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Research Article

ISOLATION, CHARACTERIZATION AND INVESTING THE INDUSTRIAL APPLICATIONS OF THERMOSTABLE AND SOLVENT TOLERANT SERINE PROTEASE FROM HOT SPRING ISOLATED THERMOPHILILIC *Bacillus licheniformis* U1

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Abstract

Protease is the largest selling enzyme in the world due to its various applications in the various industries such as detergent, food and leather, meat tenderisation and pharmaceutical industries. The aim of the study is to isolate and identify thermophilic *Bacillus licheniformis* U1 strains for thermostable protease production. The partial purified enzyme was characterized under different conditions using Anson-Hagihara's method. Casein as a substrate in the concentration of 0.6 % w/v optimum for enzyme activity and tolerant up to 2.0% casein concentration. An optimum enzyme activity was reported at pH 7 and decreased with increase in pH, while temperature optima was found to be 50 °C. The enzyme was stable at 40 °C to 50 °C for half an hour and nearly 50% residual activity was indicated at 60 °C. NaCl was not required for catalysis. Stability of enzymes in the presence of various organic solvents and different detergents was remarkable. The enzyme was stable up to 3 days into various solvents and slowly denatured with prolonged incubation. The result of the washing performance with detergent was clearly indicated. Moreover the removal of blood stains and dehairing in goat skin suggests the crucial application in the commercial production at large scale.

Key words: Protease; *Bacillus licheniformis;* Thermostable; Solvent stability

Introduction

Microbial proteases are the most important hydrolytic enzymes and have been studied extensively since the advent of enzymology. Now-a-days there seems a renewed interest in the study of proteolytic enzymes, mainly because of its important role in the cellular metabolic processes which has gained the substantial attention in the industrial community (Gupta *et al.*, 2002). So it is an important enzyme in physiological and commercial processes (Chellappan *et al*., 2011). At present proteases account for about 60% of total worldwide enzyme sales in various industrial sectors like detergent, food, pharmaceutical, leather, diagnostics, waste management and silver recovery (Gupta *et al.*, 2002, Rao *et al.*, 1998). In fact, only 2% of the microbial communities have been tested as sources of industrial important enzymes (Wiseman A, 1985). From extreme environments, only a small fraction of microorganisms is explored as an important source of enzymes as they tend to result in novel process applications (Govardhan and Margolin, 1995).

The ability of thermophilic bacteria to grow at high temperature and to produce thermostable extracellular enzymes was attributed to the probability of increasing their enzyme application. Therefore, these microorganisms were the first candidates for massive enzyme production for industrial applications. *Bacillus* species are major sources of industrial and commercial valuable enzymes, however, some of thermophilic *Bacillus* species have been reported to produce useful enzymes (Rao *et al.*, 1998, Rahman *et al.*, 1994). Few thermophilic strains such as *Bacillus stearothermophilus* (Sookkheo *et al.*, 2000), *Bacillus licheniformis* (Ferrero *et al.*, 1996), and *Bacillus pumilus* (Kumar, 2002) were explored for protease production. However, the earlier studies did not deal with the stability and the application evaluation of proteases, which offers new possibility and potential for their biotechnological applications. In fact, enzymes from such microorganisms are not only thermostable and active at high temperature, but also often resistant to organic solvents and detergents (Hawumba *et al.*, 2002). The thermal stability is not due to any specific characteristic but a result of the various changes

which contribute to the whole stability of the protein in an additive manner (Vieille *et al*., 1996).

The application of thermostable enzymes offers higher reaction rates, increased half-life, operational stability, increased resistance towards organic solvents and lower contamination risks during fermentation. 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). Alkaline protease is widely employed as technical grade enzymes in detergent and leather industries. Serine proteases have Aspartate (D) and Histidine (H) residues along with Serine (S) in their active site forming a catalytic triad. Serine proteases contribute to one third share in the alkaline protease enzyme market (Page and DI Cera, 2008).

Though *Bacillus licheniformis* is the model system for the production of thermostable alkaline protease, thermotolerant and thermophilic strains are very less reported. Moreover commercial production of protease for detergent and leather industries are in the budding stage in the developing country like India. Having based on the ideal properties of thermostable protease, the aim of the study was to isolate and identify the proteolytic thermophilic *Bacillus licheniformis* from less explored Unai hot spring water sample. It was also aimed at ascertaining its suitability through characterization for biotechnological exploitation, especially for the detergent and leather industries.

Materials and Methods

Isolation and screening of thermophile protease producer

Thermophilic bacterium U1 was isolated by enrichment culture technique from Unnai hot spring water sample, South Gujarat, India. 1 ml of water was added to a gelatin casein (GC) medium 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 8.0 followed by incubation at 50 °C for 48 h under shake flask conditions. After 48 h, a loopful of culture was streaked on same solid agar medium and morphologically distinguished colonies were selected. Potent protease producer U1 strain was selected based on gelatin hydrolysis zone on the plate.

Characterization of isolate

Biochemical characterization was carried out using various biochemical tests described in Bergey's Manual of Systematic Bacteriology along with the basic morphological and cellular characterization.

Identification of isolated by 16S rDNA sequencing

For 16S rRNA gene sequencing, the genomic DNA of the strain U1 was isolated using Hi-media bacterial genome isolation kit (Hi-Media Laboratories, Mumbai, India). After testing the purity of DNA by electrophoresis on 0.8 % agarose gel, DNA was subjected for the amplification of 765 bp long 16S rDNA fragment using universal primers followed by sequencing and similarity search using BLASTn.

Decoding the protease gene

From the purified genomic DNA, the protease was identified by amplification of the gene encoded in the protease using the species specific primers- ParpA, 5′-CATGCCATGGATGGTGAGGAAGAAGATTTTTGGC-3′ and ParpB, 5′-GAATCTCGAGTTGAGCGGCAGCTTCGACATTG-3′. The amplification was carried out using procedure describe by Tang (Tang *et al*, 2004). Nearly 445 bp long fragments amplified and decoded by Sanger sequencing method followed by BLASTn search to categorize the enzyme.

Enzyme production and activity assay

The inoculum was prepared by adding a loopful of pure culture into 25 ml of sterile GC medium pH 8.0 and incubated at 50 ºC on a rotary shaker for 24 h. A 10% inoculum from the culture (at A_{660}; 1.0) was added to the same medium and incubated at 50 ºC under shaking condition at 150 RPM. Growth was measured colometrically at 540 nm and culture was harvested by centrifugation at 8000 RPM for 10 min at 5 ºC. The cell-free extract was used as a crude preparation of enzyme to measure protease activity. The enzyme activity was estimated by the Anson-Hagihara method using casein as a substrate (Hagihara, 1958). Exactly 0.5 ml enzyme was added to 3.0 ml 0.6% w/v casein prepared in 20 mM sodium phosphate buffer, pH 8.0 and the reaction mixture were incubated at 50 °C for 10 min before the addition of 3.2 ml of TCA mixture containing 0.11 M trichloroacetic acid, 0.22 M sodium acetate, 0.33 M acetic acid. The terminated reaction mixture was incubated for 30 min at room temperature. White precipitates were removed by filtration with Whatman No. 1 filter paper and the OD of the filtrate was measured at 280 nm in UV-Visible spectrometer. One unit of protease activity (U) was taken as the amount of enzyme liberating 1 µg of tyrosine per min under the standard assay conditions. The estimations were based on a tyrosine calibration curve.

Growth kinetics and protease production

Culture was grown in gelatin casein medium as described above. Samples were withdrawn aseptically at specific time interval and growth was measured at A_{540}. Samples were centrifuged at 8,000 RPM for 10 min at 4°C and the cell free extract was used as a source of the crude enzyme to estimate protease activity. The enzyme was precipitated and fractioned by ammonium sulfate with 75% saturation under standard condition. The precipitated enzyme was further purified by dialysis. Remaining liquid was dissolved in the 2 ml of 20mM phosphate buffer pH 8 and used for the subsequent study.

Enzyme characterization

Effect of pH on protease activity and stability

In order to investigate the influence of pH on protease activity, the 0.5 ml enzyme was added to 3.0 ml of 0.6 % w/v

casein prepared in 20mM Acetate buffer (pH 4 and 5), 20mM Phosphate buffer (pH 6, 7 and 8) and 20 mm Borax–NaOH buffer (pH 9, 10 and 11) and assay was performed as described above. The pH stability was determined by the pre-incubating the protease at pH 5, 7 and 9 for 45 minutes and residual activity was measured at every 15 minute interval.

Effect of temperature on protease activity and stability

The effect of temperature on protease activity was determined by performing the standard assay at a temperature ranging from 25ºC to 60ºC. Thermal stability was determined by pre-incubating the protease at temperatures of 25ºC, 35ºC, 40ºC, 50ºC, 55ºC and 60ºC for 45 min and residual activity was determined.

Effect of salt on protease activity and stability

To evaluate the effect of salt, different concentration of NaCl ranges from 0 to1%, w/v was added to the substrate followed by determining the activity of crude enzyme using standard assays. Salt pre-treatment with same concentration for 45 min. was given to test the stability of protease and residual activity was measured at every 15 min intervals.

Effect of substrate on protease activity

In order to determine the substrate tolerance, various concentrations of casein ranging from 0.2 to 2%, w/v were prepared in phosphate buffer pH 8.0 and the activity of protease was determined by Anson-Hagihara's method.

Effect of organic solvents on the stability of crude protease

Dimethyl sulphoxide, Methanol, Butanol, n-Hexane and Benzene were used in the study along with distilled water as an aqueous solvent. 1ml of 25% v/v of each organic solvent was added separately into 3.0 ml of the crude protease in screw-capped tubes and incubated at 30°C, 120 RPM up to 10 days, Enzyme activity was measured at definite time interval under standard assay condition. Stability is expressed as the remaining proteolytic activity relative to activity at 0 day incubation (Geok *et al.*, 2003).

Compatibility of protease with commercial detergents

The enzyme was pre-treated with 0.7 %, w/v of commercial detergents namely Ariel, Tide, Surf excel, Rin and Wheel and residual activity was measured at every 15 min interval for an hour.

Destaining of blood

Clean pieces of white cloth (2x2 cm) were stained with human blood and allowed to dry for 15 min followed by soaking in 2% v/v formaldehyde for 15 min and washed twice with sterile distilled water to remove excess formaldehyde. The cloth pieces were immersed with 5 U/ml of partially purified enzyme and incubated at 50°C with shaking condition. Destaining was observed at specific time interval. After every time-interval, the cloth pieces were rinsed with water for 2 min and then dried. The same procedure was done for the control except incubation with the enzyme solution.

Dehairing of skin

Goat's skin was cut into 2x2 cm pieces and incubated with the partially purified protease (5 U/ml) in 20mM phosphate buffer (pH 8) at 50 ºC for 8 hours along with the control. The skin was observed for removal of hair after 8 hours.

Washing performance of protease as a detergent additives

Washing efficiency of the purified enzyme was studied by mixing with 0.7% Ariel detergent. White cotton cloth pieces (1x1cm) stained with blood, chocolate, and barbecue sauce separately. Cloth pieces were soaked in 2% formaldehyde for 15 min and washed twice with sterile distilled water to remove excess formaldehyde and dried. They were immersed in the following three different systems (a) 20 ml Distilled water (Control). (b) 20 ml enzymes (5 U/ml) and (c) 10 ml enzymes (10 U/ml) + 10 ml of 0.7 % Ariel detergent. Then the samples were incubated at 50 °C for 15 min with shaking condition and rinsed thoroughly with distilled water and dried. Visual examination of various pieces was carried out.

Result and Discussion

Screening and identification of protease producer thermophile

Spore-forming-rod-shaped Gram positive bacterium was isolated from the hot spring and selected as a potent protease producer based on the clear zone of gelatin and casein hydrolysis on the gelatin casein agar plate. Colonies of isolate U1 were raised and irregular shaped with an opaque appearance on gelatin casein agar media. The isolate was able to utilize the wide range of sugars. Catalase reaction, litmus milk test, H_2S production and Triple Sugar Iron agar test showed positive reaction, whereas indole production test, phenylanine deamination, urea hydrolysis, nitrate reduction, ammonium production and citrate utilization were showing negative reaction Utilization of starch, gelatin and casein indicated the catalytic potentials of isolates.

The isolate was identified as a moderate thermophilic *Bacillus licheniformis* using the morphological, biochemical characters and 16S rDNA sequence analysis. The BLASTn result shows the 99 % similarity with *Bacillus licheniformis.* Partial sequence of the 16s rRNA gene was submitted in the NCBI nucleotide database with accession No. GU979026.

Identification of protease

Nearly 445 bp long sequence of protease gene was 99% similar to serine protease of *Bacillus licheniformis* NH1 strain. The sequence was submitted to the NCBI nucleotide database with accession No. HM569711. The result suggests that the isolate produces the serine protease.

Growth kinetics and enzyme production

Growth of isolate in the gelatin casein medium with reference to the enzyme production indicated that the lag phase of the organism was up to 1 hour, after which the growth was exponential up to 42 hours followed by stationary phase (Fig. 1). Pink coloured pigmentation was

observed in fermentation medium in early stationary phase and vanished in late stationary phase. Protease secretion was found to be growth dependent and reached maximum at 42 hours during the late exponential phase. This result is supported by the investigation of Deejing *et al.* (2005) who showed that the production of protease from *Bacillus licheniformis* K61 strain was recorded maximum in exponential phase. Maximum production 403.65 U/ml is nearly 7 times higher than the alkaline protease from *Bacillus cereus* MTCC 6840 (Joshi *et al.*, 2007). About 55% enzyme activity remained in the medium after 69 hours indicating that the enzyme is quite stable and resistant to denaturation (Patel *et al.*, 2005).

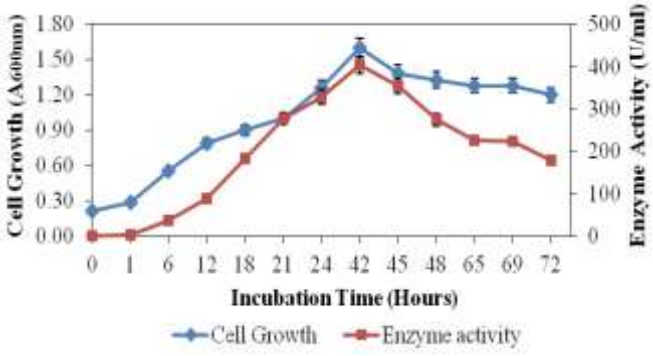

Fig. 1- Growth kinetics with references to protease production

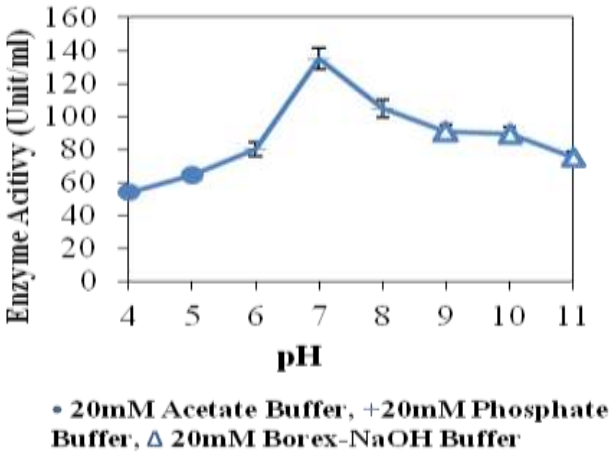

Fig. 2- Effect of pH on enzyme activity

Effect of pH on activity and stability of enzymes

The enzyme was active in a wide scale of pH ranging from 4.0 to 11.0 with an optimum at pH 7.0. The enzyme retained more than 65% of activity at pH 9.0 and 10.0 showing alkali tolerance (Fig. 2). The pH range of catalysis is relatively wide compared to reported thermophilic proteases (De Azeredo *et al.*, 2004). The enzyme was very stable in a broad pH range and maintaining over 85% of its original activity up to half an hour at pH 5.0 to 11.0 followed by decreasing activity with increasing incubation at each pH (Fig. 3).

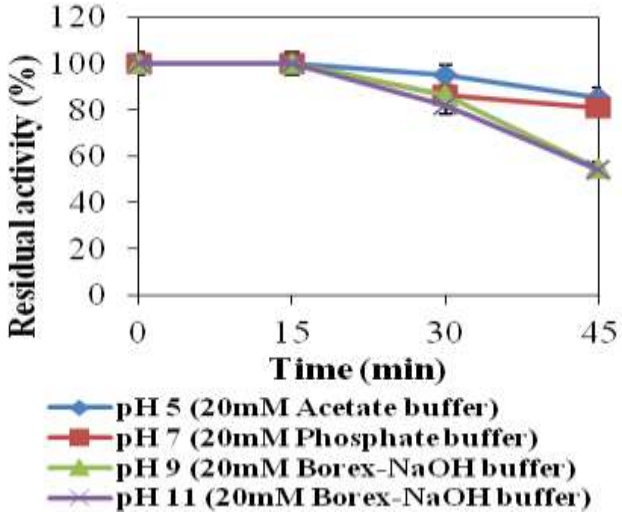

Fig. 3- pH stability of enzyme

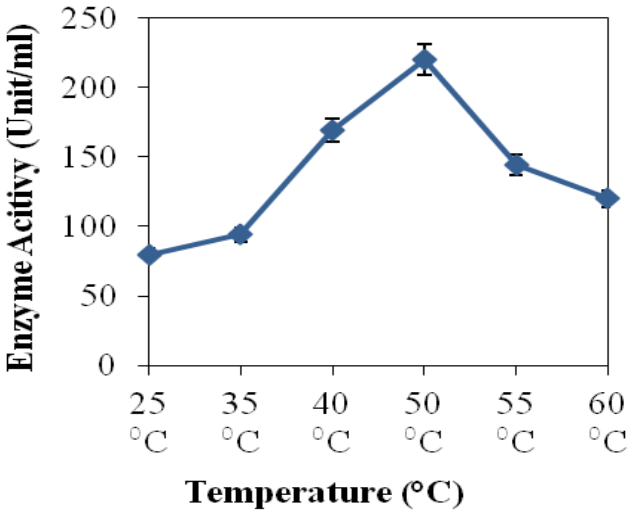

Fig. 4- Effects of temperature on enzyme activity

Effect of Temperature on activity and stability of enzymes

The enzyme was thermoactive in a temperature range of 25°C to 60°C temperature with optimum at 50°C followed by a decline 55°C however it retained about 55% activity at 60°C. Moreover, activity at 60°C was comparatively higher than activity at below 40°C (Fig. 4). The result suggests elevated temperature required for optimum catalytic activity. The thermostability of the partially purified enzyme in 20 mM phosphate buffer, pH 7.0 was remarkable as it retained 75% activity up to 30 min incubation at temperature ranging from 35°C to 60°C and prolonged incubation cause the loss of activity. Stability at elevated temperature is significantly higher than 25°C to 35°C temperature suggests the thermostable nature of enzyme (Fig. 5). The result are in agreement with earlier reports (Deejing *et al.*, 2005). Thermal stability was independently decreased at all the temperature values tested with respect to increase the incubation time. Thermostability of isolate is quite similar to many reported moderate thermophilic enzymes, but higher than those of most mesophilic bacterial proteases (Nascimento and Martins, 2006).

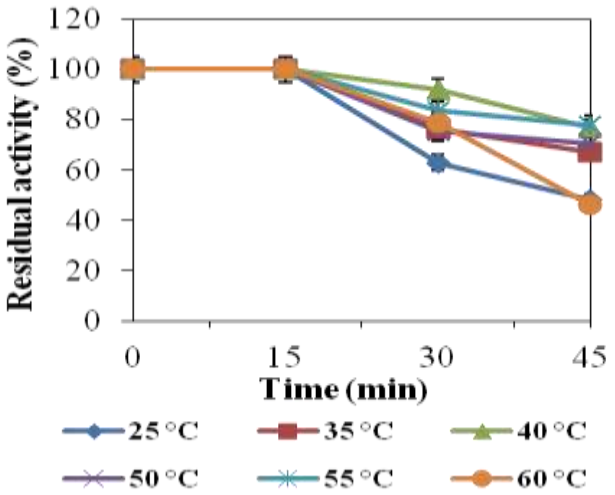

Fig. 5- Temperature stability of enzyme

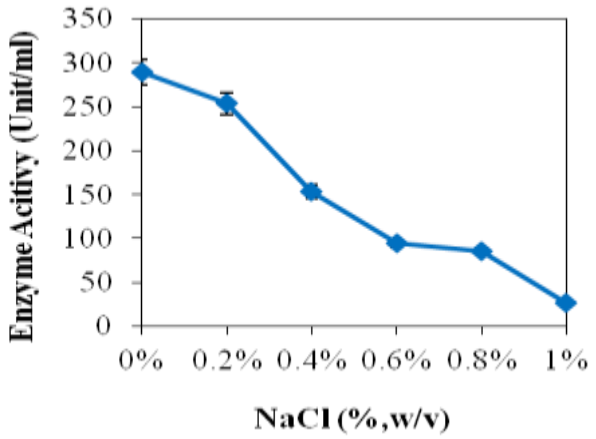

Fig. 6- Effects of Salt concentration on enzymes

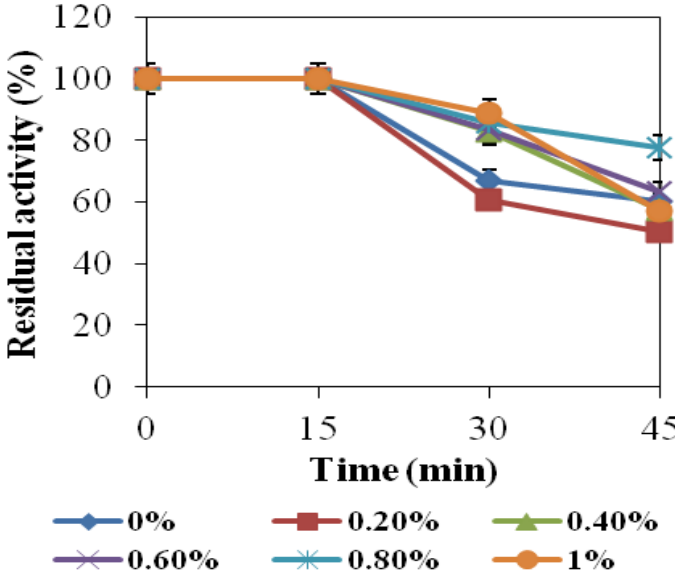

Fig. 7- Salt stability of enzyme

Effect of salt on activity and stability of enzymes

The effect of NaCl on purified enzyme was studied at 50 °C in 20 mM phosphate buffer at pH 7. NaCl was not required for catalysis and activity of the enzyme was marginally declined at 0.2% (w/v) NaCl, the further increase in salt concentrations led to decrease the activity significantly. Observations suggest the salt-independent catalysis exhibited by the enzyme (Fig. 6). However enzyme was found to remain stable for short periods in the presence of salt concentration ranging 0.2 to 1.0 % (Fig. 7). Hence, the study suggests the thermostability does not rely on salt requirements. The stability and activity of the enzyme in the absence of salt make it quite interesting for wider applications in biotechnological processes.

Effect of substrate on activity of enzymes

Optimum requirement of the substrate was observed to be 0.6 %, w/v. Lower concentration of casein dramatically decreases the activity and high concentration inhibited the enzyme activity marginally (Fig. 8). High concentration of substrate cause the substrate level inhibition of the enzymes, however in the present investigation the enzyme tolerate up to 2 % w/v casein concentration suggest the applicability of of serine protease in substrate rich systems.

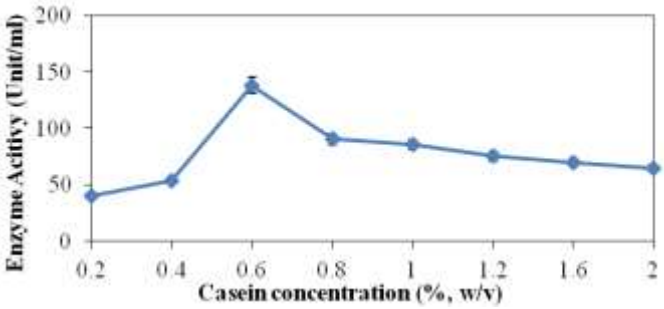

Fig. 8- Effects of substrate concentration on enzymes activity

Effect of solvent stability of enzymes

It was also desired to search a solvents stable protease for novel applications in non-aqueous systems. The protease secreted by isolate was incubated with 25% v/v of various organic solvents along with distilled water for 10 days. The enzyme showed remarkable stability in the presence of the all solvents. More than 60 % activity was retained after 3 days' incubation in all the solvents except methanol and benzene. Decline in activity was reported in all the solvent with extended the incubation. However the solvent effect is not universal and several solvents destabilize the enzyme (Table 1). Almost similar stability trends of proteases in the presence of various organic solvents were reported earlier (Ogino *et al.*, 1995, Vidyasagar *et al.*, 2009). The level of stability towards solvents is the unique properties of enzymes. Biocatalysis in organic media offers several advantages including the higher solubility of hydrophobic substrate enabling their active reactions, reduced microbial contamination and reusability leads to the development of novel application prospects (Gupta and Khare, 2006).

Table 1. Activity of enzyme in the presence of various solvents

Incubation (Days)	Residual activity (U/ml) of enzyme in various organic solvent					
	Distilled water	DMSO	Methanol	n-Hexane	Butanol	Benzene
0	232.65	212.4	207.9	234	247.05	265.05
1	227.25	186.3	142.2	203.4	205.2	208.35
3	213.75	168.3	109.35	166.5	171.19	144
5	141.75	131.4	85.05	130.05	112.5	103.5
8	132	89.1	41.4	62.1	82.35	34.83
10	122.4	23.4	32.85	34.2	28.35	31.5

Table 2. Effects of various commercial available detergents on enzyme

Detergent's Treatment Time	Control (Without detergent)	Rin 	Surf excel 	Ariel 	Tide 	Wheel 
(Min)	Residual activity (%) of enzyme at specific time interval					
0	100	100	100	100	100	100
15	99	92	90.3	92.7	90.4	97.7
30	96	88.5	86.2	89	81.5	82.3
60	92	73.78	69.05	67	62.1	75.9

Compatibility of protease with commercial detergents

Enzyme showed excellent compatibility in the presence of commercially available detergents like Rin, Ariel, Tide, Surf excel and Wheel (Table 2). The enzyme was highly compatible with the presence of all the tested detergent as they were showing more than 80% activity after 30 min treatment. After 1 hour pre-treatment, enzyme retained nearly 70% enzyme activity with Rin, Surf excel and Wheel detergent proposes the ideal characteristics of enzyme as an additives in detergents. In order to be effective during washing, a good detergent enzyme must be compatible and stable with all commonly used detergent and its formulation compounds like anionic surfactants, bleaching agents and water-softening agents (Stoner *et al.*, 2004). Therefore, the suitability of the proteases as a detergent additive is dependent on its stability and compatibility with these components at alkaline pH and wide range of temperature, and an ideal detergent enzyme should be stable and active in the detergent solution.

Destaining of blood

The enzyme was efficiently removed the blood stain within 40 min. without adding any detergent (Fig. 9). This is due to the *in vitro* protein hydrolysis of blood tissue. The result is far better comparable to the earlier reports of protease from *Pseudomonas aeruginosa* (Najafi *et al.*, 2005).

Dehairing of goat skin

Significant loss of hairs was observed in the enzyme treated sample within 8 hours, moreover hairs were removed very easily from enzyme treated skin compared to the control (Fig. 10). This observation seems superior to the protease from *Bacillus subtilis* (Ramakrishna *et al.*, 2010). Since this protease can also digest tissue collagen, the process of dehairing must be controlled to avoid the poor quality of the leather at industrial scale. However, enzymatic processes are better than harsh chemical method, because of its less use of hazardous and polluting chemicals.

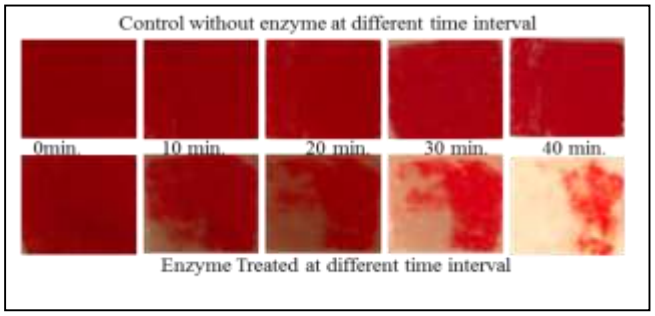

Fig. 9. Destaining of human blood in the presence and absence of enzyme

Washing performance as a detergent additive

It was seen that the protease enables to remove blood stain, chocolate and barbecue sauce very easily without addition of any detergent (Fig. 11). However, in the presence of detergent rapid removal of spots have been observed which suggests the impending use of enzyme in detergent industry. Its ability to act in the presence of solvents and detergents can also be exploited for multiple purposes.

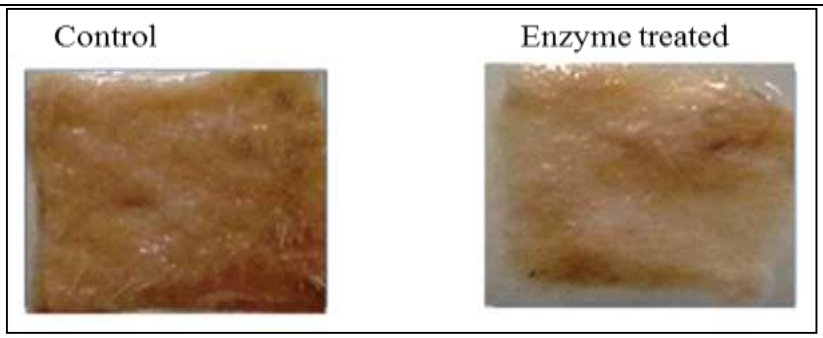

Fig. 10. Skin was incubated 50°C to observe dehairing

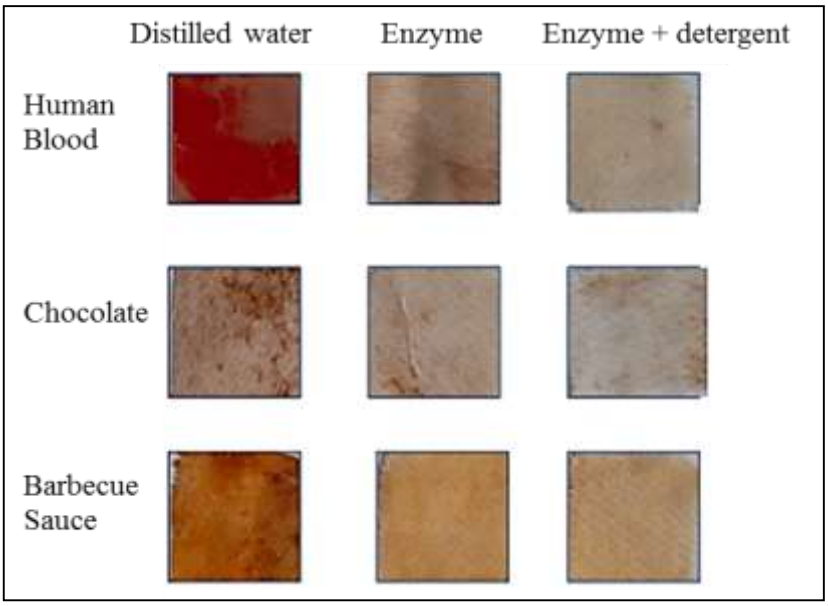

Fig. 11. Washing performance for the removal of blood, chocolate and barbecue sauce

Conclusion

Thermophiles are a potentially important source of thermozyme. Protease of *Bacillus licheniformis* U1 demonstrated the vital properties including thermostablility, alkalitolerance, organic solvent-tolerance and detergent stability. The activity and stability of crude proteases in a broad pH range, at high temperature and catalysis the substrate without salt makes it extremely attractive for detergent application. The use of partially purified enzyme is preferred against purified preparation. This avoids the cost of purification and makes the processes commercially viable. Washing performance analysis and dehairing indicated the impending application of enzyme for detergent and leather industries.

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No conflict of interest is found.

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