Effect of Alkaline Protease Produced from Fish Waste as Substrate by Bacillus clausii on Destaining of Blood Stained Fabric

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ABSTRACT

Alkaline protease or peptidases are the largest groups of enzymes in the biological industry with a variety of applications in manufacturing units used in the process of substrate stabilization, dehairing, diagnosis, extraction, food production, destaining, etc., where the pH of the environmental conditions remain above neutral pH. Because of their wider applications of alkaline proteases in industries, their demand is increasing to compete with their chemical counterparts. An alkaline tolerant bacterial strain Bacillus clausii was isolated from fish waste and used for mass production of alkaline protease using fish waste homogenate as media. Preliminary study on optimization of conditions for the mass production carried out. The optimum temperature for production ranges between 25°C and 35°C and pH determined as 9. The mass production of extracellular alkaline protease carried out using mobilized and immobilized cells of B. clausii at optimized condition using production media, the mixture of production media and fish waste homogenate and in nutrient broth. The recorded results showed that the maximum enzyme production obtained with immobilized cells in nutrient broth media and followed by fish waste homogenate media of 8900 U/ml and 8600 U/ml. Purification protease enzyme yielded 0.35 g/ml from the production media. Blood-stained cloth treated with immobilized enzyme removed the stain completely compared to treatment with non-immobilized enzyme and commercially used detergent. So, the current study suggests the usage of microbial alkaline protease in household detergents to replace the usage of synthetic detergents and save the environment from chemical pollutants.

Keywords: Alkaline protease, Destaining, Fish waste media, Immobilization, Mass production, Optimization

Introduction

Alkaline protease is one of the most important enzymes in the commercial field as its demand is increasing, so the enzyme requirement occupies a large sector in industries [1]. The enzyme required in volumes for removal of wastes and impurities, ligand formation, destaining, protein refining and blending in the leather industry, diagnosis process, extraction of silver, animal feed production, and food processing [2]. Protease enzyme are mainly derived from animal, plant, and microbial sources. Among them, microbial production from Bacillus subtilis considered as cheap. But major factor influencing the cost of microbial production is the substrate used in the production [3].

Economically cheap resources from the environment are given priority in producing microbial proteases. However, no defined medium has been established for the best production of proteases from different microbial sources. Several investigations have looked for ways of producing microbial proteases using inexpensive media. Various protein-rich wastes are a consideration as a substrate for the microbial production of a protease enzyme on a large scale [4]. Fish farming and the sale of fishes from dams and sea are the major income source of many fishermen. A huge amount of solid wastes including fish viscera, skin, fins, and liquids from drained fish storage tanks released drastically into sea or rivers which causes
severe pollution and associated problems [5]. After the usage of the edible part of the fish, the abrupt release of fish waste into the environment has detrimental effects on the environment and became an issue of public interest. The release of organic wastes determines changes in the community structure and biodiversity of the environmental assemblages. Moreover, the fish viscera are also from an animal source. Fish processing generates solid wastes that are as high as 50–80% of the original raw material [6]. These wastes are excellent raw materials for the preparation of high protein foods. About 30% of the wastes consist of skin and bone with high collagen content. So, the reuse of protein-rich fish waste as a substrate for the production of microbial proteases is under consideration. The current study revealed that the diversified utilization of fish processing waste considered as the potential media for microbial proteases production and expected to deliver an attractive and promising strategy for protease enzyme production.

Interestingly, studies demonstrated that fish processing wastes such as fish meat wastes, chitin material from cephalopods and wastewater offers good potential for used as a substrate for microorganisms [7-9]. Wastewater from fish processing industry supplemented with cuttlefish by-products powder was also tested as growth media for microbial growth and protease production by five bacterial species (Bacillus licheniformis, Bacillus subtilis, Pseudomonas aeruginosa, Bacillus cereus BG1, and Vibrio parahaemolyticus). According to Souissi et al., 2008, all the tested strains obtained their carbon and nitrogen source requirements directly with proteins from cuttlefish by-products and, interestingly, further addition of the fishery wastewater improved the protease activity. In commercial practice, still, optimization of medium composition carried out to sustain a balance between the various microbial growth nutrients [10].

So, global protease production from fish and shrimp waste has been in a steadily increasing trend over the last decade and this trend expected to continue. Bacillus species are the common producers of extracellular proteases and in industrial sectors, commonly Bacillus subtilis used for the production of various enzymes. More than 99% of are extracellular protease which has advantages of the ease of recovery and application in the industrial sector [11]. Application of protease in industries further enhanced by immobilizing on solid particle. As solid particles are the efficient delivery system at lower concentrations, the study aims to produce microbial alkaline protease using fish waste as substrate under optimized conditions, immobilize protease and compare the efficiency of immobilized and non-immobilized protease enzymes on distaining process.

Material and Methods
Preparation of fish waste as substrate
The fish waste Sardinella longiceps includes viscera, fins, scales, and bones collected from the local fish market (Palakkad, Kerala) in fresh condition and brought to the maintained at 4°C. The collected samples homogenized aseptically in a mixer (homogenizer) and used as a substrate for further studies.

Identification of alkaline protease producing bacteria
For isolation of protease producing bacteria, soil sediment from fish waste dumping the site collected aseptically, serially diluted till 10^6 dilutions and plated on sterile nutrient agar and skim milk agar plates at pH 9. Plates incubated at 37°C for 24 hours. Colonies with a clear zone of inhibition selected for the study [12]. The organism showing a large zone of clearance selected for further study and identified by microscopic (size, shape, arrangement, Gram-staining, spore staining), biochemical tests includes oxidase test, catalase test, starch hydrolysis, casein hydrolysis, gelatin hydrolysis and coagulase test. DNA from the overnight broth culture of the isolate was extracted by enzymatic lysis using bacterial DNA isolation kit, Sigma Aldrich, India, yielding 20 μg of DNA and molecular identification of isolate was carried out by 16S rRNA gene sequencing using the primer 5′-AAGAGCCGTT-3′. Amplified and sequenced nucleic acid compared with existing nucleotide databases using the BLASTN program. Then specific species identified from list of organism showing maximum homology generated by multiple sequence analysis of 16S rRNA coding region. [13].

Optimization of culture conditions for the maximum growth of isolate
Skim milk agar plates with different pH at 4, 6, 7, 9, and 11 prepared and inoculated with the isolate for determining the effect of pH on the proteolytic activity of isolates. Skim Milk Agar plates with pH 9 prepared and inoculated with selected isolate and incubated at different temperatures (15°C, 25°C, 35°C, 40°, 45°, 50° and 60°C) for determining the effect of temperature on its proteolytic activity. All experiments conducted in tripli-
Mass production of alkaline protease

For the selection of cheap substrate for the mass production, different media were tested such as production media \((\text{NH}_4)_2\text{SO}_4: 2 \text{ g/L}, \text{K}_2\text{HPO}_4: 1 \text{ g/L}, \text{KH}_2\text{PO}_4: 1 \text{ g/L}, \text{MgSO}_4: 0.01 \text{ g/L}, \text{MnSO}_4: 0.01 \text{ g/L}, \text{FeSO}_4: 0.01 \text{ g/L}, \text{Peptone: 10 g/L, and yeast extract: 1 g/L})\), homogenized fish juice media (10 g of homogenized fish waste in 100 ml of distilled water), a mixed media (100 ml production media + 2% of homogenized fish juice) and nutrient broth prepared, adjusted to pH 9, inoculated with 1 ml of overnight broth culture of normal cells and immobilized cells of isolate in separate flasks and incubated in a shaking incubator (140 rpm) at 35°C for 24 hrs for optimum production of protease enzyme.

Preparation of crude and partially purified alkaline protease

The culture media transferred to centrifugation tubes, bacterial cells removed by centrifugation at 10,000 rpm for 10 minutes at 4°C. The precipitate containing the cell debris discarded and supernatant treated with 70% ammonium sulphate and allowed to stand at 4°C overnight. Then the precipitate centrifuged at 10000 rpm for 20 mins at 4°C. The resulting precipitate dissolved in 0.82% NaCl. The samples subjected to dialysis using ammonium sulphate (60% saturation).

Immobilization of partially purified enzyme on calcium alginate beads

A slurry of calcium alginate beads prepared carefully. For enzyme immobilization, 1 ml of purified enzyme mixed with 9 ml of sodium alginate suspension (1, 2, and 3%). The preparation added dropwise into cold 0.2 M CaCl_2 solution with constant stirring. The beads obtained kept for curing at 4°C for 1h in a refrigerator. The cured beads so formed washed two to three times with sterile distilled water and stored at 4°C until use.

Assay of proteolytic activity of partially purified enzyme

The proteolytic activity of crude protease and purified extract assayed using Glycine-NaOH buffer (pH 9). The assay mixture consists of 1.25 ml of buffers, 0.5 ml of casein as substrate and 0.25 ml of enzyme extract. The reaction stopped by adding 3 ml of 5% TCA after holding at 37°C for 30 mins. The final mixture filtered and TCA soluble peptides infiltrate quantified by measuring the absorbance at 660 nm by Lowry’s method [14].

Effect of enzyme on blood stained cloth

Immobilized protease investigated as a destaining agent. For this study, four pieces (1 cm × 1 cm) of white cotton cloth stained with sheep blood and oven-dried at 95 – 100°C for 5 min. each cloth piece placed in a Petri dishes with 1 ml of distilled water. To the set-up, 1 % of commercial detergent, 1 ml of calcium alginate immobilized enzyme (8900 U/ml), and 1 ml crude enzyme (8250 U/ml) added respectively and allowed to destain for 30 minutes. The petri plate only with stained cloth and distilled water served as a control [15].

Results and Discussions

Collection of fish waste

Fresh fish waste of S. longiceps collected and stored at 4°C. The collected samples were homogenized aseptically in a mixer (homogenizer) (Figure 1). Resulted suspension appeared creamy in colour and was opaque.

Isolation and identification of alkaline protease producing bacteria

From the collected soil sediment pure culture of bacteria isolated and subjected to standard microscopic and biochemical tests. After performing standard biochemical test results, the isolated organism identified as rod shaped, non-motile, en-
dolspore-forming bacteria and positive for oxidase, catalase and casein, gelatin and starch hydrolysis test. The coagulate test was negative. So, the isolate confirmed as *Bacillus* sp., (Figure 2). Molecular studies confirmed that the isolate belongs to *B. clausii* as it showed 87.45% of sequence similarity with the already explored genome of *B. clausii* KSM-K16 [16]. *Bacillus* sp., highly resistant organism that could survive in extremes of conditions. Thereby the isolate survives at high temperature as a spore-producing organism and produced alkaline protease showing maximum activity at pH 9. These attributes considered favourable for industrial utilization of isolate for mass production of alkaline protease. Moreover, as *B. clausii* is a probiotic organism, the products can be considered as safe for commercial purposes [17].

**Optimization of pH and temperature for protease production**

The optimum pH for the proteolytic activity in skim milk agar medium with *B. clausii* at pH 4, 6, 7, 9, and 11 recorded as 0 mm, 0 mm, 24 mm, 26 mm and 0 mm respectively. So, the optimum pH for maximum production of protease determined as pH-9.0 for the isolated bacteria, and similar significant activity recorded at pH 7 also (Figure 3).

The growth and protease production influenced by different incubation temperatures. Proteolytic activity in skim milk agar medium with *B. clausii* at different temperature 15°C, 25°C, 35°C, 40°C, 45°C, 50°C and 60°C observed as 22 mm, 21 mm, 28 mm, 25 mm, 24 mm, 23 mm, and 20 mm respectively. The optimum temperature for maximum production of protease considered as 35°C (Figure 4) which was significantly higher than other temperatures of incubation. Besides 35°C, other incubation temperatures showed a proximately equivalent to enzyme production.

Similar research on the production of extracellular protease from fish waste using *Bacillus* sp., recorded as maximum in raw fish waste juice and in production medium at pH 9, incubation temperature of 35°C under shake culture the condition with 2.5 × 10⁹ cells/ml as an initial inoculum density for 36 h of incubation. The result confirms that isolates from the same raw source (fish juice) exhibited maximum protease yield when compared to the reference standard *B. subtilis* 14410. Also, the purified protease enzyme from *Bacillus* sp., exhibited maximum activity at 35°C (160.37 ± 0.18 U/ml) and beyond this temperature, the enzyme activity decreased [18]. Moreover, microbial sources of enzymes are more advantages than their equivalents from animal or vegetable sources. The advantage includes lower production costs, possibility of large-scale production in the industrial fermenter, ease of purification, production within a short period, a wide range of physical and chemical characteristics, the possibility of genetic manipulation, absence of effects brought about by seasonality, rapid culture development, and the use of non-burdensome methods. The above characteristics make microbial enzymes suitable bio-catalysts for various industrial applications [19].

**Mass production and purification of alkaline protease**

The alkaline protease enzymes purified by ammonium sulphate precipitation and dialysis and used for the enzyme assay. The below mentioned table gives enzyme yield from different media. Enzyme yield high in media inoculated with immobilized cells and maximum recovered from production medium of 0.35 g/ml. The general-purpose media, nutrient broth also yielded 0.23 g/ml and in fish homogenate media 0.19 g/ml recovered (Table 1). When organisms cultured in fish waste media, the enzyme produced is maximum consumed for hydrolysis of protein present in the media, as the rest of the enzyme was recovered, comparatively less amount of enzyme from the fish waste media. Though 40% less yield achieved from fish waste media compared to production cost in artificial media, fish waste media consiste-
Assay of activity of purified alkaline protease

Assay on enzyme activity also high by immobilized cells in nutrient broth culture as 8900 U/ml, next to that fish waste homogenate showed 8600 U/ml and in artificial production media of 8565 U/ml. Other media showed enzyme activity ranging 8240 U/ml to 8510 U/ml (Figure 5). The enzymatic range is approximately closer to the maximum activity. Comparatively, all purified enzymes showed maximum activity in an alkaline environment as the composition and purity the same, and experiments conducted under standard conditions.

The non-immobilized enzyme showed enzyme activity between 8200 to 8350 U/ml, whereas immobilized enzyme activity ranged between 8280 to 8900 U/ml. About a 1% increase in proteolytic activity of alkaline protease recorded from the study. Even 1% increase in enzyme activity also recognized as cost-effectively at an industrial scale. Immobilization helps to improve stability of the enzyme, decreases loss of enzyme activity due to changes in pH, temperature, conformational changes as a result of friction, osmotic pressure imposed by the environments of their use and a cumulative effect of all these factors as a function of the duration of their use [20]. Since enzymes are decipherable, their recovery from a mixture of substrate and product for reuse is not economically practical rendering the enzymatic process even more costly. However, the advent of immobilized enzyme technology has led to increasing efforts to replace conventional enzymatic processes with immobilized preparations [21].

Similar research on immobilization of alkaline protease enzyme depends on the enzyme permeability and rigidity of beads. For this purpose, concentration of agar, sodium alginate and polyacrylamide need to be optimized. Research on optimization of the concentration of immobilizing agents for immobilization revealed that the maximum entrapped protease activity obtained with 2% agar, 3% sodium alginate and 10% polyacrylamide beads. Results indicated that alkaline protease activity was minimum when the concentrations of different matrices low. Due to the high permeability of different matrices at low concentration. The reusability of the immobilized enzyme is very important to reduce the cost of the enzyme. This is an important factor while considering its suitability for commercial applications [22].

The reuse of the immobilized enzyme after five reuses, 25.63, 22.05, and 34.04 % activity obtained as compared to their first use with calcium alginate-, agar-, and polyacrylamide entrapped enzymes, respectively [20]. So, the reusability of enzyme more than 3 times ensured by immobilization on calcium alginate beads. Anwar et al., in 2009 also reported that entrapment of protease in calcium alginate beads and decreases in protease activity after three reuses. Another study stated that α-amylase entrapped on calcium alginate beads could be reused for six cycles with about 30% loss in activity [23].

Alkaline proteases have wide-scale industrial applications including food processing, leather processing as a dehairing agent, textile industry,
diagnostic reagents, household waste management, recovery of silver from X-ray film, and bioremediation [24]. As alkaline protease has wider applications, the study suggests to use alkaline protease from fish waste media for commercial usage at the industrial level.

**Destaining of blood stain from fabric using protease enzyme**

The visual observation of results revealed that destaining of bloodstain from the cotton fabric under standardized condition (Figure 6). Bloodstains completely removed from cloths treated with immobilized and non-immobilized alkaline protease enzymes within 30 minutes. Whereas the superior detergent at the same concentration unable to remove the stain. Cloth treated with immobilized enzyme completely removed stain compared to the non-immobilized enzymes (Figure 5). So, the current study suggests the usage of microbial alkaline protease in house hold detergent to replace chemical detergent and save the environment from chemical pollution.

Similar research on the role of immobilized alkaline protease produced by *B. amyloliquefaciens* as detergent studied. Rapid bloodstain removal noticed with supplementation of commercially available detergents with immobilized alkaline in less than 25 mins. However, individual treatment of distilled water, 1 % detergent, and the immobilized enzyme were not able to remove stains [25]. There are few reports showed the use of immobilized protease as a detergent additive; however, similar results noticed with the protease of *B. alveayuensis* CAS [26]. The removal of blood stains from cloth recorded that the protease enables to removal of bloodstain very easily without the addition of any detergent. The novel alkaline protease showed a high-capacity to remove proteins and stain from cloth so it could be used as a destainer.
in detergent powder or solution. So, the enzyme exploited as solvents and detergents. Similarly, Anwar and Saleemuddin, 1997 reported the usefulness of protease from *Spirosoma obliqua* for removal of blood stains from cotton cloth in the presence and absence of detergents but the purified protease is more effective [27].

**Conclusion**

Fish and fish waste is available throughout the year and fish waste is abundantly disposed of abruptly into the environment. Recycle of naturally available, organic resources especially fish waste remains the most practical option to slow down the exhaustion caused by their diminution. Alkaline protease has wider applications in industry, so their demand is increasing drastically. As fish waste is a rich source of proteins and nutrients required for the growth of bacteria, an experiment to exploit it as a substrate carried out. Indigenous alkaline protease producing, a thermotolerant strain isolated and identified by molecular techniques as *Bacillus clausii*. *B. clausii* has the potential of producing alkaline protease from fish waste as a substrate in an eco-friendly way. Alkaline protease mass-produced in fish waste media and the average yield of enzyme recorded, when it purified and immobilized on calcium alginate beads, their stability increased. The immobilized enzymes showed maximum activity, so their practical application to remove stain evaluated on blood-stained cloth. Compared to chemical detergent, both immobilized and non-immobilized enzymes completely removed the bloodstain and proved as a potential candidate for industrial application. Also, the current study suggests the large scale production of alkaline protease using the probiotic and thermotolerant strain *B. clausii* and the usage of microbial alkaline protease in household detergent to replace chemical surfactant and save the environment from chemical pollutants.

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