphate buffer pH 7.5. The activity of the enzyme was measured as described previous.

### *Stability of pH, Temperature and Salt on Protease Activity*

The protease was preincubated at three different pH i.e., 5.0 (acidic), pH 7.0 (neutral) and pH 9.0 (alkali) at 50°C for 45 min. The enzyme assay was carried out after 15, 30 and 45 min interval under standard assay conditions and residual activity were measured. Thermostability of protease was measured by pre-incubation of the protease up to 45 min at 25°C to 60°C and residual activity of the enzyme was calculated using standard assay. Salt pretreatment was given to the protease using 0 to 1% w/v NaCl concentration followed by the incubation up to 45 min and residual enzyme activity was determined by assay.

### *Solvent Stability of Protease*

Five organic solvents including DMSO, Methanol, Butanol, n-Hexane, Benzene along with Distilled water were used for solvent stability. One ml of 25% v/v organic solvent was added to 3.0 mL of the protease in screw-capped tubes and incubated at 30°C in ashaker at 120 rpm up to 5 days. Enzyme activity was measured at defined time interval under standard assay condition. Stability is expressed as the remaining proteolysis activity relative to the activity in the presence of distilled water (Geok *et al.*, 2003).

### *Compatibility of Protease with Commercial Detergents*

The Protease was pre-treated with 0.7%, w/v of commercial detergents including Ariel, Tide, Surf excel, Rin and Wheel and residual activity was measured after 15, 30 and 60 min.

### *Digestion of Natural Proteins*

Fresh goat hide purchased from the local market was prewashed with 1% formaldehyde and sterile distilled water. The hide was cut in 2×2 cm size and incubated with the 10 U/mL protease in phosphate buffer (pH 8) at 50°C for two hours. Similarly, 10 U/mL protease was incubated with blood saturated cloth and incubated at 50°C for two hours. Natural proteins hydrolysis observed at regular time interval.

## **Results**

### *Isolation and Identification of Organism*

U3 strain was optimally grown at pH 8.0 at 50°C temperature on GC media. The isolate was Gram-positive, rod shape, spore-forming bacterium. Isolate was identified as a moderate thermophilic *Bacillus cohnii* by 16s rRNA sequence analysis. The BLASTn result shows the 99% similarity with *Bacillus cohnii* strain GS47 (accession number KP053306.1). The 16S rRNA gene sequence-based phylogeny is presented in Fig. 1.

### *Protease Production and Optimization*

After 12 h, the isolate indicates the exponential growth and it maintains up to 66 h followed by a stationary phase. Maximum biomass was reported at 72 h and protease secretion corresponded with the growth. The highest enzymes secretion was found 344 U/mL at the interphase of the late exponential to early stationary phase (Fig. 2). Enzymes activity after the 72 h was found to slightly decrease due to the proteolysis of enzymes. Production medium after 42 h appeared very dense and turned in dense yellow color due to the high concentration of biomass and enzyme.

### *Characterization of Protease*

#### *Effect of pH, Temperature, Salt and Substrate on Protease Activity*

The protease was optimally active at alkaline pH with maximum activity (255.25 U/mL) at pH 9.0 and showed low activity at less than 9.0 pH values. Protease activity at pH 10 was 203.85 U/mL that is double than the activity at pH 7.0. Moreover the activity at pH 4.0 and 5.0 is very less. So it is clear that the enzyme is alkaline protease required the alkaline pH for the catalysis. Protease was found to active in wide range of temperature; however the optimum activity (169.20 U/mL) was reported at 50°C and nearly identical activity (155.0 U/mL) at 55°C. The protein catalysis between 35°C to 55°C was reported maximum. The enzyme appears thermo-active as there was better catalysis at 60°C than the 25°C temperature. The protease was optimally active in the absence of the salt, however activity of the enzyme was marginally declined to 0.2% (w/v) NaCl and the further increase the salt concentrations led to the significant reduction in the activity. The optimum requirement of the substrate was reported 0.6% w/v, less than 0.6% dramatically decreases the activity. More than optimum substrate was also inhibited the enzymes activity and negligible activity was reported at 1% substrate (Table 1).

#### *Stability of pH, Temperature and Salt on Protease Activity*

Protease was stable for prolonged time and maintaining over 85% of its original activity at pH 5.0, 7.0 and 9.0 during 30 min incubation followed by decreasing activity with increasing incubation at each pH (Fig. 3). The protease was retained nearly 70% of activity after 45 min incubation at each temperature ranging from 25°C to 55°C. At 60°C protease activity was drastically reduced (Fig. 4). Prolong incubation decreased the enzyme activity. Loss of activity was high at low temperature. The enzyme was found to unstable in the presence of salt as the maximum stability was found in the absence of salt. Stability of the enzyme was decreased by an increase the incubation time at each tested salt concentration. In 1% salt concentration after 30 min only 62% activity was remained (Fig. 5).

### *Solvent Stability of Protease*

Protease showed remarkable stability in all tested solvents. Very less reduction in enzyme stability was reported after 24 h incubation in the presence of all solvents except benzene. After 5 days' incubation, stability in the presence of n-Hexane and DMSO were more than 92 and 85% respectively. Greater than 70% activity was found after 3 days' incubation with all the solvents (Table 2).

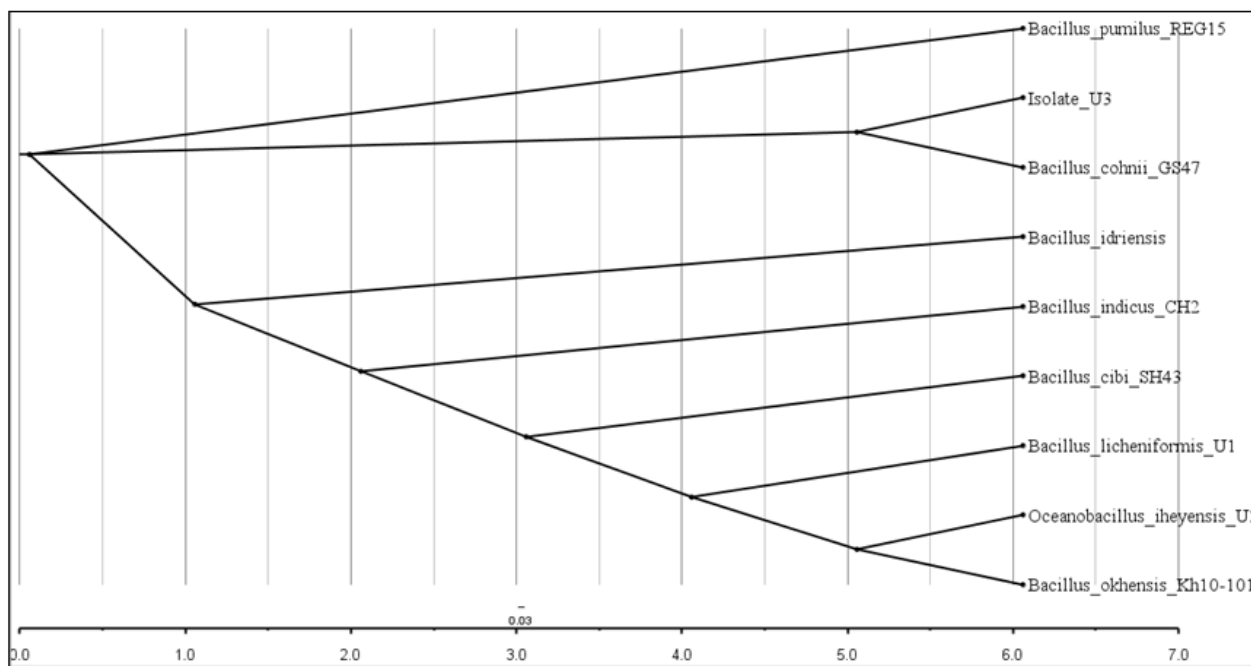


Fig. 1. 16S rRNA gene sequence based phylogenetic analysis of *Bacillus cohnii* strain U3. The phylogenetic tree constructed by the neighbor-joining method using ClustalW2 showing the position of isolate U3 with scale axis

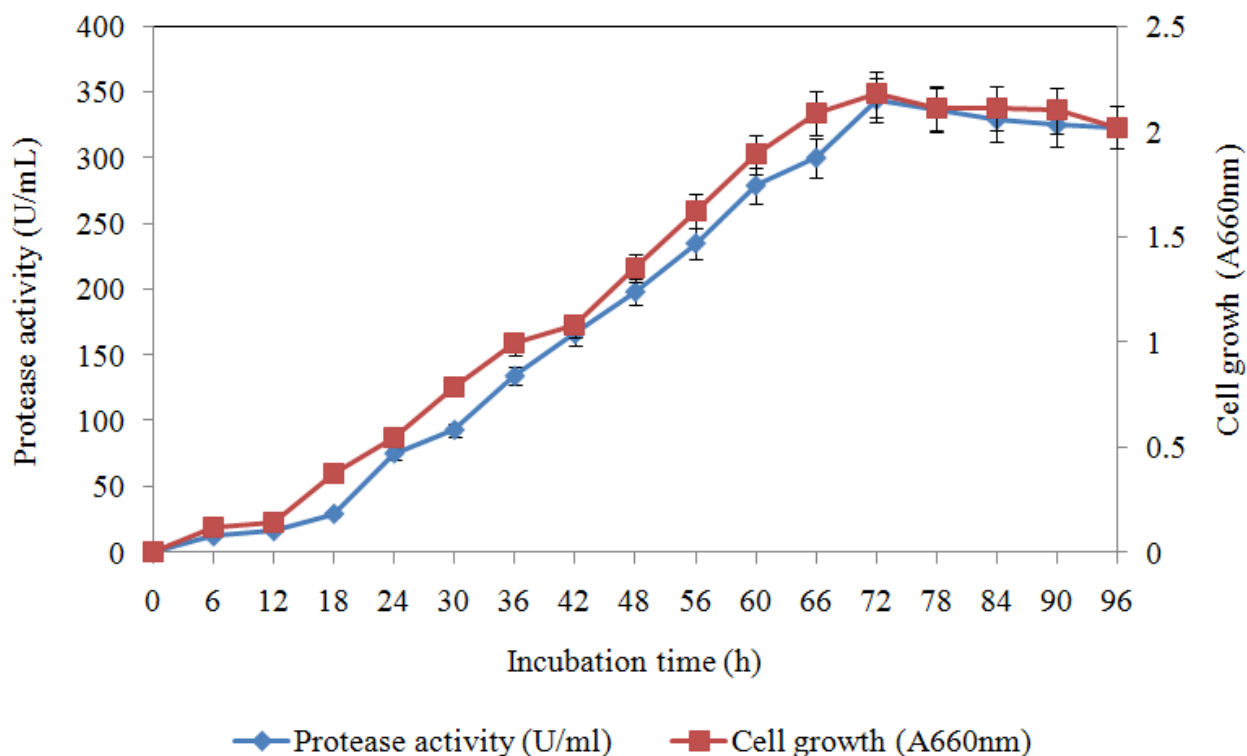


Fig. 2. Growth profile of *Bacillus cohnii* strain U3. The bacterium was grown in gelatin casein medium as described in text. Samples were withdrawn aseptically at every 6 h interval for the determination of cell growth and protease activity. Results represent the means of three experiments and bars indicate  $\pm$  standard deviation. Absence of bars indicates that errors were smaller than symbols

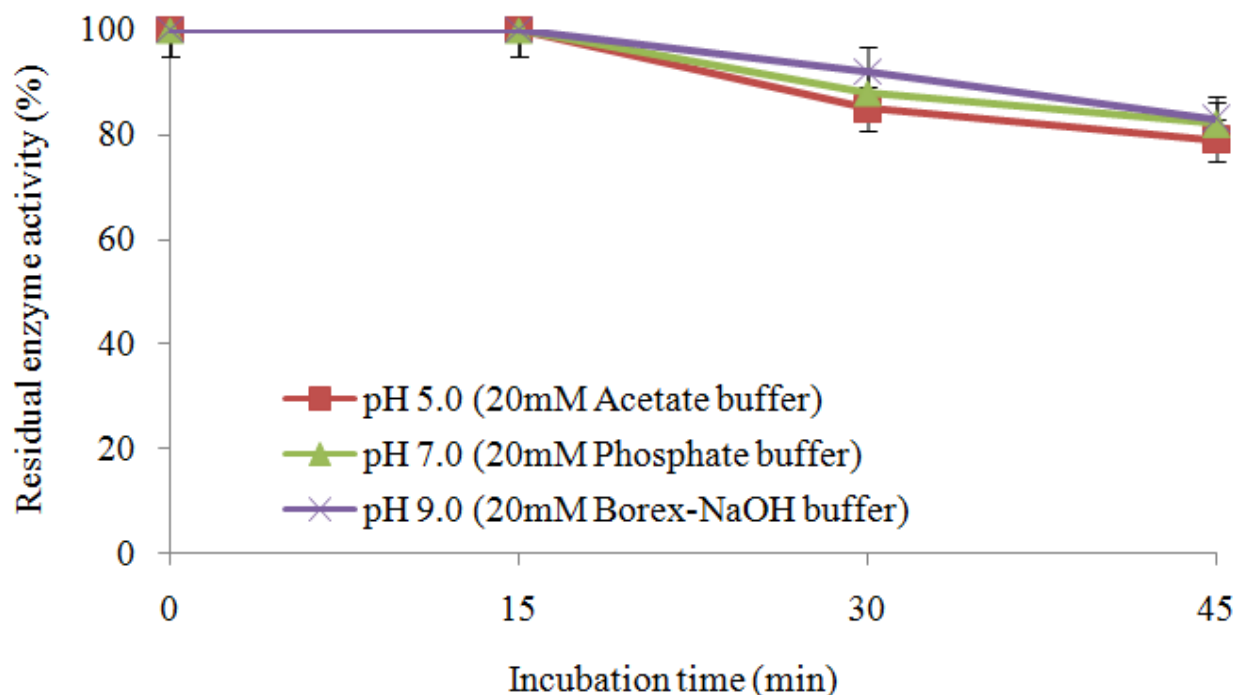


Fig. 3. Effect of pH on the stability of the protease. The enzyme was pre-incubated at 5.0, 7.0 and 9.0 pH and residual activity was determined from 0 to 45 min at 15 min intervals. Activity of protease after 15 min was considered as the control (100%). Results represent the means of three experiments and bars indicate  $\pm$  standard deviation

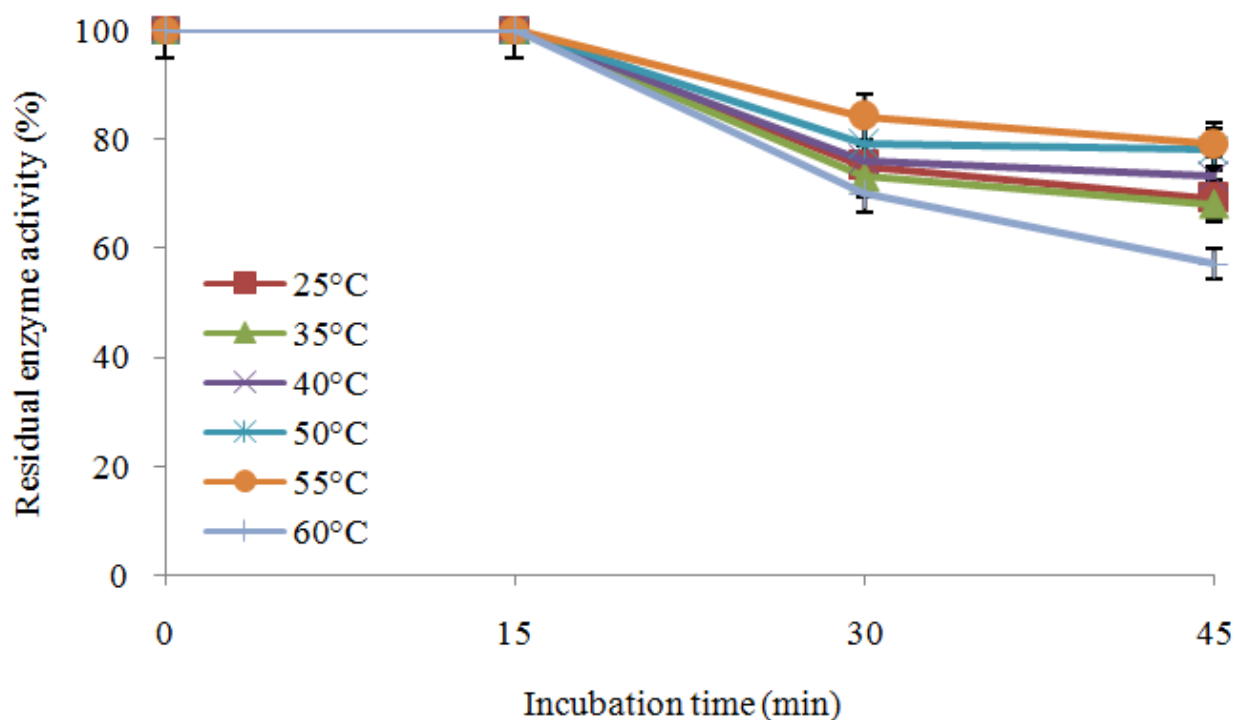


Fig. 4. Effect of temperature on the stability of the protease. The enzyme was pre-treated at 25, 35, 40, 50, 55 and 60°C and residual activity was determined from 0 to 45 min at 15 min intervals. The activity of protease after 15 min was considered as the control (100%). Results represent the means of three experiments and bars indicate  $\pm$  standard deviation

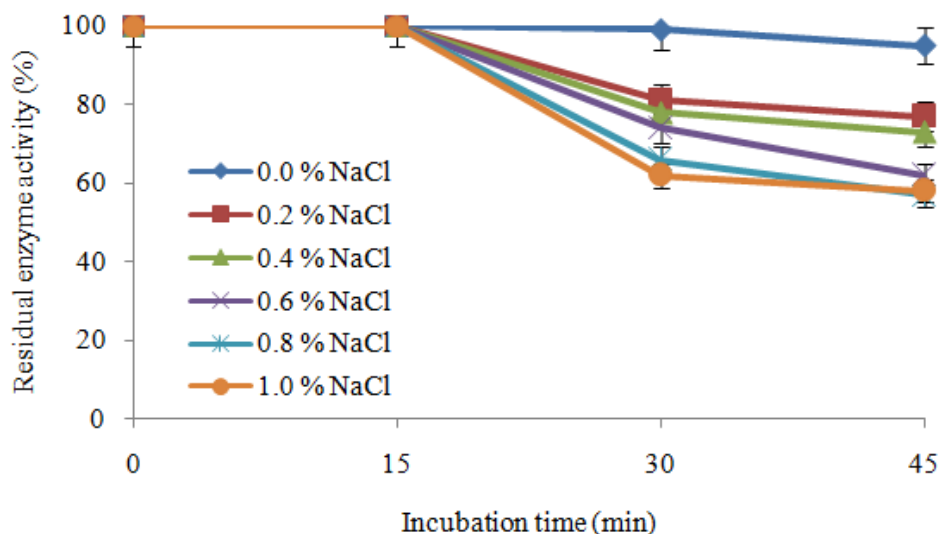


Fig. 5. Effect of NaCl concentration (%) on the stability of the protease. The enzyme was pre-treated at 0.0 to 1.0% salt concentration and residual activity was determined from 0 to 45 min at 15 min intervals. The activity of protease after 15 min was considered as the control (100%). Results represent the means of three experiments and bars indicate  $\pm$  standard deviation

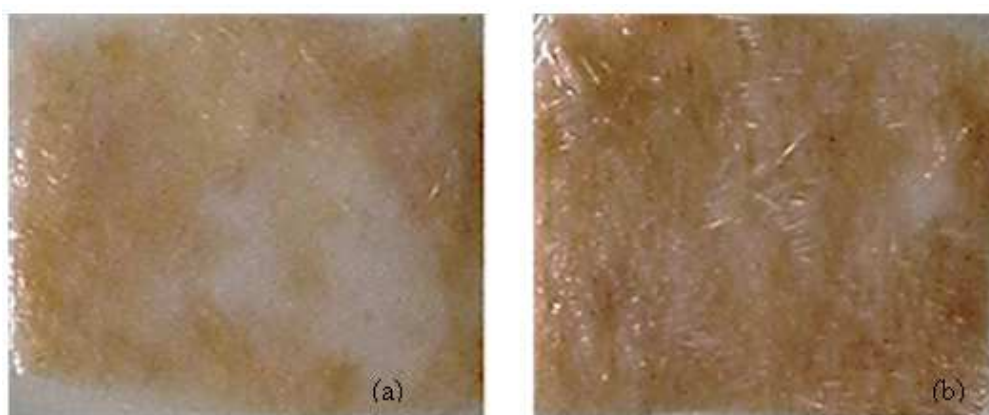


Fig. 6. Dehairing of goat hides (a) Protease treated (10 U/mL) hide showing dehairing (b) No dehairing in control (without protease) hide

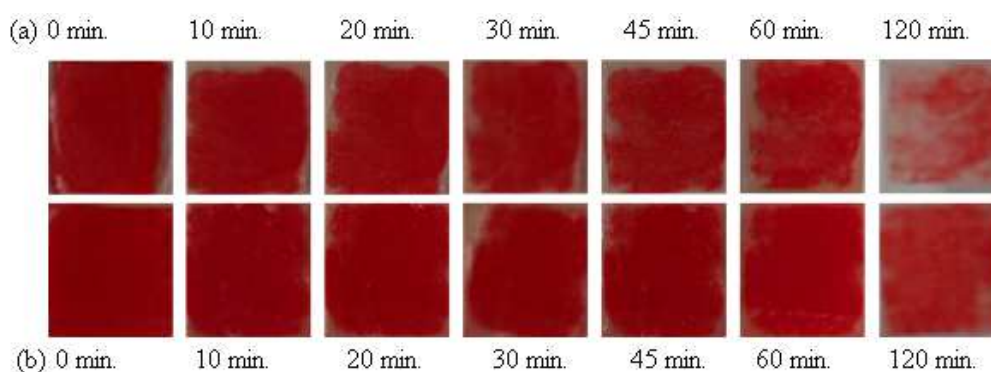


Fig. 7. Destaining of blood on cloths (a) Protease treated (10 U/ml) cloth showing the removal of blood (b) negligible removal of blood showed in control (without protease)

Table 1. Effects of pH, temperature, salt and substrate on protease activity. It values in the table represent the mean of three experiments

| Physico-Chemical Parameters | Scale            | Enzyme activity (U/mL) |
|-----------------------------|------------------|------------------------|
| pH                          | 4.0              | 44.10                  |
|                             | 5.0              | 75.15                  |
|                             | 6.0              | 89.55                  |
|                             | 7.0              | 93.15                  |
|                             | 8.0              | 155.25                 |
|                             | 9.0              | 255.15                 |
|                             | 10.0             | 203.85                 |
|                             | 11.0             | 99.45                  |
| Temperature (°C)            | 25.0             | 75.15                  |
|                             | 35.0             | 109.35                 |
|                             | 40.0             | 134.55                 |
|                             | 50.0             | 169.20                 |
|                             | 55.0             | 155.00                 |
|                             | 60.0             | 94.50                  |
| NaCl (%w/v)                 | 0.0              | 297.00                 |
|                             | 0.2              | 244.35                 |
|                             | 0.4              | 144.00                 |
|                             | 0.6              | 104.40                 |
|                             | 0.8              | 81.00                  |
|                             | 1.0              | 31.50                  |
|                             | Substrate (%w/v) | 0.2                    |
| 0.4                         |                  | 58.05                  |
| 0.6                         |                  | 144.45                 |
| 0.8                         |                  | 113.85                 |
| 1.0                         |                  | 103.95                 |
| 1.2                         |                  | 85.05                  |
| 1.6                         |                  | 64.35                  |
| 2.0                         |                  | 45.90                  |

Table 2. Solvent stability of protease. Residual enzyme activity (%) before the solvent mixing was considered as 100%. Relative to the residual activity of the distilled water at each day was kept as control to calculate the residual activity of five tested organic solvent

| Sr. No. | Solvents        | Log P value | Residual enzyme activity (%) after incubation (days) |       |       |       |
|---------|-----------------|-------------|--|-------|-------|-------|
|         |                 |             | 0  | 1     | 3     | 5     |
| 1       | Distilled water |             | 100  | 98.33 | 96.99 | 95.67 |
| 2       | DMSO            | -1.35       | 100  | 93.66 | 88.78 | 85.36 |
| 3       | Methanol        | -0.82       | 100  | 82.21 | 75.99 | 71.33 |
| 4       | Butanol         | 0.83        | 100  | 81.19 | 72.66 | 68.99 |
| 5       | Benzene         | 2.45        | 100  | 79.56 | 72.33 | 67.73 |
| 6       | n-Hexane        | 3.6         | 100  | 99.33 | 95.79 | 92.48 |

Table 3. Stability of protease in the presence of 0.7% laundry detergents. Activity of protease without the detergent was considered as 100%

| Detergent treatment time (min) | Residual enzyme activity (%) at specific time interval in the presence of 0.7% commercial available laundry detergents |        |        |            |        |        |
|--------------------------------|--|--------|--------|------------|--------|--------|
|                                | Control (Without detergent)  | Ariel  | Rin    | Surf excel | Tide   | Wheel  |
| 0                              | 100.00   | 100.00 | 100.00 | 100.00     | 100.00 | 100.00 |
| 15                             | 98.30  | 93.66  | 91.33  | 90.99      | 90.10  | 95.68  |
| 30                             | 95.57  | 89.10  | 84.93  | 84.42      | 83.99  | 90.33  |
| 60                             | 92.29  | 81.33  | 77.66  | 76.67      | 75.45  | 83.23  |

### *Compatibility of Protease with Commercial Detergents*

Good detergents stability was testified in the 0.7% concentration of each detergent. The enzyme was more compatible with Wheel and Ariel detergent as there was a greater than 80% activity after 60 min. Moreover, protease stability in all the detergent was more than 84% after 30 min (Table 3). Negligible loss of activity in the control is evident due to the prolong shaking.

### *Digestion of Natural Proteins*

The result of dehairing in goat hide in 2 h was remarkable. The visible difference of dehairing between enzyme treated and control hide can be observed (Fig. 6). Blood stain in fabric was lessened gradually with prolong incubation and removed within 2 h by enzyme treatment (Fig. 7).

## **Discussion**

Hot spring is the bio-prosperity sink of the thermophiles and industrial important thermoenzyme. Recently, culture-independent methods revealed the presence of many enzyme producing bacteria in the Indian subcontinent's hot springs (López-López *et al.*, 2013; Ghelani *et al.*, 2015; Mangrola *et al.*, 2015). Thermophilic microorganisms have the adaptability to survive in harsh environmental conditions. The enzymes from thermophiles hold the structural secrets to remain stable at elevated temperature (Panda *et al.*, 2013). The strain *Bacillus cohnii* was isolated from the less explored Unnai hot spring, where the temperature usually reported between 57°C to 61°C. Earlier, thermophilic protease producing *Bacillus licheniformis* was reported from the Unnai hot spring (Dudhgara *et al.*, 2014). *Bacillus* species are attractive industrial tools for a variety of reasons, including their high growth rates leading to short fermentation cycle times, their capacity to secrete proteins into the extracellular media and the Generally Regarded As Safe (GRAS) status with the food and drug administration (Manikandan *et al.*, 2014).

The relationship between growth and enzyme secretion has been well studied in *Bacillus* species with maximum secretion reported in early stationary phase (Patel *et al.*, 2005; Karan *et al.*, 2011), which is concur with our result. Enzyme activity in late stationary phase and decline phase was remarkable; suggest the excellent structural stability of the enzyme and remains unfolded for prolong time. Use of the crude and partially purified enzymes is preferred over purified preparation to avoid the cost of purification and make the processes commercially viable. Recently, the applications of surfactant and detergent compatible crude protease from *Bacillus mojavensis* have been studied (Anissa *et al.*, 2010).

The optimum pH and temperature for casein-degrading activity were 9.0 and 50°C respectively. It is indicative of alkaline and moderately thermoactive the nature of enzyme (Kirankumar *et al.*, 2009). The pH and temperature optimum of protease is quite better than the alkaline protease of spring isolate *Bacillus* species (Badhe *et al.*, 2009). The Salt-independent catalytic activity is suggestive of the cations in dependent thermostability nature of the enzyme. A good catalytic activity with various concentration of substrate is well suitable for the many industrial processes.

Stability at the various pH, temperature and salt concentration is the key properties to explore the enzyme at industrial scale. The results of stability study correspond to earlier reports (Nejad *et al.*, 2010; Nascimento and Martins, 2006). The enzyme exhibited good solvent tolerance to all the tested organic solvent. Almost similar stability trends for proteases in the presence of various organic solvents were reported in *Pseudomonas aeruginosa* and *Bacillus licheniformis* (Gupta and Khare, 2006; Sareen and Mishra, 2008). However, the effect of organic solvents on protease activity differs depending on types of protease and bacterial strains. The protease may be solvent stable because of the replacement of some water molecules in an enzyme with organic molecules leads to stabilizing the structure of the enzyme (Ghorbel *et al.*, 2003).

The enzyme preparation exhibited the dehairing in goat hide reveals the keratinolytic nature of the enzyme. A similar application was evaluated in hot spring isolate *Bacillus subtilis* (Priya and Archana, 2008). This finding is crucial in enzymatic dehairing of hide in leather industries. The enzyme may have an impending application as a dehairing agent (Vijay Kumar *et al.*, 2011). The result of blood destaining with the protease in 2 h supports the impending eco-friendly application (Rao *et al.*, 2009).

## **Conclusion**

Hot spring is the rich source of the protease producing bacteria. The isolate can grow at alkaline pH and elevated temperature so contamination free system can be designed for mass production of protease. The partial purified protease from the *Bacillus cohnii* was found thermostable alkali stable and clearly indicate the robust nature and good stability in the presence of wide range of physical and chemical parameter. Additionally, suitable solvent stability along with compatibility with detergents proved the extreme character of the enzyme. It is a valuable enzyme and can be utilized for the large-scale processes at a leather processing. Enzyme holds remarkable catalysis in harsh condition suggest the impending application in hide dehairing and detergent additives.



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## Author's Contributions

**Pravin R Dudhgara:** Designed the research plan and concept, Conduct the study and complete the data-analysis, Prepared the manuscript, Communicating the manuscript.

**Sunil Bhavsar:** Take part in all the experiments starting from sampling to result of each experiments and performed the data analysis.

**Anjana Ghelani:** Participate and coordinate the in experiments, Reporting the result and collection of data, Manuscript preparation and correction.

## Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

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