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CHARACTERIZATION AND POTENTIAL APPLICATION OF ALKALINE PROTEASE FROM THE GUT MICROBIAL ISOLATE OF *Portunus pelagicus* AS DETERGENT ADDITIVE

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AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration among all authors. Author KP designed the study, wrote the protocol and wrote the first draft of the manuscript. Author EG collected the source for methodology and review. Authors JJ and MGR performed the statistical analysis and proofread the manuscript. Authors JJ and MGR managed the literature searches, read and approved the final manuscript.

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Original Research Article

ABSTRACT

Proteases are the foremost important hydrolytic enzyme that plays a vital role in the metabolism and physiological activities of every organism. There is an increasing demand for protease enzyme worldwide. An extracellular protease producing strain was isolated from the gut of *Portunus pelagicus* and was identified as *Bacillus subtilis*. The stability of crude protease in various solid laundry detergents was investigated. The enzyme had an optimum activity at pH 9.0-10.0 and temperature 55-60°C. The protease was found to be stable towards non-ionic (Triton X-100, Tween 20, Tween 80, Brij 35) and ionic detergents (0.1% SDS) and were relatively stable in bleaching agents such as 1% H_2O_2 and 1% sodium perborate which retained 74% and 63% of their initial activity respectively. Except for acetone, all other organic solvents showed more than 70% of total activity in 10% of solvents (methanol, 2-propanol, xylene, toluene, and benzene). Moreover, the alkaline crude enzyme extract showed its stability and compatibility with commercial solid detergents. Wash performance analysis, stability in pH, temperature, thermal stability and the stability in the presence of surfactants, bleaching agents and organic solvents, suggest its application as a laundry additive.

Keywords: Proteases; *Portunus pelagicus*; *Bacillus subtilis*; detergents; stability; laundry additive.

1. INTRODUCTION

Recent advancements in enzymology rendered scientists to study microbial proteases among various hydrolytic enzymes. In addition to their vital role in cellular metabolic processes, these enzymes gained more attention in the industrial sectors. These enzymes have been used as an additive in detergents since 1941. A wide range of microorganisms such as bacteria, yeasts, mould, produce protease and are also found in plants and various animal tissues [1]. Bacterial proteases possess many significant properties as they are mostly extracellular, easily produced in larger quantities, thermos table and active at wider pH range [2] which enabled them to be most suitable for wider industrial application.

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Almost half of all industrial enzymes are proteases, mostly used in the detergent, leather, and food industries [3]. To employ proteases as a detergent additive, it should stable as well as active in the presence of typical detergents such as bleaching agents, surfactants, fillers, fabric softeners and various other formulation adjuncts. To meet this demand, the proteolytic enzyme with appropriate specificity and stability to various parameters such as pH, temperature, ionic, non-ionic and oxidative surfactants, polar and non- polar compounds. An interesting and fascinating application of alkaline protease was developed by Fujiwara and co-workers [4].

The proteolytic enzymes provide a gentle and selective debridement which supports the natural healing process in the successful local management of skin ulcerations by the efficient removal of the necrotic material [5]. Proteases are also useful and important components in biopharmaceutical products such as contact-lens enzyme cleaners and enzymic debriders [6]. Base on their activity under acidic, neutral or alkaline conditions and in the presence of a distinctive active group of the enzyme, microbial proteases are classified into various groups such as metalloproteases, aspartic proteases, cysteine or sulfhydryl or serine proteases. Alkaline proteases are defined as those proteases which are active in a neutral to alkaline pH. Among all the proteases, alkaline proteases are the most important group of enzymes which is being exploited commercially.

Only a few works have been carried out in the field of the gut microbe of the crab of *Portunus pelagicus*. Therefore, the present study has been initiated to investigate and screen alkaline protease producing bacteria and examine the potential of protease enzyme to be used as detergent additives.

2. MATERIALS AND METHODS

2.1 Microorganism

2.1.1 Isolation and screening of alkaline protease producing bacteria

About 30 crab samples of *Portunus pelagicus* were collected from Pulicat Lake, Tamil Nadu, for the present study. Crab specimens were dissected with sterilized dissecting materials and the whole gut was removed [7]. The gut of crab samples was homogenized in 10 ml of sterilized nine-salt solution (NSS) [8]. The prepared gut sample was used for the screening of protease producing isolates. From the prepared gut sample, 0.2 ml was spread on casein agar plates (nutrient agar with 1% casein) and incubated at 30°C for 3 days. The enriched sample was plated over nutrient agar containing 0.4% gelatin [9] and then the plates were incubated for 24 hr. Colonies showing clear zone were picked and purified. About 17 isolates were screened using casein digestion method for protease production. An isolate PP16 which showed maximum activity was selected and maintained on nutrient agar at 4°C.

2.1.2 Identification of selected bacterial isolate

The culture was examined for various morphological and biochemical characteristics [10]. Further, the higher-yielding strain was identified by taxonomic characterization based on the nucleotide sequence of the 16S rRNA gene. The sequence similarity search was done for the16SrRNA sequence using an online search tool called MOLE-BLAST (https://blast.ncbi.nlm.nih.gov/moleblast). The unknown organism was identified using the maximum aligned sequence through MOLE-BLAST.

2.2 Cultivation and Media for Enzyme Production

Production of protease from PP16 was carried out in the medium containing (g/l) Dextrose 10 g, peptone 7.5 g, $FeSO_4$ 1 g, K_2HPO_4 2 g, $MgSO_4$ $7H_2O$ 5 g. The inoculated medium was incubated at 37°C, pH 7 on a rotary shaker. At the end of the incubation period, the culture medium was centrifuged at 5,000 rpm for 10 min to obtain a crude extract, which was used as an enzyme source for the estimation of protein content.

2.3 Assay of Proteolytic Activity and Protein Content

Protease activity was analyzed according to the slightly modified method described by Anson [11] where casein was used as the substrate. The enzyme was assayed in the reaction mixture containing 2.0 ml of 0.5% casein solution in 0.1 M Carbonate – Bicarbonate buffer pH 9.5 and 1ml enzyme solution in a total reaction mixture was incubated for 5 min at 37°C. The reaction was terminated by adding 3 ml of 10% ice-cold trichloroacetic acid (TCA). The tubes were incubated for 1 hr at room temperature. The precipitate was centrifuged at 5000 rpm for 15 min and after then the supernatant was collected. 5 ml of 0.4 M Sodium carbonate and 0.5 ml of Folin phenol reagent were added to 1 ml of supernatant, vortexed immediately, then incubated for 30 min at room temperature and OD was taken at 660 nm. The concentration of tyrosine in the supernatant was read from a standard curve for tyrosine. The total protein content of the samples was determined according to the described method [12]. One unit of protease

activity was defined as the amount of enzyme required to liberate 1 µg of tyrosine ml-1min-1 under experimental conditions.

2.4 Partial Purification of Protease

2.4.1 Ammonium sulphate fractionation

Solid ammonium sulphate was added slowly to the crude enzyme extract and constantly stirred for 2 hr. Then the solution was centrifuged at 10000 rpm for 10 min at 40°C and the pellet was resuspended in cold double distilled water and buffer (Tris buffer, pH 8.0, 25 mM). Hence the dialyzed sample was used for further studies.

2.5 Characterization of Protease

2.5.1 Effect of pH on activity and stability of protease

To study the optimum pH of the crude enzyme preparation, the pH range was adjusted from 7.0 – 13.0 at 60°C with casein 1% (w/v) as a substrate. Stability of alkaline protease was determined by pre-incubating the enzyme in different buffers at various pH range of 6.0 – 13.0 for 1 hr at 40°C. Aliquots were withdrawn to determine the residual proteolytic activity of an enzyme at pH 9.5 and 60°C. The buffer systems which were used for adjusting pH were 100 mM potassium phosphate buffer for pH 6.0–7.0; Tris - HCl buffer for pH 8.0 – 9.0 and glycine-NaOH buffer for pH 9.0 – 13.0.

2.5.2 Effect of temperature on activity and stability of protease

To study the effect of temperature on the enzyme activity, the crude enzyme was incubated at various temperatures for 15 min at pH 9.5. Thermal stability was analyzed by incubating the enzyme preparation for 60 min at 50, 55, 60 and 65°C at pH 9.5. To determine the residual activity of an enzyme at standard conditions, aliquots were drawn at different intervals. The non-heated enzyme was kept as control (100%).

2.5.3 Effect of surfactants and bleaching agents on protease activity

To investigate the effect some non-ionic and ionic detergents on enzyme stability, both types of agents (Triton X-100, Tween 20, Tween 80, Brij 35 and SDS) with different concentrations were added to the enzyme and buffer mixture. Bleach stability of the enzyme was also tested in the presence of sodium perborate and hydrogen peroxide with different concentrations. All mixtures were preincubated for 1 hr at 40°C. The residual activity was measured. The activity of the enzyme without any surfactants was taken as 100%.

2.5.4 Effect of different organic solvents on the stability of protease

To study the stability of the protease against different solvents (2-propanol, methanol, acetone, toluene, xylene), 2 ml of enzyme solution in 100 mM Tris - HCl buffer were mixed with 1 ml of 10%, 20%, 40% organic solvents and left for 1 hr at 40°C with constant stirring. All the reactions were analyzed in triplicate. Controls had no solvents in the reaction mixture. Residual activity of an aliquot of 300 ml was measured after 1 hr by assay method.

2.5.5 Washing test with crude protease enzyme preparation

The compatibility of *Bacillus subtilis* PP16 protease with solid laundry detergents was analyzed using Surf Excel, Tide, Mr White, Fena and Wheel. To study the application of crude protease enzyme as a detergent additive, white cotton clothes (5*5 cm) were stained with human blood and egg yolk separately [13,14].

The following set of preparations were made and studied:

- Each stained cloth was dipped in a separate flask containing only 100 ml distilled water.
- Each stained cloth was dipped in a separate flask containing 100 ml distilled water with 1ml detergent (7 mg/ml)
- Each stained cloth was dipped in a separate flask with 100 ml distilled water, 2 ml enzyme solution and 1ml detergent (7 mg/ml)
- Untreated stained cloth was taken as a control.

The above-mentioned flasks were incubated at 40°C-60°C for 1 hr. After incubation, the clothes were rinsed with water and dried and the effect of enzyme compatibility was investigated. The effect of removal of stains was also analyzed by visual examination.

3. RESULTS AND DISCUSSION

3.1 Isolation and Screening of Alkaline Protease Producing Bacteria

The crab samples (Fig. 1) were used to isolate the gut which contains Protease producing bacterial isolates. Then, primary screening was carried out for maximum protease producing bacterial isolate by inoculating on a case in agar plate. Based on the large

Fig. 1. Blue crab *Portunus pelagicus*

hydrolytic zone on casein agar plates, single isolate PP16 was selected and used for further experimental studies to find the industrial potential application.

3.2 Identification of Selected Bacterial Isolate

Morphological and physiological characteristics of an organism was investigated [10]. Results of phenotypic and biochemical characterization of the isolate PP16 represented the isolate as aerobic, spore-forming, Gram-positive rod-shaped bacteria. Based on the morphological, physiological and biochemical characterization the isolate PP16 belongs to the genus Bacillus. The organism was further identified by 16S rRNA gene sequencing analysis, the genomic DNA of the isolate PP16 was amplified and obtained the product length of 812bp nucleotides. The 16S ribosomal RNA gene was sequenced and submitted to NCBI GenBank and the accession number is MN173351. The blast result of the isolate (Fig. 2) was found sequence similarity with *Bacillus subtilis*. From the overall, physiological, biochemical and molecular characterization, the isolate was identified as *Bacillus subtilis* PP16.

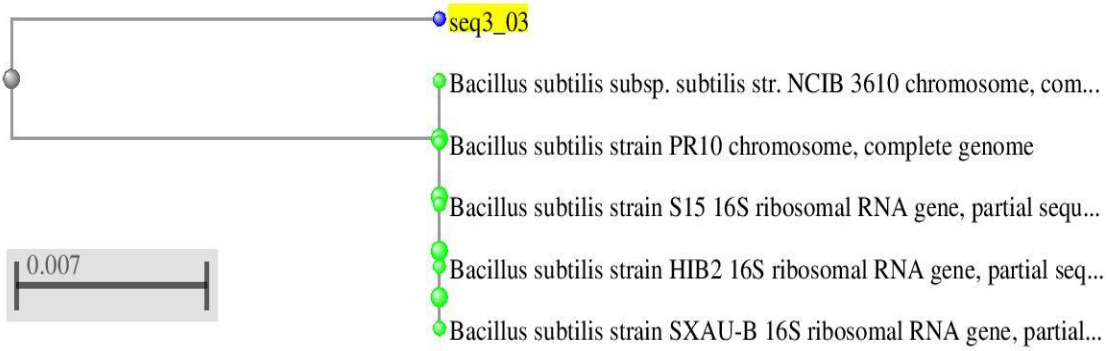

Fig. 2. Phylogenetic tree showing the position of an isolate, based on the partial 16S rRNA sequence comparison, obtained by the neighbor-joining method

3.3 Characterization of Protease

3.3.1 Effect of pH on activity and stability of protease

The detergent proteases should have high activity at high pH solutions for their effective role during washing. In general, bacteria belonging to Bacillus genus are known to secrete mostly two types of extracellular proteases, a neutral or metalloprotease which exhibits optimum activity at pH 7.0 and an alkaline protease having pH optima between 9.0 and 11.0 [15]. The PP16 protease was highly active in the pH range of 7.0–11.0 with an optimum at pH 9.0–10.0 (Fig. 3). It also indicates that this enzyme belonged to alkaline protease group. These findings are in accordance with several earlier reports showing the optimum pH range at 11.0 [16] and this is a very important characteristic feature for its eventual use in detergent formulations [17]. The relative activities at pH 7.0, 8.0 and 11.0 were about 74, 80.5 and 60.44% respectively. To determine the stability of an enzyme in different pH, the protease enzyme was incubated for 1 hr at 40^{o} C and the residual enzyme activities were analyzed. The pH activity of the crude protease extract is shown in Fig. 4. Protease PP16 was highly stable over a wide range of pH, maintaining 100% of its original activity between 7.0 to 9.0. The enzyme retained 98 and 77% of its original activity at pH 10.0 and 11.0 respectively.

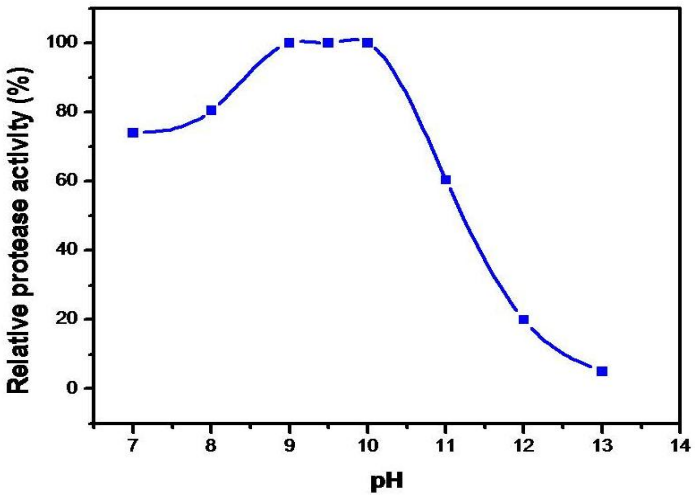

Fig. 3. Effect of pH on alkaline protease activity

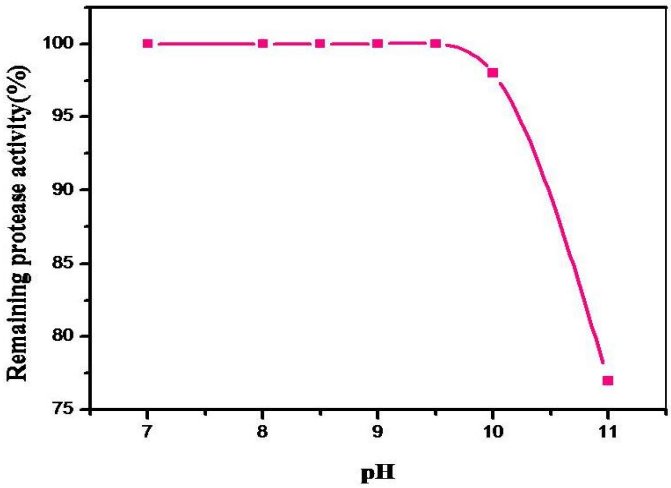

Fig. 4. Effect of pH on the stability of alkaline protease

3.3.2 Effect of temperature on activity and stability of protease

The effect of temperature on protease activity was determined by assaying enzyme activity at different temperatures (Fig. 5). The PP16 protease was active at temperatures ranging from 45 to 65°C and had an optimum at 55-60°C. The relative activities at 45, 50 and 65°C were about 65, 80 and 70% respectively. The optimum temperature for crude protease extract was similar to that of protease from *B. brevis* [18]. The rapid decrease in enzyme activity was observed above 65°C.

The thermal stability profile of the crude protease extract showed that proteases were highly stable at temperatures such as 50 and 55°C (Fig. 6). The enzyme retained more than 86.8% and 80.1% of its initial activity after 60 min incubation at 50 and 55°C, respectively. The enzyme retained only 32.3% of its initial activity after an hour at 60°C. These findings were comparable with results of several earlier reports [19]. Residual activity of an enzyme was almost lost at 65°C after an hour which remains as only 5%.

3.3.3 Effect of surfactants and bleaching agents on protease activity

Earlier reports states that it is crucial to search protease from different sources that are more appropriate to be used with washing systems. These properties have already been studied in trypsin-like enzymes in tropical fish [20]. A good detergent protease must be compatible and stable with all

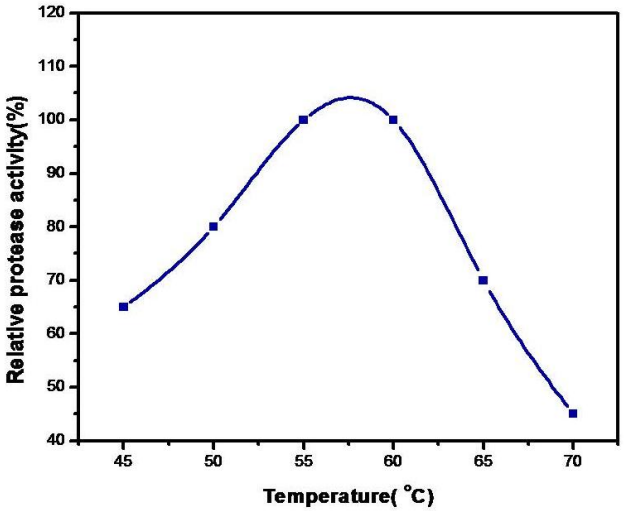

Fig. 5. Effect of temperature on alkaline protease activity

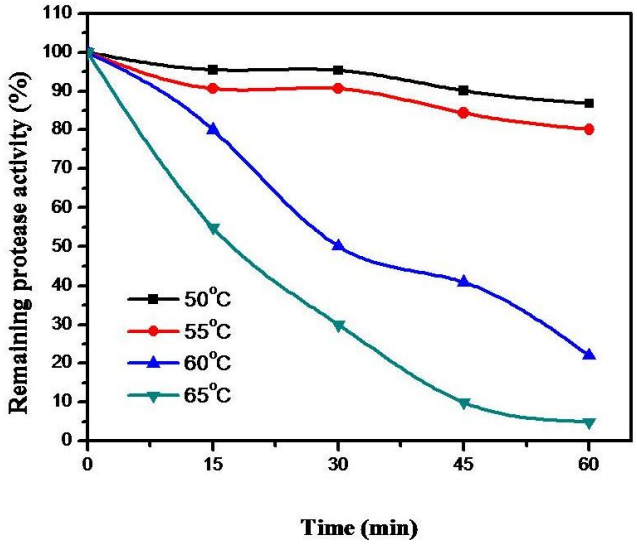

Fig. 6. Effect of temperature on the stability of alkaline protease

commonly-used detergent compounds such as surfactants, bleaches, oxidizing agents and other additives, which might be present in the formulation [21] along with their activity and stability in high pH range and high temperatures [22].

Table 1. Stability of PP16 alkaline protease in the presence of various surfactants and bleaching agents

Additives	Concentration (%)	Residual activity (%)
None		100
Triton X-100	1 (v/v)	10
Triton X-100	5	100
Tween 20	1 (v/v)	100
Tween 20	5	100
Tween 80	1 (v/v)	97
Tween 80	5	84.3
Brij 35	1 (v/v)	100
Brij 35	5	100
SDS	0.1 (w/v)	76.4
SDS	0.5	33.5
Hydrogen peroxide	1	74.1
Hydrogen peroxide	2	62
Sodium perborate	1	63.4
Sodium perborate	2	51.7

To determine the residual activity of crude protease extract at pH 9.5 and 60°C, it was preincubated for 60 min at 40°C in the presence of Triton X-100, Tween 20, Tween 80, Brij 35, SDS, H_2O_2 and sodium perborate (Table 1). The enzyme was highly stable in the presence of the non-ionic surfactants like Triton X-100, Tween 20, Brij 35. It showed inhibition of 3% and 15.7% at 1% and 5% Tween 80 respectively. Stability towards SDS is indispensable because SDS stable enzymes are also not generally available except for a few [23]. In the present study, the activity of an enzyme in the presence of 0.1% and 0.5% SDS was determined as 76.4% and 33.5% respectively. In addition, bleach stability of hydrogen peroxide and sodium perborate was investigated. The activity of PP 16 protease decreased with increasing concentration of H_2O_2. It was 74.1% with 1% H_2O_2 and was 62% after incubation with 2% H_2O_2. Sodium perborate at 1% caused 36.6% inhibition and 2% concentration caused 48.3% inhibition. Only very few studies are reported on Bleach stable enzymes [24,25].

The alkaline crude protease extract was preincubated with surfactants and bleaching agents for 60 min at 40°C and the remaining activity was measured at pH 9.5 and 60°C. The enzyme activity is expressed as a percentage of the activity level in the absence of additives.

3.3.4 Effect of different organic solvents on the stability of protease

It is observed from the earlier investigation that the organic solvent-stability of enzymes depends on the kind of enzyme and organic solvent [26]. The protease can act in the presence of a different concentration of solvents in the reaction system (Fig. 7). The enzyme showed 80-90% of maximum activity for 20% methanol, benzene, xylene and toluene. Rest of the solvents used here showed more than 70% of total activity in 10% of solvents except acetone. Moreover, studies on a novel tolerant strain of *P. aeruginosa* ST-001 reports that it can grow even in the presence of an excess of toluene [27].

Further, it is also reported that the thermostable protease from a strain of *Bacillus* was stable in the presence of solvents including methanol, petroleum and ethanol [28]. An activity of a solvent stable endopeptidase from a marine crab is increased eight-fold in the presence of 2-propanol [29].

3.5.5 Washing test with the crude protease enzyme preparation

The enzyme showed extreme stability from 40-50^o C in the presence of all detergents tested (Fig. 8). Though the enzyme is incubated for 1 hr at 40 and 45^o C, it retained 87.9% of its original activity in all the commercial detergents tested. In Surf excel and Tide, about 92% of original activity was detected after 1 hr incubation at 40, 45 and 50^o C. Protease showed finest stability and compatibility at 50^o C with most of the detergents. The enzyme retained about 93, 91 and 86.4% of its initial activity in the presence of Surf excel, Tide and Mr. White respectively. Similarly, detergents like Fena and Wheel retained 82.5 and 79% of its activity respectively.

It is concluded from these experimental results that PP16 protease enzyme is more efficient and active at a temperature from 40°C-50°C for 1 hr. These findings were compared with the reports represented by other workers from different strains of Bacillus sp. [30]. The results (Fig. 9) of the present study shows that alkaline protease from *B. subtilis* exhibited high efficiency for the removal of blood and egg yolk stains at 40 and 45°C with all detergents. Therefore, it is reported that subsidized alkaline protease isolated from *Bacillus subtilis* could effectively cleanse the stains and brings complete removal even at high temperature (50°C).

Limited stain removal was observed if detergent alone was used, further stains supplemented with detergent

and crude proteases performed finer stain removal. The obtained results denote that the crude protease enzyme can potentially remove a variety of stains such as blood and egg yolk. There are reports on the use of bacterial proteases for removal of stain [31]. However, only very few information is available in the literature regarding the use of active proteases from the bacterial isolates of crab for detergent additive in the laundry industry.

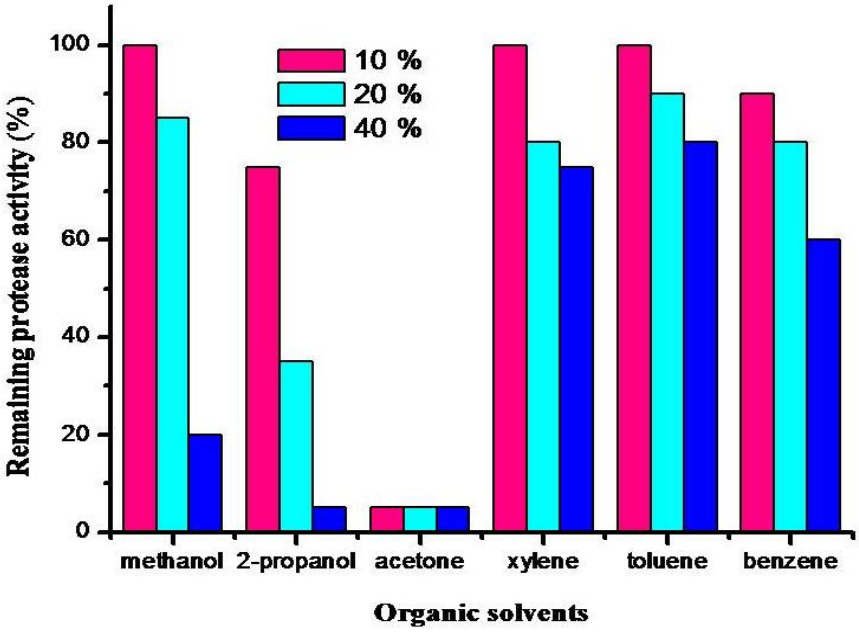

Fig. 7. Effect of organic solvents on the stability of alkaline protease

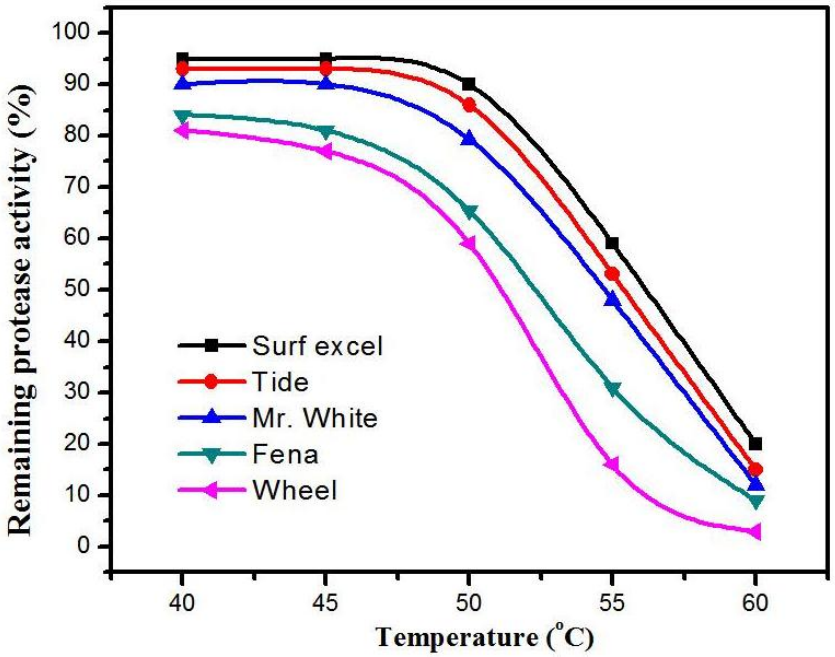

Fig. 8. Stability of the alkaline protease from *Bacillus subtilis* in the presence of various commercial solid detergents

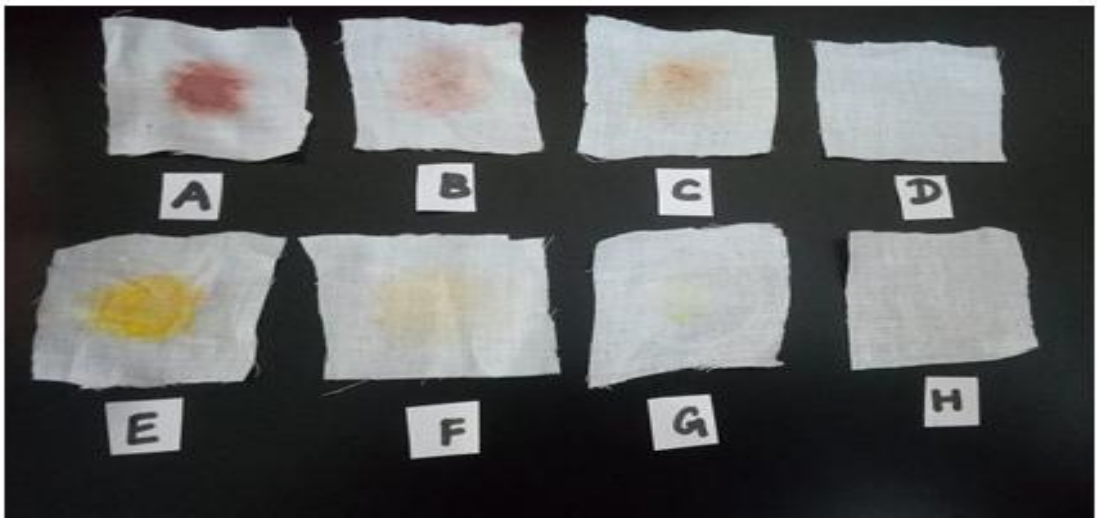

Fig. 9. Washing performance analysis of alkaline protease from *Bacillus subtilis* in combination with commercial detergents where (A) Cloth stained with blood, (B) Bloodstained cloth washed with water only (C) Bloodstained cloth washed with detergent only, (D) Bloodstained cloth washed with detergent and enzyme, (E) Cloth stained with egg yolk, (F) Egg yolk stained cloth washed with water only (G) Egg yolk stained cloth washed with detergent only, (H) Egg yolk stained cloth washed with detergent and enzyme

4. CONCLUSION

In the present study, the alkaline protease producing gut microbial isolate *Bacillus subtilis* is isolated and identified based on morphological, biochemical and molecular characterization. The enzyme shows more stability and high activity at pH range of 7.0–11.0 with an optimum at pH 9.0–10.0 and temperature 55-60°C. The enzyme is highly stable in the presence of the non-ionic surfactants like Triton X-100, Tween 20, Brij 35 which showed inhibition of only 3% and 15.7% at 1% and 5% Tween 80 respectively. The analysis also proves the stability of protease in the presence of bleaching agent's where1% Hydrogen peroxide shows 74.1% activity and 1% sodium perborate shows 63.4% activity. The enzyme shows 80-90% of maximum activity for 20% methanol, benzene, xylene and toluene. Further, the alkaline protease extract reveals high stability in the presence of various commercial solid detergents even at 50^{o} C and effectively removes the stains of blood and egg yolk. Concerning the significant and promising properties of crude protease enzyme from *Bacillus subtilis*, it may find its potential applications in laundry detergents as an additive.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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