



**Characterization of *Geobacillus stearothermophilus* protease for detergent industry**  
**Caracterización de la proteasa *Geobacillus stearothermophilus* para su uso en la industria de detergentes**

I. Iqbal<sup>1</sup>, M.N. Aftab<sup>2\*</sup>, M.S. Afzal<sup>3</sup>, A. Zafar<sup>4</sup>, A. Kaleem<sup>5</sup>

<sup>1</sup>Department of Zoology, Lahore College for Women University, Lahore, Pakistan.

<sup>2</sup>Institute of Industrial Biotechnology, Government College University, Lahore, Pakistan.

<sup>3</sup>Department of Life Sciences, School of Science, University of Management and Technology (UMT), Lahore 54770, Pakistan.

<sup>4</sup>Faculty of Life Science, University of Central Punjab, Lahore, Pakistan.

<sup>5</sup>Department of Biotechnology, Lahore College for Women University, Lahore, Pakistan.

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**Abstract**

The Thermostable alkaline serine protease gene has potential applications in many industrial processes such as detergent, feed, etc. Cloning of thermostable alkaline serine protease gene from a thermophilic strain of *Geobacillus stearothermophilus* (B-1172) was carried out in *E. coli* BL 21, and its expression was studied. The expressed protease was purified followed by its identification. A 16.9-fold purification with 55.68% recovery of the protease was achieved by ammonium sulfate precipitation and gel filtration chromatography. The protease specific activity was 120 U mg<sup>-1</sup>. The purified enzyme remained stable at 90 °C at a pH range 6-9. Its interaction with EDTA, different metal ions, inhibitors, surfactants and detergents was also mapped. Its interaction with EDTA showed no significant effect on the activity of the enzyme confirming its metaloprotease nature. Metal ions, i.e., Ca<sup>2+</sup>, Mg<sup>2+</sup>, Ni<sup>2+</sup>, Cd<sup>2+</sup>, Cu<sup>2+</sup>, Zn<sup>2+</sup> showed no significant effect on the stability of protease. Its compatibility was checked with different commercial detergent (6 mg/mL) such as Surf Excel Ariel, Bonus, wheel and Shine. It retained more than 80% proteolytic activity in all detergents after incubation at 50 °C for 1 h. Wash performance analysis of the protease of *G. stearothermophilus* showed good results of de-staining of blood sample at various temperatures. Therefore, recombinant protease could prove as good candidate for commercial use in detergents.

**Keywords:** *Geobacillus stearothermophilus*, protease, thermophile, detergent, purification.

**Resumen**

El gen de serina proteasa alcalina termoestable tiene aplicaciones potenciales en muchos procesos industriales tales como detergente, alimentación, etc. La clonación del gen de serina proteasa alcalina termoestable de una cepa termófila de *Geobacillus stearothermophilus* (B-1172) se llevó a cabo en *E. coli* BL 21, y se estudió la expresión. La proteasa expresada se purificó seguido de su identificación. Se consiguió una purificación de 16,9 veces con una recuperación del 55,68% de la proteasa mediante precipitación con sulfato de amonio y cromatografía de filtración en gel. La actividad específica de la proteasa fue de 120 U mg<sup>-1</sup>. La enzima purificada permaneció estable a 90 °C en un rango de pH 6-9. También se mapeó su interacción con EDTA, diferentes iones metálicos, inhibidores, tensioactivos y detergentes. Su interacción con EDTA no mostró un efecto significativo sobre la actividad de la enzima confirmando su naturaleza metaloproteasa. Los iones metálicos, es decir, Ca<sup>2+</sup>, Mg<sup>2+</sup>, Ni<sup>2+</sup>, Cd<sup>2+</sup>, Cu<sup>2+</sup>, Zn<sup>2+</sup> no mostraron ningún efecto significativo sobre la estabilidad de la proteasa. Se comprobó su compatibilidad con diferentes detergentes comerciales (6 mg / ml) como Surf Excel Ariel, Bonus, wheel y Shine. Retenía más del 80% de actividad proteolítica en todos los detergentes después de la incubación a 50 °C durante 1 h. El análisis del rendimiento del lavado de la proteasa de *G. stearothermophilus* mostró buenos resultados de la tinción de la muestra de sangre a varias temperaturas. Por lo tanto, la proteasa recombinante podría ser un buen candidato para uso comercial en detergentes.

**Palabras clave:** *Geobacillus stearothermophilus*, proteasa, termófilo, detergente, purificación.

**1 Introduction**

Proteases are proteolytic enzymes, which have commercial importance and are functional at or near

neutral pH and at 40-60 °C (Lika-Ida *et al.*, 2016; Razzaq *et al.*, 2019). Proteases and exochitinases with unique properties have also been identified from certain bugs like stink (omate & Bonning, 2016; Erban *et al.*, 2016), and from a number of mesophilic bacteria (Yang *et al.*, 2016).

\* Corresponding author. E-mail: nauman535@yahoo.com

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Proteases that can withstand high temperature and pH are commercially more important than their counterparts produced by fungi, because of their easy handling and stability. In addition, a number of thermophiles have been explored for thermophilic proteases (Chatterjee *et al.*, 2016; Thebti *et al.*, 2016; Sammond *et al.*, 2016; Mahajan *et al.*, 2016; Maharaja *et al.*, 2018; Salem *et al.*, 2005).

The current worldwide enzyme sale of industrial enzymes is around \$4.2 billion (Singh *et al.*, 2016). Total enzyme market is expected to reach \$6.5 billion by year 2024. Projected share of protease is estimated around \$3 billion by year 2024 that hold around 50% share worldwide (Sharma *et al.*, 2019), Microbial proteases accounts for the largest share in the market in terms of value (Proteases Market by Source, 2019), where they are used in manufacturing of products such as food, leather and detergents etc. (Asmaa *et al.*, 2011). The detergent industry is one of the largest market for various enzymes like protease, where they are used in dishwashers, laundries and industrial cleaning products. Generally four types of enzymes are being used in cleaning industry including serine proteases, lipases, cellulases and amylases. The proteases remove protein stains which have a tendency to stick strongly to textile fiber such as egg, blood and human sweat (Sarmiento *et al.*, 2015, Marathe *et al.*, 2018; Oztas Gulmus *et al.*, 2020).

The efficiency of protease depends on several factors such as ionic strength, pH of detergent, bleaching agent, washing temperature, mechanical handling and composition of the detergent. Although high yielding alkaline protease strains have been widely studied, but bleach-stable enzymes are still not available where only few studies have been reported (Mei and Jiang, 2005; Srinubabu *et al.*, 2007; Lakshmi *et al.*, 2014; Ochi *et al.*, 2017; Bano *et al.*, 2019). Due to their broad specificity, numerous alkaline proteases have been identified and purified from many *Bacilli* strains (Bhaskar *et al.*, 2007; Doddapaneni *et al.*, 2007; Muhammad *et al.*, 2019; Osire *et al.*, 2019), such as subtilisin, which has been produced by *Bacillus licheniformis* (Jacobs 1985) and *Bacillus amyloliquefaciens* (Wells *et al.*, 1983). However, production of thermostable enzymes has been the focus of attention for many years (Chen *et al.*, 2004), because of their high stability and sustainability at elevated temperature. They play an important role in achieving optimal conditions, such as increase in solubility, and reaction rate, with a decrease in microbial contamination rate (Sookkheo *et al.*, 2004).

The present study report purification, characterization and wash performance analysis of a thermostable alkaline serine protease cloned from *Geobacillus stearothermophilus* and it is suggested that thermophilic recombinant serine protease has potential applications in many industries especially in detergent industry for removal of blood and other protein stains.

## 2 Materials and methods

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### 2.1 Expression of recombinant protease enzyme

Lyophilized culture of bacterial strain *Geobacillus stearothermophilus* B-1172 was obtained from American Type USDA ARS Culture Collection maintained at the USDA National Center for Agricultural Utilization Research, Peoria, US. The identification of the culture *Geobacillus stearothermophilus* was carried out by biochemical tests. Primers were designed using Vector NTI software and synthesized from Integrated DNA Technologies, USA. The cloning of gene into *E. coli* BL21 and expression of the recombinant enzyme was studied earlier (Iqbal *et al.*, 2015).

### 2.2 Purification

#### 2.2.1 Ammonium sulphate precipitation

A saturation of 10-100% of the 50 mL enzyme sample was prepared by dissolving 0.6-14.3 g ammonium sulphate in the enzyme sample. The enzyme placed on ice (4 °C) was constantly stirred on a magnetic stirrer plate, ammonium sulfate salt was added pinch by pinch with 5 min interval until it was completely dissolved. Centrifugation was carried out at 12,000 rpm for 20 min at 4 °C and pellet collected was finally dissolved in 10 mL Tris-HCl phosphate buffer (10 mM, pH 7.2).

### 2.3 Dialysis

Dialysis of the sample was performed with the help of dialysis membrane having molecular weight cut-off of 12 kDa. The dialysis membrane along with sample was suspended in sodium citrate buffer (50 mM, pH 7) in a large beaker for 24 hrs (4 °C) and stirred constantly with periodic change of the buffer.

## 2.4 Gel filtration chromatography

After dialysis, 5 mL sample was loaded in SuperDex 75 column Hi Load 16/60 (GE Health care Life Sciences) with 120 mL. pre-equilibration of column was performed with gel filtration buffer (10 mM EDTA, 150 mM NaCl, 50 mM Tris-HCl pH 7.0 and 1 mM DTT) to stabilize the bed. After loading the sample, column elution was carried out at flow rate 0.5 mL/min by 500 mM Tris-HCl buffer (pH 7). Pump pressure limit was adjusted at 0.45 MPa. The elution fractions were collected (2 mL each) and analyzed by SDS-PAGE. The purified enzyme was then used for determination of the enzyme characteristics.

## 2.5 Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis

For gel preparation, gel casting system was used. The gel was assembled by inserting spacers between each plate and was grabbed with clamps. A 12% resolving gel was prepared and the solution was quickly poured between the glass plates and space was left for stacking gel (5%) that was poured after polymerization of resolving gel. After polymerization of the stacking gel, protease samples were mixed with loading dye in 1:1 (v/v) heated at 70 °C for 5 min. A 60 Amp voltage was given after loading of samples for 40 min and voltage was increased to 100 Amp voltage when samples reached to resolving gel for 2 hrs. Gel was stained with Coomassie Brilliant Blue with gentle agitation at room temperature for 30 min and destaining was carried out overnight. The gel was observed in gel documentation system under UV light.

## 2.6 Protease assay

Protease assay was performed by a method of McDonald and Chen (1965) and casein was used as a substrate. Incubation of the enzyme extract (1 mL) was carried out with 4 mL casein (1%) at 60 °C for 40 min. A 5 mL trichloroacetic acid was added which resulted in precipitation of the residual protein that was centrifuged at 6,000 rpm for 10 min. Clear supernatant obtained (1 mL) was mixed with alkaline reagent (Folin reagent, 1:1 (v/v) 5 mL, 1 N NaOH) and incubation was carried out for 30 min. Finally absorbance was measured through spectrophotometer (Cecil-CE7200, Aquarius, UK) at 700 nm while blank (without enzyme) was used as control. One unit of protease activity is defined as “one unit that hydrolyze

casein to produce color equivalent to 1.0  $\mu$ mole (181  $\mu$ g) of tyrosine per minute at pH 7.5 at 37 °C”.

$$\text{Units/mL} = \frac{\mu\text{mol of Tyr} \times \text{reaction vol (mL)}}{\text{Samples vol (mL)} \times \text{reaction time} \times \text{vol (mL) assayed}} \quad (1)$$

## 2.7 Protein estimation

Estimation of protein was performed by Bradford (1976), using bovine serum albumin as standard. A 5.0 mL Bradford reagent was added to a tube that contain 0.2 mL enzyme extract. A blank was run in parallel using distilled water that was used as control. Absorbance was measured by spectrophotometer (Cecil-CE7200, Aquarius, UK) at 595 nm. For measurement of total proteins, standard BSA curve was used.

## 2.8 Enzyme characterization

### 2.8.1 Effect of pH and temperature

Effect of temperature and pH was studied for enzyme stability. A wide range of temperature and pH was used to determine the protease enzyme stability. Protease stability was studied by measuring its residual activity. Protease was incubated at pH 6.0-11.0 and temperature ranging between 40 - 100 °C for 1 - 5 hrs to determine the effect of pH and temperature on enzyme characterization.

### 2.8.2 Effect of EDTA, metal ions and inhibitors

Effect of metal ions ( $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Zn}^{2+}$ ) and EDTA at different concentration (1, 5 and 10 mM) and inhibitors like glutathione, leupaptin, DNTB (Dinitrothiocyanobenzene), pepstain was found by standard assay method.

### 2.8.3 Effect of surfactants, oxidizing agents and detergents

The effect of ionic surfactant (SDS) and non-ionic surfactants (Tween 20, Tween 60, Tween-80 and Triton-X 100, hydrogen peroxide, PMSF (phenyl methyl sulphonyl fluoride) were studied by incubating the enzyme for 1-5 hrs and relative activity was determined by standard assay method.

### 2.8.4 Detergent compatibility

Compatibility of the purified recombinant protease enzyme activity in the crude extract was studied

with locally available commercial solid laundry detergents. The solid detergents tested include Surf Excel (anionic and nonionic surfactants, sodium carbonate and sodium aluminosilicate, sodium perborate, anti-redeposition agent, perfume, sodium silicate, sodium sulfate), Ariel (alcohol ethoxylate, alkyl (or alcohol) ethoxy sulphate (aes) and alkyl sulphate (as), amine oxide, carboxymethyl cellulose, citric acid, cyclodextrin, ethanol, diethyl ester dimethyl ammonium chloride, ethylene diamine disuccinate, hydrogen peroxide, linear alkylbenzene sulfonate, mono ethanol amine (mea), 2-aminoethanol or ethanolamine, ercarbonate, polyvinyl alcohol, propylene glycol, sodium carbonate, sodium disilicate, sodium hypochlorite, sodium triphosphate, tetra acetyl ethylene diamine, titanium and titanium dioxide, zinc phthalocyanine sulphate), Shine (isopropyl alcohol, ethyl alcohol, sodium xylene sulphate, citric acid, alkoxyated alcohol, deionized water), Bonus (ethyl alcohol, propylene glycol, citric acid, alkoxyated alcohol, sodium silicate, perfume) and Wheel (sodium LAS, soda ash, calcium carbonate, CI 74260, CI 11680, Perfume). To confer a final concentration of 6 mg/mL, detergents were diluted using tap water. Inactivation of the endogenous enzymes present in laundry detergents was carried out by heating the diluted detergents at 70 °C for 1 h prior to the addition of 200 units of purified protease. Assay for protease was performed and standard conditions were used for determining the residual activities. The enzyme activity of a control, incubated under the similar conditions without detergent, was assumed as 100%.

## 2.9 Wash performance analysis

Equal volume of blood was used for staining cotton cloths (4 cm x 4 cm) that were dried in an oven at 50 °C. The stained cloths were washed with commercially available detergent Surf Excel Ariel, Shine, Bonus and Wheel. The detergent was diluted with tap water at a concentration of 6 mg/mL. The endogenous proteases were denatured by heating the diluted detergent solution at 90 °C for 1 h. After treatment, the diluted detergent was cooled down and supplemented with and without recombinant crude protease. The stained cloth was treated with

three different types of detergents, one is Surf Excel detergent (6 mg/mL) diluted in a tap water, second is Surf Excel solution supplemented with the *G. stearothermophilus* enzyme (81 U) in water and third is the detergent (6 mg/mL) supplemented with (81 U) enzyme in a tap water. Dilution of different mixtures was carried out in 100 mL of tap water in 250 mL flask and incubated at three different temperatures, 4, 20 and 50 °C under shaking condition (200 rpm) for 20 min.

## 3 Results and discussion

### 3.1 Confirmation of bacterial strain

#### 3.1.1 Cloning and expression of protease enzyme

Amplification of the protease gene from *G. stearothermophilus* was carried out by PCR using specific primers. The amplified fragment was ligated with pET-22b(+). The recombinant plasmid was transformed into *E. coli* BL21 and cloned gene was expressed.

### 3.2 Purification

Purification of the recombinant protease was carried out in three steps; ammonium sulphate precipitation, dialysis and gel filtration chromatography. In previous study, DEAE chromatography was used for the purification of protease (Iqbal et al., 2015) but in this study mainly gel filtration chromatography was used. Purification was carried out according to the procedure described in methods and materials. Fractional ammonium sulphate precipitation (10-100% saturation) was carried out for crude filtrate of protease. Fig. 1 shows expression of proteins after treatment with 80, 70, 60, 50, 40 and 30% ammonium sulphate. After ammonium sulphate precipitation, specific activity was increased to 43.6 U/mg and enzyme activity was calculated as 48 U/mL (Table 1). SDS-PAGE was carried out by running samples in parallel with protein marker. The result indicates the appearance of 55.4 kDa band of recombinant protease along with some unwanted protein bands only in 70 and 80% ammonium sulphate precipitation.

Table 1. Summary of overall purification of protease enzyme by ammonium sulphate precipitation, and gel filtration chromatography.

Sample	Volume (mL)	Enzyme Activity (U/mL/min)	Protein Concentration (mg/mL)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude extract	180	64	8.9	7.1	100	1
[NH <sub>4</sub> ] <sub>2</sub> SO <sub>4</sub> precipitation	105	48	1.1	43.6	75	6.1
Gel filtration chromatography	60	42	0.4	110	68.75	15.4

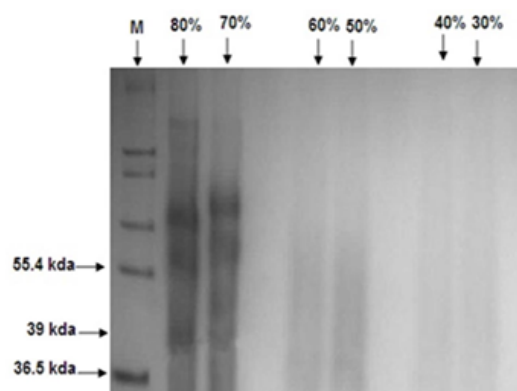


Fig. 1. SDS PAGE after ammonium sulphate precipitation. Lane 1, protein marker (Mark12™ Unstained Protein Standard on a Bolt™ 4-12% Bis-Tris Plus Gel with MES SDS Running Buffer); Lane 2, expression of proteins after treatment with 80% ammonium sulphate; Lane 3, treatment with 70% ammonium sulphate; Lane 4, treatment with 60% ammonium sulphate; Lane 5, treatment with 50% ammonium sulphate, Lane 6, treatment with 40% ammonium sulphate and Lane 7, treatment with 30% ammonium sulphate.

The two fractions (70 and 80%) obtained were pooled together and purified utilizing dialysis and gel filtration chromatography. The results of step wise purification are given in Table 1. After purification, the enzyme activity was dropped to 42 U/mL/min but specific activity was drastically increased to 110 U/mg. With 15.4 fold purification, the yield of enzyme turned to be 68.75% (Table 1). The purified fraction of the protease was examined by SDS-PAGE and a single band of 39 kDa was observed indicating high grade purity for the enzyme. No band was seen in

the control sample (*E. coli*) transformed with vector without protease gene and control sample (*E. coli*) transformed with vector plus protease gene (Fig. 2).

The protease enzyme (39 kDa) was isolated with 15.4 fold purification. Enzyme activity was increased from 7.1 U/mg to 110 U/mg after purification. In the literature, there are variable reports about purification and specific activity of the proteases. Asmaa et al. (2011) cloned and later purified protease from *Bacillus stearothermophilus* with specific activity (36.7 U/mg), while Purohit et al. (2014) reported specific activity (0.0159 U/mg) of cloned thermostable protease in *E. coli* Rosetta (DE3).

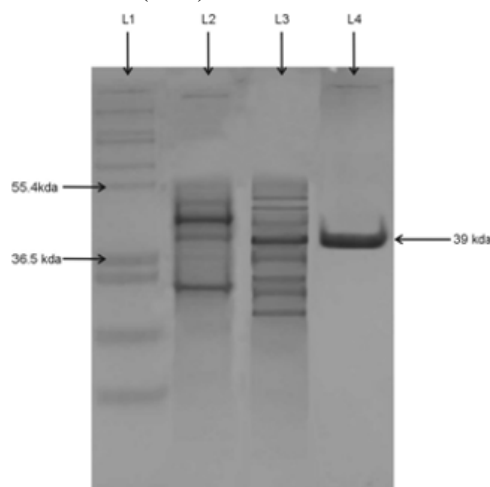


Fig. 2. SDS-PAGE of purified protease sample. Lane 1 indicates protein marker, lane 2, shows expression of control (*E. coli*) transformed with vector without protease gene, Lane 3 indicates expression of control (*E. coli*) transformed with vector plus protease gene, and Lane 4 shows cloned *E. coli* extract (protease gene).

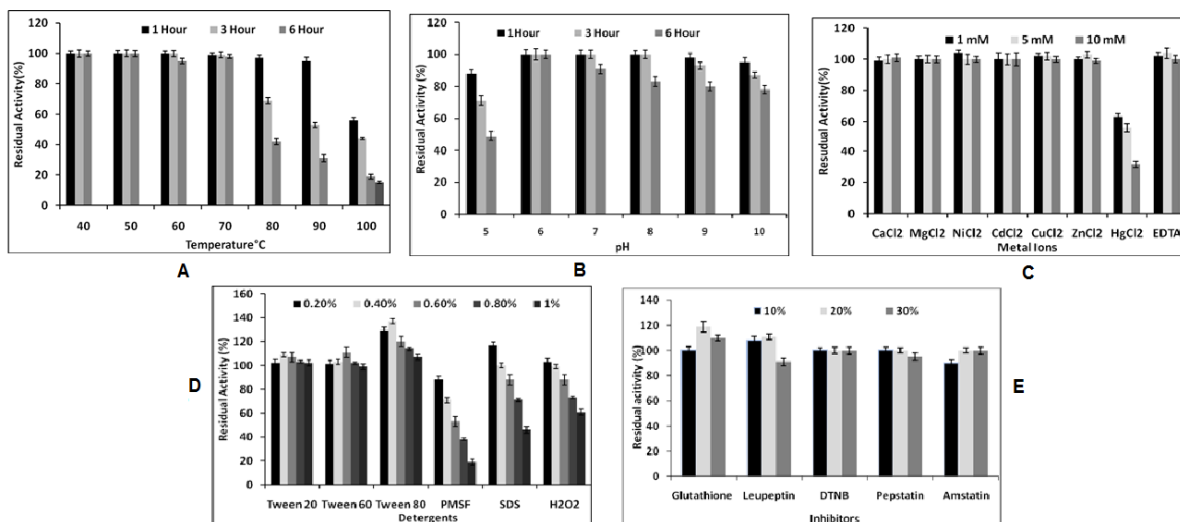


Fig. 3. (A) Characterization of expressed protease enzyme. Residual activity of protease after incubation with temperature (B) Residual activity of protease after incubation with different concentrations of pH (C) Residual activity of protease after incubation with variable metal ion (D) Residual activity of protease after incubation with Different detergents (E) Residual activity of protease after incubation with organic solvents.

### 3.3 Characterization of protease enzyme

#### 3.3.1 Temperature and pH stability

*G. stearothermophilus* protease was highly stable for 6 hrs after pre-incubation at 70 °C and its residual activity was 100%. For heating enzyme for 3 hrs at 90 °C, residual activity was calculated to be 95%. However, heating protease enzyme for 6 hrs at 100 °C, the enzyme residual activity was dropped to 19% (Fig. 3 A). The protease was stable at a wide range of alkaline pH. Incubation at acidic pH 6 for 1 hr, and at alkaline pH 11, the residual activity of 88% and 95% was observed, respectively. For longer incubation period (6 h) at pH 6, there was a decrease in residual activity of the enzyme (51%), while at highly alkaline pH (pH 11) the protease enzyme showed 78% stability (Fig. 3B). These results indicate the high stability of enzyme at alkaline pH as compared to stability at acidic pH. On the basis of characterization of *G. stearothermophilus* protease, it is classified as alkaline protease.

The protease described in this study was stable at higher temperature and highly alkaline pH compared with the proteases identified from *B. amyloliquefaciens*, *B. subtilis*, *B. horikoshii*, *B. lehensis* and *Pseudoaltermonas haloplanktis* that showed stability at 50-90 °C and pH range 5-10, after 1 hr of incubation (Hang-seung and Jan, 2012;

Joshi and Satyanarayana, 2013; Wells *et al.*, 1983; Liu *et al.*, 2020; Wu *et al.*, 2015; Yang *et al.*, 2016). However, some proteases from *Oceanobacillus iheyensis* are known to increase their activity with an increase in their incubation temperature (Purohit *et al.*, 2014). The protease stability at elevated temperature has many biotechnological advantages i.e. it shows higher reactivity, higher process yield (increased in solubility of substrates and products, equilibrium displacement in endothermic reactions), lower risk of contamination and important influence on the bioavailability and efficient bioremediation (Haki and Rakshit 2003). Stability of the proteases at high temperature allow them to withstand harsh reaction conditions and industrial processes. Alkaline protease with high temperature and alkaline pH optima is also desirable in detergents industry (Hang-seung and Jan, 2012). In a study protease produced from *Bacillus safensis* have shown optimal activity at at pH 8.0 and at 40 °C and serine protease from *Bacillus licheniformis* showed stability at pH 7.0 (Ji *et al.*, 2019; Shakir *et al.*, 2019; Martínez-Pérez, 2019). In another study protease enzyme from *Bacillus* sp. exhibited stability at wide range, pH 7-10. Similarly, the protease remained stable at 50°C (Ariyaei *et al.*, 2019). It was reported that extremophilic protease from *Pseudomonas aeruginosa* showed stability between pH 5 and 8 that was almost 100% of their activity up to 24 hours (Flores- Fernández *et al.*, 2019).

Table 2. SDS-PAGE of purified protease sample. Lane 1 indicates protein marker, lane 2, shows expression of control (*E. coli*) transformed with vector without protease gene, Lane 3 indicates expression of control (*E. coli*) transformed with vector plus protease gene, and Lane 4 shows cloned *E. coli* extract (protease gene).

S. No	Name of organism	pH	Temperature	References
1.	<i>Geobacillus stearothermophilus</i>	6-11	70°C	This work
2.	<i>Bacillus</i> sp.	7-10	50 °C	Ariyaeia et al. (2019)
3.	<i>Bacillus safensis</i>	7-12	40 °C	Rekik et al. (2018)
4.	<i>Bacillus lehensis</i>	8-12	30-60°C	Joshi and Satyanarayana (2013)
5.	<i>Pseudoalteromonas</i> sp.	6-11	10-60°C	Wu et al. (2015)
6.	<i>Bacillus amyloliquefaciens</i>	11	50°C	Yang et al. (2016)
7.	<i>Bacillus horikoshii</i>	5-10	50-90°C	Hang-seung and Jan (2012)

pH stability study of protease from *Bacillus safensis* indicated that the enzyme remained stable at pH ranging from 7 to 12 at 40 °C for 180 min (Rekik et al., 2018). The activity of protease from *Barrientosimonas* sp. V9 showed the highest relative activity around 100% in the pH range from 4.0 to 10.0. However, thermal stability assay showed 50% loss of activity at 60 °C (Flores-Santos et al., 2020). A summary of the pH and temperature stability of recombinant protease enzymes produced in this study as compared to recombinant enzyme of previous studies is shown in table 2.

### 3.3.2 Effect of metal ions and EDTA

Previously effect of various metal ions on protease enzyme was tested that include  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Na}^{+}$  and  $\text{Hg}^{2+}$  (Iqbal et al., 2015). In the present research work, effect of  $\text{Ni}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Zn}^{2+}$  metal ions on protease enzyme stability was studied in addition to study of  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$ . The effect on new metal ions has generated new information about behavior of protease enzyme.

To determine the effect of metal ions and EDTA on the activity of the purified protease, hydrolytic activity of the enzyme was studied at 60 °C, pH 9.0. The protease was pre-incubated for 1 h with different concentrations (1-10 mM) after addition of different metal ions.  $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$  showed no effect on the protease activity. By an addition of 10 mM  $\text{Hg}^{2+}$  ions, there is marked decrease in the enzyme activity. The  $\text{Hg}^{2+}$  can establish stable toxic compounds by combining with proteins and enzymes. The  $\text{Hg}^{2+}$  also inhibit proper folding of the proteins and distort the protein structure that could not be refolded in the presence of EDTA chelator (Sharma et al., 2008). Further, Heavy metals are known to bind with the enzymes causing their precipitation and deactivation (Pandey et al.,

2013). The enzyme activity was decreased to 32% while addition of  $\text{Ca}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Ni}^{2+}$  and  $\text{Cu}^{2+}$  ions showed no alteration in the protease activity confirming that this enzyme was not a metalloenzyme (Fig. 3 C).

The chelating agent EDTA had no influence on the activity of the purified protease enzyme that remained stable after incubation with EDTA and due to protease stability, it may prove as better additive in detergent industry. Proteases stability with EDTA was observed by Hirata et al. (2013), Sanatan et al. (2013) and Hutadilok-Towatana et al. (1999). The purified protease showed stability at high concentration of various metal ions ( $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Cu}^{2+}$ , and  $\text{Zn}^{2+}$ ) and verified that it is not a metalloenzyme and do not need metal ions for its catalytic activity. Proteases that show stability at higher concentration of metal ions are usually suitable in detergent and leather processes as well as sewage treatment etc (Valls et al., 2011). Recently, protease from thermophilic *Anoxybacillus kamchatkensis* has been reported with increased activity by 130%v and 150% respectively by  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$ . On the other hand,  $\text{Co}^{2+}$  and  $\text{Cd}^{2+}$  showed complete inhibition of protease (Mechri et al., 2019).

### 3.3.3 Effect of surfactants, oxidizing agents and detergents

Effect of  $\text{H}_2\text{O}_2$ , Tween 60 and protein inhibitors (glutathione, leupeptin, DNTB, pepstatin and Amstatin) on protease was determined that was not studied in our previous study. This evaluation has provided additional information about the recombinant protease enzyme. All detergents have amphipathic structure and are either anionic, cationic or non-ionic and are able to solubilize proteins. Different ionic detergents including SDS, nonionic detergent Tween (20, 60, and 80) and  $\text{H}_2\text{O}_2$  were

studied to assess the activity of the protease. The enzyme was pre-incubated in sodium phosphate buffer (pH 7; 50 mM) along with 0.2, 0.4, 0.6, 0.8 and 1% (w/v) detergents. Effect of 50 mM PMSF (phenyl methyl sulphonyl fluoride) was studied at pH 7. At 0.2-1% concentration of Tween 20 and Tween 60, the enzyme activity remained unaffected. The protease activity was increased by 139% after an addition of Tween 80 at a concentration of 0.2-1% (w/v) (Fig. 3D). However, the enzyme activity was decreased to 38% and 19% after a treatment with PMSF concentration of 0.8% and 1% w/v respectively. The enzyme activity was reduced to 46% by the addition of 1% SDS, however, the activity remained unaffected at low concentrations, 0.2% of SDS. H<sub>2</sub>O<sub>2</sub> being a strong oxidant, enzyme activity was reduced to 61% by addition of 1% H<sub>2</sub>O<sub>2</sub>. While addition of lower concentrations (0.2%) had no effect on the activity of the enzyme. The inhibitory effects of different concentrations (10-30%) of glutathione, leupeptin, DNTB, pepstatin and Amstatin on protease activity was studied. After incubation the protease remained stable for 1 h (Fig. 3 E). Residual activity of protease was increased (119-115%) after addition of leupeptin and glutathione at a concentration of 20% (v/v) respectively. By addition of 10% of pepstatin, 95% residual activity of the enzyme was observed. However, enzyme activity was reduced to 90% by an addition of 10% amstatin.

Activity of the protease is not affected by the addition of Tween 20 and Tween 60 (Esakkiraj *et al.*, 2016), while there was an increase in the protease activity after addition of non-ionic detergent Tween 80 (0.2-1% w/v). Padmapriya *et al.* (2012) have also reported an increase in the activity of the protease from marine Bacillus species. Strong effect on enzyme activity was observed with SDS at 1% [w/v] concentration, while reduction in the protease activity to 61% was observed with increasing concentration of 1% H<sub>2</sub>O<sub>2</sub>. The inhibition in enzyme activity could be resulted due to reduction in protein-protein interaction and in hydrophobic interactions (Traut *et al.*, 1989). Addition of PMSF (0.6 w/v) reduced the enzyme activity to 53%, while increased concentration (1% w/v) resulted in the decrease of the enzyme activity to 19%. Proteases showed consistency in the presence of glutathione, leupeptin, DNTB, pepstatin and amstatin. Protease activity was increased and caused changes in the structure, which resulted

in increased catalytic activity (Peek *et al.*, 1992). Protease is very sensitive to PMSF as it is completely inhibited in the presence of PMSF confirming that it belong to a group of serine protease (Peek *et al.*, 1992). PMSF is a known sulphonateine residue at the active site of protease resulting in complete loss in its enzyme activity (Hang-seung and Jan 2012).

### 3.4 Wash performance analysis

The wash performance was carried out in detail and studied the efficiency of protease enzyme to remove blood stains from cloth. This was not performed in previous study (Iqbal *et al.*, 2015). The wash efficiency of protease enzyme is the hallmark of this study that depicts its effectiveness against blood stains. The study revealed that recombinant protease can be added in detergents to increase the washing ability of detergents to remove various protein stains from the cloth.

The wash performance of *G. stearothermophilus* protease supplemented in detergent was determined by incubation at 4, 20 and 50 °C for 20 min. To analyze its stain removing ability, a blood tarnished cloth was used for its wash ability. The protease enzyme (81 U) displayed best result for de-staining performance at 50 °C, while considerable loss of stain was observed at 20 °C. By supplementing the recombinant protease enzymes in the commercial detergent, the wash performance of the detergent was enhanced and blood stains were completely removed. Moreover, *G. stearothermophilus* protease alone has shown considerable de-staining ability (Fig. 4). These observations suggested that recombinant protease enzyme could be used as a detergent additive. These results show the efficiency of *G. stearothermophilus* protease in the removal of proteinaceous stains (Table 3).

Table 3. Wash Performance analysis of thermostable alkaline serine protease with solid detergents and enzyme against blood stained cloth.

Solid Detergents + 200 units of enzyme	Relative protease activity (%)
Surf Excel	114
Ariel	109
Shine	91
Bonus	89
Wheel	105



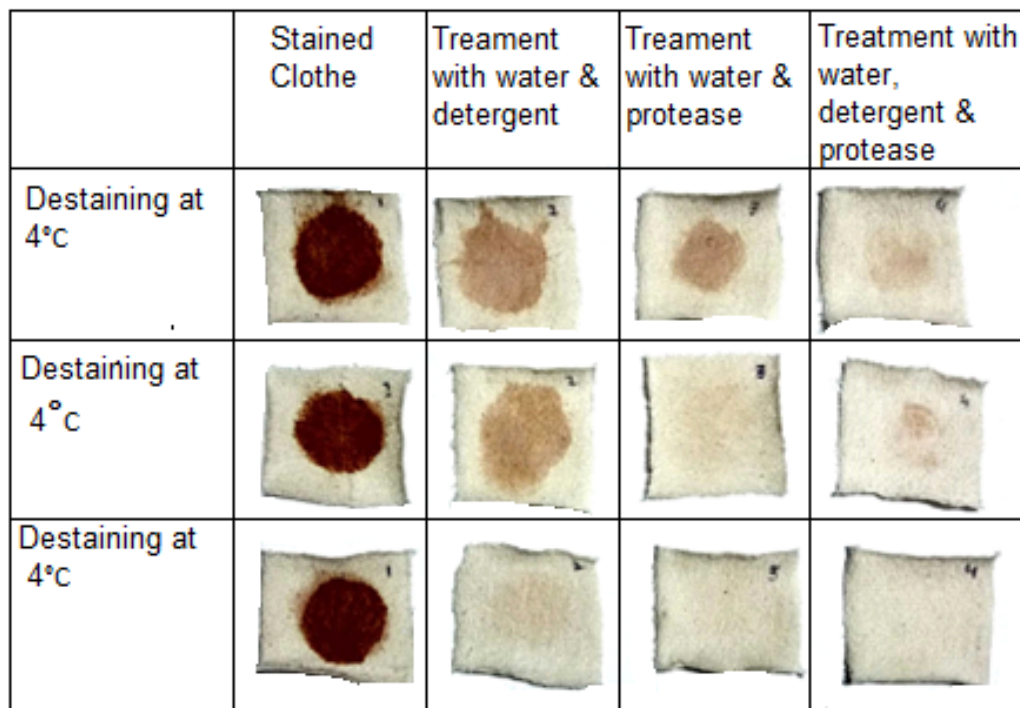


Fig. 4. Wash Performance analysis of stained cloth with protease enzyme, water and solid detergents at 50 °C, 20 °C and 4 °C.

The results obtained were exceptional and was highly compatible with the exogenous proteases present in the commercially available detergents. Cavello *et al.* (2012) and Abidi *et al.* (2008) obtained the similar results by adding proteolytic enzyme of *Paecilomyces lilacinus* and *Botrytis cinerea* in laundry detergent for the removal of blood spots on the fabric. Jellouli *et al.* (2011) reported that *Bacillus licheniformis* MP1 showed a good result in blood stains removal from a cloth. On the other hand, similar results have been reported by Savitha *et al.* (2011) for the blood stain removal from the fungus protease as compared to control.

## Conclusions

In conclusion, the alkaline serine protease produced by a thermophilic strain of *G. stearothermophilus* B-1172 was successfully purified and characterized using the *E. coli* BL 21 expression system. Wash performance analysis of the purified protease exhibited good results in the removal of blood stains from the cloth at various temperatures. Therefore, this alkaline serine protease

could prove as good candidate for use in detergents.

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