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Original Article

Isolation, partial purification, biochemical characterization and detergent compatibility of alkaline protease produced by *Bacillus subtilis*, *Alcaligenes faecalis* and *Pseudomonas aeruginosa* obtained from sea water samples

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ABSTRACT

In the current study, bacteria isolated from sea water samples of Murdeshwar, Karnataka, were screened for the production of alkaline protease by culturing them onto skim milk agar media. Of the isolated bacteria, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Alcaligenes faecalis* showed distinct zones of hydrolysis due to enzyme production. They were each inoculated into enzyme production media under submerged fermentation conditions at 37 °C for 48 h with a constant agitation of 120 rpm. Partial purification of alkaline protease was carried out by isoelectric precipitation. Enzyme activity was determined under varying conditions of pH, incubation temperature, different substrates, carbon and nitrogen sources and salt concentrations using sigma's universal protease activity assay. Enzyme immobilization was carried out using 2% Sodium alginate and 0.1 M ice cold $CaCl_2$ and its activity under varying pH, temperature conditions and detergent compatibility was assayed. Efficacy of enzyme in stain removal was tested and haemolysis was observed within of 60 s which resulted in removal of the stain. Among the three organisms, enzyme from *Bacillus subtilis* showed highest activity in all cases indicating that it was the most ideal organism for enzyme production.

1. Introduction

Detergents are very important in fabric cleaning process because they disperse well in water, do not damage the fabric or our body on exposure and cleanse different types of stains. Detergents can clean stains of protein, chemical, fat, carbohydrate or any other origin. A Laundry detergent formulation contains a multitude of components and comes in solid powder form to liquid formulations [1]. A typical laundry detergent powder, contains the following components: Builders such as Sodium tripolyphosphate (Inorganic), nitrilotriacetic acid (Organic) or polycarboxylates (Polymer) to eliminate hardness and maintain alkalinity, anionic, cationic or non-ionic surfactants, bleaching agents like percarbonates or perborates and lastly additives like enzymes to make the cleansing process more efficient and perfuming agents to impart fragrance [2,3]. Detergents remove stains by electrostatic interactions and steric hindrance. The negatively charged micelles of detergent bind to the dirt particles and also to fabric and imparts a negative charge to them. Hence in presence of water, these micelles repel each other by electrostatic repulsions which prevent dirt particles from sticking to the fabric surface again. These micelles are then washed away [4]. Enzymes constitute an important part of detergent formulations. Enzymes are highly beneficial because they reduce activation energy of a reaction thereby making a reaction process more efficient with reduced energy consumption. By optimizing parameters we can ensure that enzymes function most efficiently and deliver the desired. The various parameters that can be optimized are pH, temperature of reaction, dosage, etc. The use of enzymes in chemical processes saves substantial amount of water [5]. Enzymes find application in various industries such as tanning, pharma, brewery, dairy, detergent and many more. Particularly in the detergent industry, the types of enzymes used in detergent formulations range from lipases, amylases, cellulases to proteases. Proteases are group of enzymes

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1687-157X/

Table 1
Biochemical tests used to identify bacteria.

Tests	*Bacillus subtilis*	*Pseudomonas aeruginosa*	*Alcaligenes faecalis*	*Escherichia coli*	*Shigella flexneri*
Gram staining	Gram positive rods	Gram negative Rods	Gram negative coccobacilli	Gram negative Rods	Gram negative rods
Catalase	+	+	+	+	+
Oxidase	–	+	+	–	–
Indole	–	–	–	+	–
Methyl Red	–	–	–	+	+
Voges-Proskauer	+	–	–	–	–
Citrate	+	+	+	–	–
Urease	–	+	–	–	–
TSI agar (Slant/Butt)	Red/Yellow	Red/Red	Red/Red	Yellow/Yellow	Red/Yellow
Glucose fermentation	+	–	–	+	+
Lactose fermentation	–	–	–	+	–
H_2S production	–	–	–	–	–
Starch hydrolysis	+	–	–	–	–
Gelatin liquefaction	+	+	–	–	–
Nitrate reduction	+	Denitrifying	–	+	+

that bring about the hydrolysis of peptide bonds. Depending on the type of protease involved, peptide bonds adjacent to specific amino acids are cleaved. Among different proteases, alkaline protease has gained utmost utility in the detergent industry as it functions optimally above pH 8 and detergent formulations also have very high pH ranging from 9.0 to 12.0. Microorganisms serve as an important source of this enzyme in contrast to neutral proteases (with optimum activity around pH 7), which are mainly of plant origin [6]. Enzymes could be used as dry lyophilized beads in detergent powder formulations or they could be used as liquid enzymes. However, stability of liquid enzymes is an important concern and they are used as insolubilized enzymes in dispersed formulas, such as liquid dispersion products (LDPs) which contain components such as polyols, sugars, organic acids, metal salts, and water, to balance enzymatic stability. Loss in activity of enzymes could not only be caused by proteolysis but also by oxidation, or degradation by other chemical components of detergent or even by other enzymes. Therefore, enzymes must be highly tolerant to proteolysis and must have a long shelf life [7]. In the current study as well, determination of most optimum conditions for production of alkaline protease from halophilic *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Alcaligenes faecalis* is done and application of this enzyme in detergent formulation has been focused on.

2. Materials and methods

2.1. Isolation and biochemical characterization of bacteria from sea water sample

Sea water sample (undiluted) was subjected to serial dilution [8]. Five distinct bacterial colonies were obtained after incubation and identified by biochemical characterization and maintained as pure cultures [9,10]. Table 1 lists all biochemical tests conducted.

2.2. Screening for alkaline protease producing bacteria

Each organism was screened for production of extracellular alkaline protease by inoculation onto skim milk agar with some modification which consisted of Skim milk powder – 5%, Peptone – 0.25%, Yeast extract – 0.5%, Glucose – 1%, Agar – 2.5% and pH 8 and incubated at 37 °C for 24 h [11]. Skim milk powder and glucose was however sterilized separately and then added to autoclaved medium. It was observed that only colonies of *Bacillus subtilis*, *Alcaligenes faecalis*, *Pseudomonas aeruginosa* showed distinct zones of hydrolysis around them while *E. coli, Shigella flexneri* showed no zone of casein hydrolysis. Fig. 1 shows casein hydrolysis by *Alcaligenes* on skim milk agar.

Fig. 1. Casein hydrolysis seen as zone of clearance around colonies on skim milk agar.

2.3. Cultivation of bacteria in enzyme production media

Loop full of cultures of *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Alcaligenes faecalis* were inoculated into separate flasks containing alkaline protease production media which was made up of the components of Horikoshi-I alkaline medium (pH 9) i.e. D-glucose-10 g, Peptone-5 g, Yeast extract-5 g, KH_2PO_4-1 g, $MgSO_4 \cdot 7\ H_2O$-0.2 g, Na_2CO_3-5 g and Distilled water-1000 ml [12]. Glucose and sodium carbonate were autoclaved separately and then added to rest of the autoclaved medium and incubated at 37 °C for 48 h with continuous agitation of 120 rpm. After 48 h, matt growth of bacteria was seen in the liquid media. The media was filtered using Whatman® filter paper in order to remove the microbial matt. The filtrate so obtained was further centrifuged at 4000 rpm for 10 min to remove any residual particles that may be present in it. The filtrate was then separated and used for further process [13].

2.4. Separation and partial purification of alkaline protease by isoelectric precipitation

The filtrate obtained after centrifugation was subjected to isoelectric precipitation in order to obtain crude alkaline protease

enzyme. Protein solubility in any solution is dependent on the pH of the solution. The pH at which the protein is least soluble in its solution and has a net charge of zero is called the isoelectric pH of the protein. When the pH of a solution is below the isoelectric pH of protein, the protein is positively charged and above isoelectric pH, the protein becomes negatively charged [14]. Isoelectric precipitation is one of the many methods used in protein purification. In the current study, the filtrate after centrifugation was first transferred to a clean beaker. As the pH of enzyme production media was maintained alkaline (pH 9), only those enzymes which were stable at high pH would be produced by the bacteria (i.e. alkaline enzymes). Therefore, as alkaline proteases are active only above pH 8, 0.5 N NaOH was used to raise the pH of the filtrate. Prior to addition of NaOH, the initial pH of the filtrate was noted. Following this, using a micropipette, 0.5 N NaOH was added dropwise, and filtrate was stirred each time till precipitates began to form in it (isoelectric point). The pH of the solution was then determined and it was found that for alkaline protease obtained from *Bacillus*, the isoelectric point was attained close to pH 10 while for the enzymes obtained from *Pseudomonas* and *Alcaligenes,* the isoelectric point was attained close to pH 9. The filtrates containing the precipitates were allowed to stand for 30 min, after which the protein free solution in the upper region of the beaker was carefully decanted using a micropipette. The filtrate at the bottom of the beaker which contained the precipitates, was then transferred into Eppendorf tubes and subjected to centrifugation at 3000 rpm for 10 min. The filtrate so obtained was discarded and a suitable buffer serving as an enzyme diluent was added to the pellet and gently mixed [15].

2.5. Ninhydrin test to detect hydrolysis of casein by enzyme alkaline protease

Ninhydrin test detects the presence of free amino acids in solution by producing a coloured reaction. The colour of the chromophore varies depending on reacting entities such as primary amino groups or imines or amides and so on [16]. This test was conducted to identify whether any free amino acids were present in the enzyme diluent solution that could give false positive results. 0.5 g of Ninhydrin was weighed and dissolved in 30 mL acetone. 20 mL of acetate buffer (pH 5.5) was added to this solution and the mixture was stored in a dark bottle [17]. Two separate reactions were set up. The first tube contained only enzyme diluent. For every 1 mL of enzyme solution, 3 mL of ninhydrin solution was added. The tube was swirled thoroughly for 30 min. No colour reaction was observed indicating presence of whole protein and no free amino acids in the enzyme diluent solution. This is because ninhydrin reacts only with amino acids and not whole (undegraded) protein. In the second reaction set up, 1 mL of enzyme diluent was taken and 1 mL of 1% casein solution was added and the tube was incubated at 37 °C for 15 min. To this tube, 1 mL of 5% Trichloroacetic acid was added in order to stop the reaction and the contents of the tube were subjected to centrifugation at 3000 rpm for 10 min. To 1 mL of supernatant obtained, 3 mL of ninhydrin was added and the tube was swirled thoroughly for 30 min. The incubation time can be considerably reduced if heat is applied. Soon, a colour change to purple was seen because the supernatant contained free amino acids which were released as a result of casein hydrolysis by the enzyme alkaline protease. Casein was used because it is known to be the best substrate for alkaline protease enzyme. Ninhydrin test represented in Fig. 2.

2.6. Estimation of alkaline protease activity

Activity of protease was estimated by Sigma's non-specific protease activity assay:

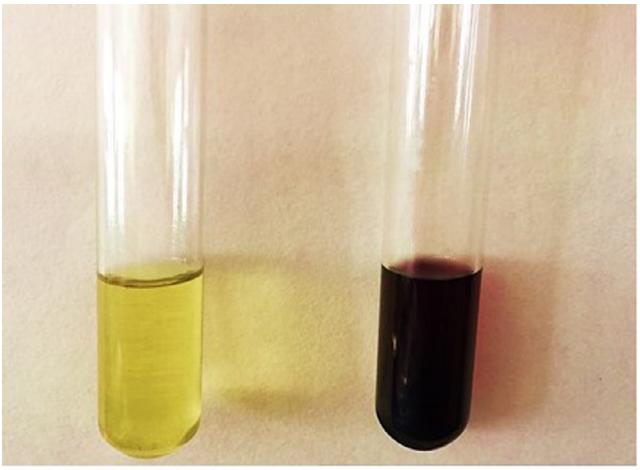

Fig. 2. Ninhydrin test to detect presence of alkaline protease.

Preparation of test samples: For enzyme isolated from each organism, this procedure was carried out. A series of empty test tubes were taken out of which, one test tube was labelled as blank and the remaining were labelled as test samples. In all the tubes, 5 mL of 0.65% of substrate was added and the tubes were incubated at 37 °C for 5 min. Following this, varying volumes of enzyme (<1 mL) was added to all the tubes except blank and the tubes were swirled in order to mix the enzyme and substrate evenly. The tubes were then incubated at 37 °C for 15–20 min. After incubation, 5 mL of 110 mM TCA was added to all tubes to stop the reaction. Then remaining enzyme solution was added to all tubes such that the final volume of enzyme in each tube was 1 mL. The tubes were then incubated at 37 °C for 30 min. After incubation, 2 mL of each test solution and blank was obtained by filtration using syringe filter and transferred to new unused tubes. Following this, 5 mls of sodium carbonate was added to all tubes and 1 ml of Folin's reagent was added immediately after. The tubes were incubated again at 37 °C for 30 min. After incubation, 3–4 mL of solution from each tube was obtained by filtration and used to check absorbance at 660 nm. Preparation of standard: 1.1 mM Tyrosine standard solution was prepared. Six test tubes were taken and to the first tube, 50 μL of standard solution (corresponding to 0.055 μM Tyrosine) was added followed by 100 μL (0.111 μM Tyrosine), 200 μL (0.221 μM Tyrosine), 400 μL (0.442 μM Tyrosine) and 500 μL (0.553 μM Tyrosine) respectively to the remaining tubes except blank. Then, to each tube including blank, distilled water was added such that the total volume came up to 2 mL in all tubes. The tubes were left aside for incubation at 37 °C for 30 min. Then 5 mls of sodium carbonate was added to all tubes and 1 ml of Folin's reagent was added immediately after. The tubes were incubated again at 37 °C for 30 min. After incubation, 3–4 mL of solution from each tube was obtained by filtration and used to check absorbance at 660 nm. These values were used to plot the standard graph.

In order to generate standard curve, difference between standard and standard blank was calculated and value of ΔA_{660}(standard) was obtained. Using this value, standard curve was created. Following this, in a similar manner ΔA_{660}(Test sample) was calculated for each test sample and plotted on the standard curve in order to obtain the μM of tyrosine generated in each test sample. The higher the protease activity, more was

the μM of tyrosine generated. Enzyme activity was calculated using the following formula [18]:

$$\text{Protease activity (U/mL)} = \frac{\mu\text{Moles of tyrosine equivalents released} \times \text{Total volume of assay (mL)} \times \text{Dilution factor}}{\text{Total volume of enzyme used in the assay (mL)} \times \text{Time of assay (min)} \times \text{Volume in cuvette (mL)}}$$

2.7. Optimization of enzyme production media by different parameters

2.7.1. Effect of pH on alkaline protease activity

Effect of different pH on the activity of non-immobilized enzyme was tested. Enzyme from each organism was tested with two different buffers. The enzyme was pre incubated with the respective buffer for 20 min at 37 °C. The buffers used for assay were sodium acetate buffer with calcium (10 mM Sodium acetate buffer with 5 mM Calcium) and 0.1 M Potassium phosphate buffer of pH 7, pH 8, pH 9, pH 10 and pH 11. After incubation, activity of protease was assayed as per Sigma's non-specific protease activity assay using 0.65% casein as substrate.

2.7.2. Effect of temperature on alkaline protease activity

Effect of temperature on the activity of non-immobilized enzyme was tested. The enzyme was diluted using an optimum pH buffer. The activity of protease was assayed as per Sigma's non-specific protease activity assay using 0.65% casein, as substrate. Incubation time of enzyme substrate mixture was maintained for 20 min but at different incubation temperatures each time. The temperatures for which activity of enzyme was assayed were 25 °C, 30 °C, 35 °C, 40 °C, 45 °C, 50 °C, 55 °C, 60 °C, 65 °C and 70 °C.

2.7.3. Effect of different substrates on alkaline protease activity

Effect of different substrates on the activity of non-immobilized enzyme was tested as per Sigma's non-specific protease activity assay. Enzyme diluted in a suitable buffer was incubated with different substrates each time. The substrates tested were 0.65% casein, 0.65% Soya protein, 0.65% Gelatin and 0.65% BSA as substrate.

2.7.4. Effect of salt concentration on alkaline protease activity

As the organisms were obtained from sea water, effect of salt concentration on the activity of non-immobilized enzyme was tested by Sigma's non-specific protease activity assay using 0.65% casein as substrate. Different enzyme production media was prepared for each organism, and these different media comprised of a range of concentration of NaCl (1–10%). The culture flasks were then incubated at 37 °C for 48 h with an agitation of 120 rpm. Enzyme obtained was then separated and tested for protease activity.

2.7.5. Effect of carbon source on alkaline protease activity

Effect of five different carbon sources was tested on the activity of non-immobilized enzyme. The enzyme was diluted using an optimum pH buffer. The activity of protease was assayed as per Sigma's non-specific protease activity assay using 0.65% casein, as substrate. Different enzyme production media was prepared for every organism, each time with a different carbon source such as Glucose, Sucrose, Maltose, Lactose and Fructose.

2.7.6. Effect of nitrogen source on alkaline protease activity

Effect of five different nitrogen sources was tested on the activity of non-immobilized enzyme. The enzyme was diluted using an optimum pH buffer. The activity of protease was assayed as per Sigma's non-specific protease activity assay. Different enzyme production media was prepared for every organism, each time with a different nitrogen source such as Yeast extract, Beef extract, Ammonium sulphate, Urea and Gelatin.

2.7.7. Enzyme immobilization: effect of pH and temperature on immobilized enzyme

Effect of pH and temperature was studied using immobilized enzyme as well. For pH studies, the buffers used were potassium phosphate and sodium acetate buffers of pH 7, pH 8, pH 9, pH 10 and pH 11. Enzyme was first diluted with the respective buffer to bring the total volume to 1 mL and pre incubated for 20 min at 37 °C. To this, equal volume of 2% sodium alginate was added and the tube was inverted two-three times for even mixing. Using a micropipette, this solution was then transferred in drops into ice cold 0.1 M $CaCl_2$ solution. The beads so formed were collected and stored in an Eppendorf tube and used for further assays. The activity of protease was assayed as per Sigma's non-specific protease activity assay using 0.65% casein, as substrate. For temperature studies, the incubation time of immobilized enzyme substrate mixture was maintained at 20 min and the temperatures for which activity of enzyme was assayed were 25 °C, 30 °C, 35 °C, 40 °C, 45 °C, 50 °C, 55 °C, 60 °C, 65 °C and 70 °C.

2.7.8. Detergent compatibility and alkaline protease activity using immobilized enzyme

Compatibility of immobilized enzyme with lab based detergents like Tween 20, SDS and Triton X-100 and commercial detergents like Surf Excel, Wheel, Ariel and Patanjali was tested. For lab based detergents, 5% solutions of each were first prepared. Immobilized enzyme beads were then directly added to 1 mL of each of these lab detergents and protease activity was estimated using 'Sigma's non-specific protease activity assay' using 0.65% casein as substrate. The incubation time was maintained at 20 min. For commercial detergents also, 5% solutions of each were prepared. Following this, each detergent solution was subjected to a temperature of 80 °C for 45 min in order to ensure that the inherent enzyme in the commercial detergent formulation had denatured. After incubation, ninhydrin test was carried out on 1 mL of this detergent using 1 mL of 1% casein as substrate. Successful enzyme denaturation in the commercial detergent formulation was concluded due to absence of colour change in the ninhydrin test even in presence of substrate. Following this, the immobilized enzyme beads were added to 1 mL of each of the pre heated commercial detergent solutions and protease activity was estimated using 'Sigma's non-specific protease activity assay' using 0.65% casein as substrate. The incubation time was maintained at 20 min.

2.7.9. Efficacy of alkaline protease enzyme in stain removal

In order to test the ability of the enzyme to remove stains, three drops of blood were placed onto three separate pieces of cloth. The first was noted as control (with no treatment on the stain). To the second cloth with blood stain, treatment with water was given and to the last piece of cloth with the blood stain, treatment with crude enzyme was given. The start of the reaction was noted as 0th second.

3. Results

3.1. Effect of pH on alkaline protease activity

Non-immobilized enzyme: Alkaline protease isolated from *Bacillus*, recorded highest enzyme activity of 103.26 ± 2.11 U/ml at pH 10 in sodium acetate buffer, and 92.25 ± 1.82 U/mL at pH 10 in phosphate buffer, while the enzyme isolated from *Alcaligenes* recorded highest enzyme activity of 96.12 ± 1.59 U/ml at pH 9 in sodium acetate buffer and 89.5 ± 1.89 U/mL at pH 9 in phosphate buffer and lastly enzyme isolated from *Pseudomonas* recorded highest activity of 90.34 ± 1.09 U/ml at pH 9 in sodium acetate buffer and 85.4 ± 1.82 U/ml at pH 9 in phosphate buffer (Figs. 3 and 4). Results clearly indicated that in all cases, optimum pH of enzyme remained unchanged irrespective of the type of buffer used and also that the activity of enzyme in sodium acetate buffer was higher than that of phosphate buffer. (Enzyme activity indicated as Mean ± Standard Deviation).

Immobilized enzyme: Enzyme activity of immobilized enzyme in both buffers was higher than its non-immobilized counterpart. Alkaline protease isolated from *Bacillus*, recorded highest enzyme activity of 132 ± 1.83 U/ml at pH 10 in sodium acetate buffer and 104.24 ± 1.64 U/mL at pH 10 in phosphate buffer, while the enzyme isolated from *Alcaligenes* recorded highest enzyme activity of 115.9 ± 1.6 U/ml at pH 9 in sodium acetate buffer and 91.47 ± 1.95 U/mL at pH 9 in phosphate buffer and lastly enzyme isolated from *Pseudomonas* recorded highest activity of 106.07 ± 1.96 U/ml at pH 9 in sodium acetate buffer 84.7 ± 2.04 U/ml at pH 9 in phosphate buffer (Figs. 5 and 6). Here again, it was observed that optimum pH of enzyme remained unchanged irrespective of the type of buffer used but the activity of enzyme in sodium acetate buffer was higher than that of phosphate buffer (Enzyme activity indicated as Mean ± Standard Deviation).

3.2. Effect of temperature on alkaline protease activity

Non-immobilized enzyme: Alkaline protease isolated from *Bacillus*, recorded highest enzyme activity of 126.77 ± 1.73 U/ml at 55 °C, and enzyme isolated from *Alcaligenes* recorded highest enzyme activity of 112.64 ± 2.46 U/ml at 55 °C and lastly enzyme isolated from *Pseudomonas* recorded highest activity of 114.38 ± 2.36 U/ml at 50 °C as represented in Fig. 7 (Enzyme activity indicated as Mean ± Standard Deviation).

Immobilized enzyme: Enzyme activity of immobilized enzyme from all three organisms was higher than its non-immobilized counterpart. Alkaline protease isolated from *Bacillus*, recorded showed an increase in temperature tolerance by 5 deg and recorded highest enzyme activity of 135.21 ± 2.12 U/ml at 60 °C. The enzymes isolated from *Alcaligenes* and *Pseudomonas* showed no increase in temperature tolerance on immobilization and recorded highest enzyme activity of 123.01 ± 2.2 U/ml at 55 °C and 117.9 ± 2.28 U/ml at 50 °C respectively. Although significant increase in temperature tolerance was not seen in the latter two cases, there was an increase in enzyme activity at the same temperature as represented in Fig. 8 (Enzyme activity indicated as Mean ± Standard Deviation).

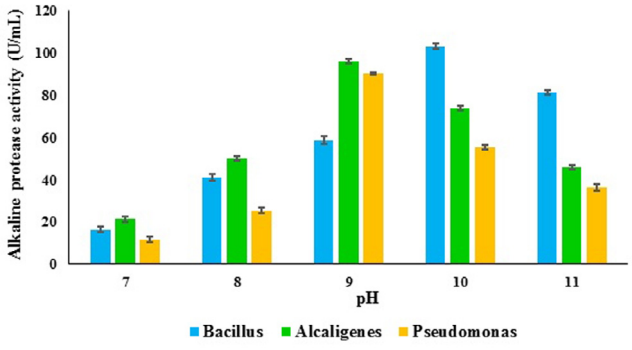

Fig. 3. Non-immobilized enzyme: Effect of pH on alkaline protease activity (U/mL) using sodium acetate buffer.

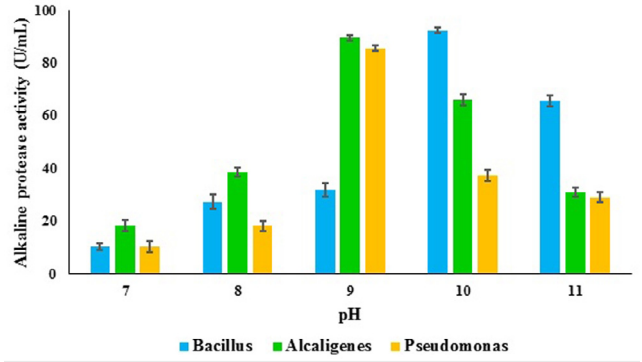

Fig. 4. Non-immobilized enzyme: Effect of pH on alkaline protease activity (U/mL) using potassium phosphate buffer.

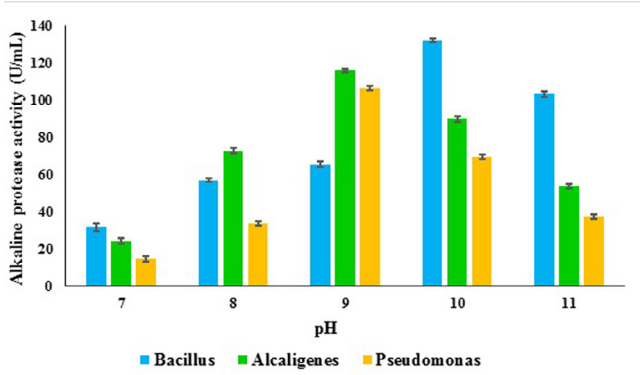

Fig. 5. Immobilized enzyme: Effect of pH on alkaline protease activity (U/mL) using sodium acetate buffer.

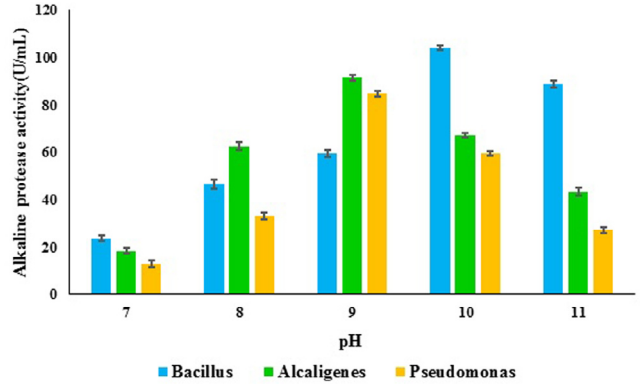

Fig. 6. Immobilized enzyme: Effect of pH on alkaline protease activity (U/mL) using potassium phosphate buffer.

3.3. Effect of different substrates on alkaline protease activity

Among the substrates tested, non-immobilized alkaline protease isolated from *Bacillus*, recorded highest enzyme activity of

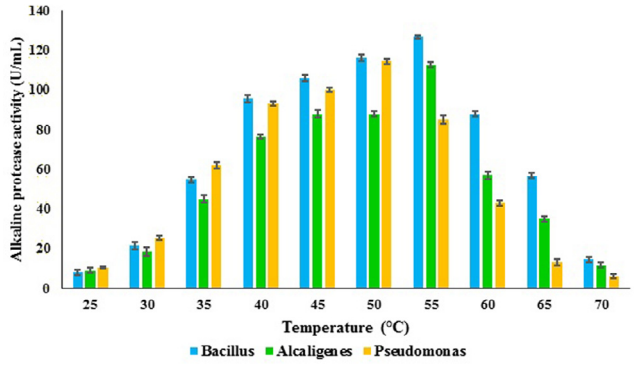

Fig. 7. Non-immobilized enzyme: Effect of temperature on alkaline protease activity (U/mL).

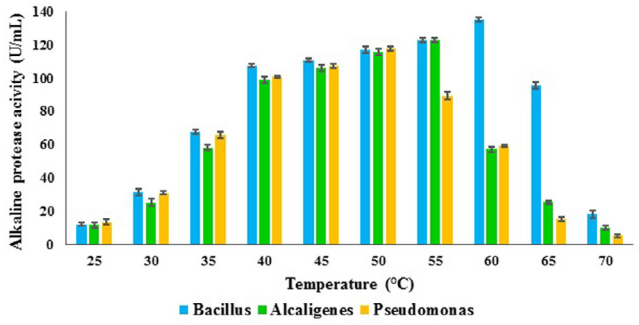

Fig. 8. Immobilized enzyme – Effect of temperature on alkaline protease activity (U/mL).

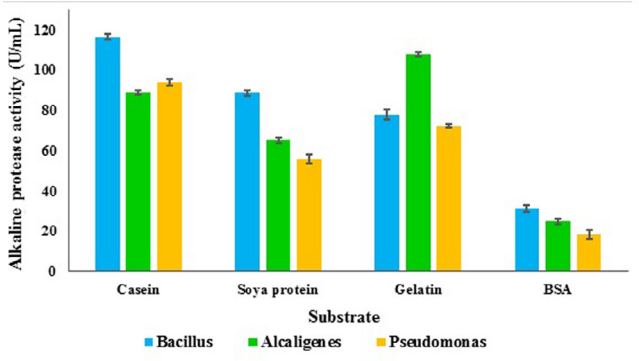

Fig. 9. Non-immobilized enzyme: Effect of different substrates on alkaline protease activity (U/mL).

116.41 ± 2.69 U/ml with 0.65% casein as substrate while enzyme isolated from *Alcaligenes* recorded highest enzyme activity of 108.07 ± 1.85 U/ml with 0.65% gelatin as substrate and enzyme isolated from *Pseudomonas* recorded highest enzyme activity of 93.99 ± 3.14 U/ml with 0.65% casein as substrate. In all cases, activity was lowest with BSA as substrate as represented in Fig. 9 (Enzyme activity: Mean ± Std. Dev.).

3.4. Effect of salt concentration on alkaline protease activity

In case of salt concentration ranging from 0 to 10% it was observed that the alkaline protease from *Bacillus* showed protease activity and salt tolerance up to 7% NaCl beyond which no activity was observed. Alkaline protease from *Alcaligenes* showed protease

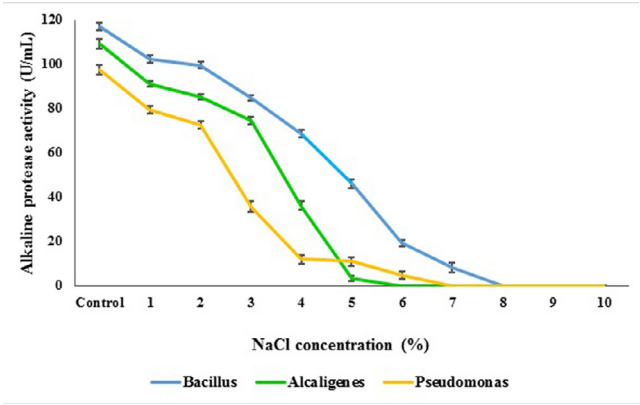

Fig. 10. Non-immobilized enzyme: Effect of salt concentration on alkaline protease activity (U/mL).

activity and salt tolerance up to 5% NaCl beyond which no activity was observed and alkaline protease from *Pseudomonas* showed protease activity and salt tolerance up to 6% NaCl beyond which no activity was observed. The control had no NaCl in the media and the enzyme from *Bacillus* showed highest protease activity of 116.77 ± 2.83 U/mL, that of *Alcaligenes* with 109.09 ± 3.52 U/mL and that of *Pseudomonas* with 97.46 ± 3.9 U/mL (Enzyme activity indicated as Mean ± Standard Deviation). Data represented in Fig. 10.

3.5. Effect of carbon and nitrogen sources

Alkaline protease isolated from *Bacillus*, recorded highest enzyme activity of 115.7 ± 1.95 U/ml with glucose as a carbon source, and lowest when maltose was used as a carbon source with an activity of 46.68 ± 1.56 U/mL. Activity of enzyme from *Bacillus* was found to be 112.97 ± 1.72 U/mL, which was highest when yeast extract was used as nitrogen source. Enzyme isolated from *Alcaligenes* recorded highest enzyme activity of 109.11 ± 1.81 U/ml with yeast extract as nitrogen source and comparable high activity with glucose and fructose as a carbon source which was 106.55 ± 1.89 U/mL and 105.52 ± 1.91 U/mL respectively and lowest with an activity of 38.07 ± 1.75 U/mL when maltose was used as carbon source. Lastly enzyme isolated from *Pseudomonas* recorded highest activity of 100.05 ± 1.91 U/mL when beef extract was used as nitrogen source, and an activity of 98.22 ± 1.91 U/ml with glucose as a carbon source and lowest of 10.84 ± 1.03 U/mL when lactose was used as carbon source. Also, in all cases, the enzyme activity was lowest when ammonium sulphate was used as nitrogen source. Data represented in Figs. 11 and 12 (Enzyme activity indicated as Mean ± Standard Deviation).

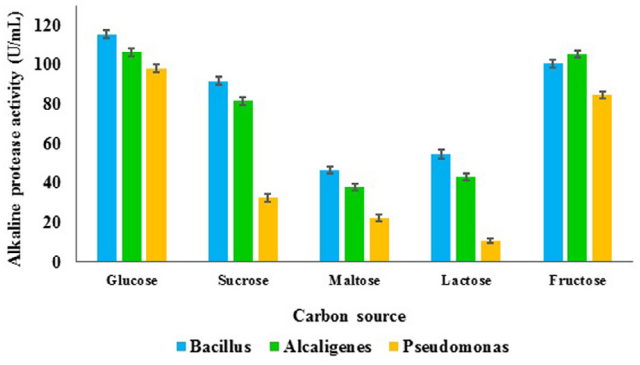

Fig. 11. Non-immobilized enzyme: Effect of different Carbon sources on alkaline protease activity (U/mL).

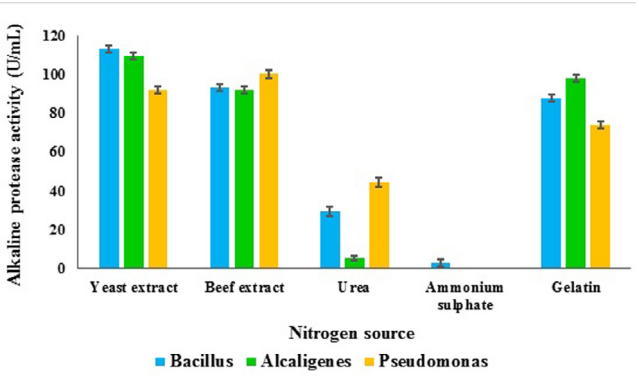

Fig. 12. Non-immobilized enzyme: Effect of different Nitrogen sources on alkaline protease activity (U/mL).

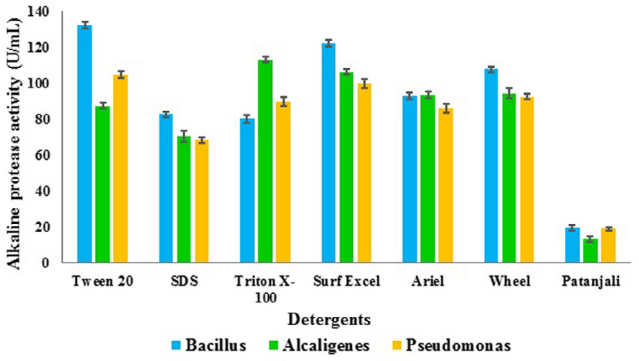

Fig. 13. Immobilized enzyme: Compatibility of alkaline protease with detergents and subsequent protease activity (U/mL).

3.6. Detergent compatibility and alkaline protease activity

Among the lab detergents, immobilized enzyme from *Bacillus* and *Pseudomonas* showed highest activity of 131.96 ± 1.75 U/mL and 104.69 ± 1.75 U/mL respectively with Tween 20 (5%) while immobilized enzyme from *Alcaligenes* showed highest activity of 112.78 ± 1.53 U/mL with Triton-X 100 (5%). Among commercial detergents, enzyme from *Bacillus*, *Alcaligenes* and *Pseudomonas* showed greatest compatibility with Surf excel with highest activities of 122.03 ± 1.90 U/mL, 106.14 ± 1.65 U/mL and 99.50 ± 2.49 U/mL respectively. Activity with Patanjali detergent was found to be lowest in all cases. It was observed that enzyme isolated from *Bacillus*, *Alcaligenes* and *Pseudomonas* showed an activity of 19.70 ± 1.735 U/mL, 13.51 ± 1.71 U/mL and 19.15 ± 1.04 U/mL respectively. Data represented in Fig. 13 (Enzyme activity indicated as Mean ± Standard Deviation).

3.7. Efficacy of alkaline protease in stain removal

Stain removal was successfully observed on applying crude enzyme over blood stain. Haemolysis was seen within 60 s of treatment of enzyme. Blood stain was retained on the cloth to which water treatment was given even after 60 s. (Fig. 14).

4. Discussion

From our study, among the three organisms, *Bacillus* was found to be most beneficial in all aspects Alkaline protease from *Bacillus subtilis* showed highest activity when the conditions for enzyme activity were maintained at pH 10, 55 °C to 60 °C incubation temperature of enzyme substrate mixture, substrate used was casein and when enzyme production media was optimized with glucose and yeast extract as carbon and nitrogen source respectively. Having isolated it from sea water, it showed a salt tolerance of up to 7% NaCl beyond which protease activity declined and finally ceased. Pant et al. [19] also conducted experiments on alkaline

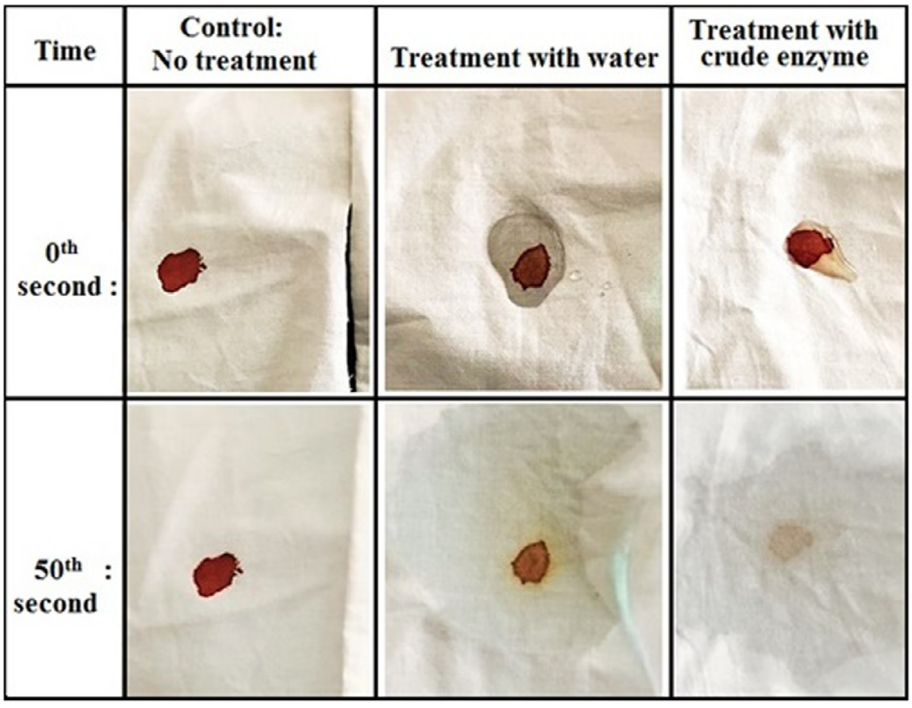

Fig. 14. Efficacy of enzyme in stain removal.

protease from *Bacillus subtilis* and reported that pH 10 and 45 °C were most optimum conditions for high enzyme activity. Also, in our study enzyme from *Alcaligenes faecalis* showed highest protease activity at pH 9, 55 °C incubation temperature of enzyme substrate mixture using gelatin as substrate and when enzyme production media was optimized with glucose or fructose as carbon source and yeast extract as nitrogen source. The enzyme showed a salt tolerance of up to 5% NaCl beyond which protease activity declined and ceased. Annamalai et al. [20], also concluded from their study that the enzyme from *Alcaligenes* showed optimum activity at pH 9 and 55 °C. Finally, in our current study, the enzyme from *Pseudomonas aeruginosa* showed highest protease activity at pH 9, 50 °C incubation temperature of enzyme substrate mixture using casein as substrate and when enzyme production media was optimized with glucose as carbon source and beef extract as nitrogen source respectively. The enzyme showed a salt tolerance of up to 6% NaCl beyond which protease activity declined and ceased. Meena et al., [21] established that *Pseudomonas* alkaline protease was active at pH 9 and 45 °C. Bayoudh et al., [22] identified the optimum pH and temperature of protease activity of enzyme from *Pseudomonas* as pH 8 and 60 °C. Abd-Rahman et al., [23], identified a organic solvent tolerant alkaline protease from *Pseudomonas* whose optimum pH and temperature of protease activity was found to be pH 10 and 70 °C. The variation in results could indicate that this organism adapts to variation in its environment and these factors also affect properties of the enzyme produced by it. Also, from our study, it was observed that immobilized alkaline protease from *Bacillus* and *Pseudomonas* was most compatible with the lab detergent Tween 20 and consequently showed highest protease activity, while that from *Alcaligenes* was most compatible with Triton-X 100 and also the enzyme from all organisms showed highest compatibility with the commercial detergent Surf Excel and consequently showed highest protease activity. Mala et al. [24] studied biocompatibility of alkaline protease from *Bacillus* sps. with commercial detergents such as Kite, Tide and Aerial and reported that the enzyme showed 70% activity even after three hours. Jaswal et al. [25] studied compatibility of enzyme from *Bacillus cirulans* with six commercial detergents and reported the highest compatibility with Fena, Rin and Tide. Finally, in our study, stain removal was very efficient and haemolysis of blood occurred within 60 s of enzyme action on the blood stain. Anupama et al., [26] also studied the blood stain removal potency of alkaline protease isolated from *Bacillus licheniformis* and reported the successful removal of stain on application of enzyme. Vishalakshi et al., [27] reported the successful removal of blood stain by alkaline protease from *Streptomyces gulbargensis.* Further, the purification of crude enzyme by ion exchange chromatography or gel permeation chromatography is recommended as it would yield the enzyme in its purest form. Studies carried out previously related to purification of alkaline protease by chromatography have yielded enzyme with high specific activity [28]. Purification also ensures thermostability and pH stability to the enzyme [29]. In conclusion, out of the several microorganisms screened for the production of this enzyme, members of the genus *Bacillus* have been identified as chief sources of alkaline proteases, viz., *Bacillus amyloliquefaciens, Bacillus subtilis, Bacillus thuringiensis, Bacillus alcalophilus, Bacillus proteolyticus, Bacillus licheniformis*. Some examples of commercial detergents which contain enzyme from *Bacillus* are: Dynamo, Era plus (Procter & Gamble), Tide (Colgate Palmolive), Alkazym, Terg-a-zym [30].

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