Journal on New Biological Reports
JNBR 9(2) 246 – 253 (2020)

ISSN 2319 – 1104 (Online)

Published by
www.researchtrend.net

# Effect of alkaline protease produced from fish waste as substrate by *Bacillus clausii* on destaining of blood stained fabric

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| **Received**: 26 April 2020 | **Accepted**: 19 June 2020 |

**How to cite**: Saravana Kumari P, Reshma R. 2020. Effect of alkaline protease produced from fish waste as substrate by *Bacillus clausii* on destaining of blood stained fabric. J New Biol Rep 9(2): 246 – 253.

## ABSTRACT

Alkaline protease or peptidases are the largest group of enzymes in biological industry with variety of application in various industries to process of raw material, dehairing, diagnosis, extraction, food production and processing, destaining, etc., where the conditions prevails above neutral pH. Because of the wider applications the demand also increases for alkaline proteases, so required to produce in large scale cost effectively. 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 protease production ranges between 25°C and 35°C and pH determined as 9. Along with fish waste homogenate, the mass production of extracellular alkaline protease from mobilized and immobilized cells of *B. clausii* carried out in production media, mixture of production media and fish waste homogenate and nutrient broth as standards. The recorded results showed that the maximum enzyme production obtained immobilized cells in nutrient broth media and followed by fish waste homogenate of 8900 U/ml and 8600 U/ml respectively. Purified enzyme yield was maximum obtained from production media 0.35 g/ml. Blood stained cloth treated with immobilized enzyme completely removed stain compared to treatment with non-immobilized enzyme and commercially used detergent. So the current study suggests the usage of microbial alkaline protease in house hold detergent to replace usage of chemicals and save the environment from chemical pollutants.

**Key words:** Alkaline protease, mass production, optimization, fish waste media, immobilization, Destaining.

## INTRODUCTION

Alkaline protease is one of the most important enzymes in the commercial field as its demand is increasing, so the enzyme requirement occupies large sector in industries (Singhal et al. 2012). The enzyme required in large volumes for removal of wastes and impurities, ligand formation, destaining, protein refining and blending in leather industry, diagnosis process, extraction of silver, animal diet production and food processing. Proteases are mainly derived from animal, plant and microbial sources. Among them, microbial production from *Bacillus subtilis* considered as cheap. But major factor influencing cost of production is substrate used in the production media.

Economically cheap resources from the environment are given priority to produce 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 consideration as substrate for the microbial production of protease enzyme in large scale. Fish farming and sale of fishes from dams and sea are the major income source of many fisherman. 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 problems. After usage of edible part of the fish, abrupt release of fish waste into the environment has detrimental effects on the environment and became an issue of public interest. Release of organic wastes determines changes in the community structure and biodiversity of the environmental assemblages. Moreover the fish viscera also consists of protease from animal source. Fish processing generates solid wastes that are as high as 50– 80% of the original raw material (Zynudheen, 2010). These wastes are an excellent raw material for preparation of high protein foods. About 30% of the wastes consists of skin and bone with a high collagen content. So the reuse of protein rich fish waste as substrate for production of microbial proteases are 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, chitinous material from cephalopods and wastewater offers good potential for used as substrate for microorganisms (Vazquez et al. 2006; Wang and Yeh 2006; Haddar et al. 2010). 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 to satisfy their carbon and nitrogen source requirements directly from proteins of the 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.

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 industrial sectors commonly use *Bacillus subtilis* for the production of various enzymes. More than 99% of are extracellular protease which has advantage of ease of recovery and application in industrial sector. Application of protease in industries further enhanced by immobilizing on solid particle. As solid particles are efficient delivery system at lower concentrations the study aims to immobilize protease and compares the efficiency of immobilized and non-immobilized protease enzymes on destaining processes.

## 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 laboratory maintained at 4$^{o}$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 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$^{o}$C for 24 hours. Colonies with clear zone of inhibition selected for the study (Ramakrishna, et al. 2010). The organism showing large zone of clearance selected for further study and identified by microscopic, biochemical and molecular technique by 16S rRNA gene sequencing using the primer 5'-AAGAGCCCGT-3'. Amplified and sequenced nucleic acid compared with existing nucleotide databases using BLASTN program. Then sequence with homology showing specified species identified by multiple sequence alignment of 16S rRNA generated as described previously by Wattiau et al. (2000).

### 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 triplicates.

### Mass production of alkaline protease

For the comparison of mass production of protease enzyme by inoculation of isolate in production media ($(NH_4)_2SO_4$): 2g/l, K2HPO4: 1g/l, KH2PO4: 1g/l, $MgSO_4$: 0.01g/l, $MnSO_4$: 0.01g, $FeSO_4$: 0.01g/l, Peptone: 10g/l and yeast extract: 1g/l), homogenized fish juice media (10g 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 (140rpm) at 35°C for 24hrs 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 10000 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 for 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**

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 drop wise into cold 0.2M $CaCl_2$ 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 consist of 1.25ml of buffers, 0.5ml of casein as substrate and 0.25ml of enzyme extract. The reaction stopped by adding 3ml of 5% TCA after holding at $37^{O}$C for 30minutes. The final mixture filtered and TCA soluble peptides in filtrate quantified by measuring the absorbance at 660 nm by Lowry's method **(**Liu et al. 2008).

**Effect of enzyme on blood stained cloth**

Immobilized protease investigated as destaining agent. For this study, four pieces (1 cm × 1 cm) of white cotton cloth stained with sheep blood and oven dried at 95°C –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 de-stain for 30 minutes. Last one served as a control (Geethanjali et al. 2016)**.**

## RESULTS AND DISCUSSION

**Collection of fish waste**

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


**Fig. 1.** Preparation of fish waste homogenate

**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 non- motile, endospore forming bacteria and positive for oxidase, catalase and casein, gelatin and starch hydrolysis test. So, the isolate confirmed as *Bacillus* sp., (Fig. 2). Molecular studies confirmed that the isolate belongs to *Bacillus clausii* as it showed 87.45% of sequence similarity with already explored genome of *Bacillus clausii* KSM-K16 (Kageyama, et al. 2007). *Bacillus* sp., highly resistant organism that could survive in extremes of conditions. Thereby the isolate survive at high temperature as a spore producing organism and produced alkaline protease showing maximum activity at pH 9. These attributes considered as favourable for industrial utilization of isolate for mass production of alkaline protease.

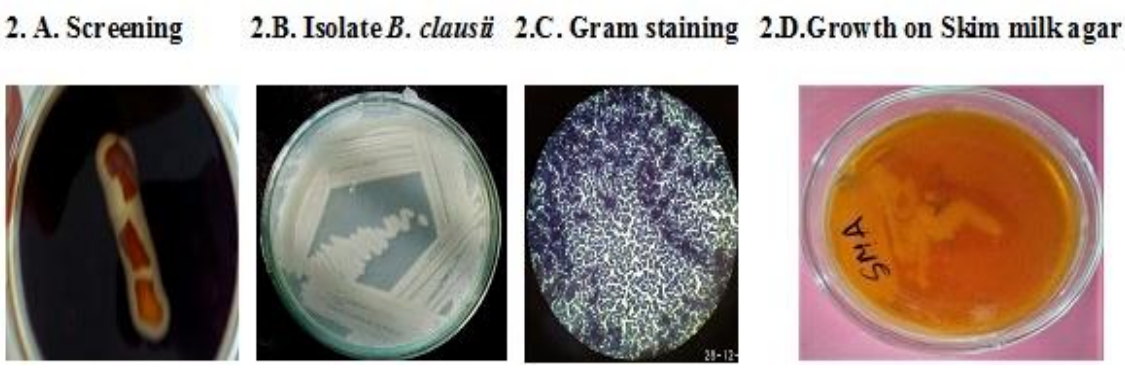
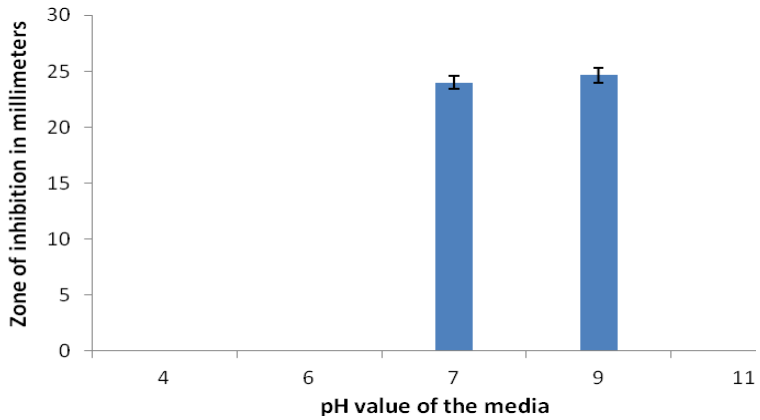



**Fig. 2.** Isolation of alkaline protease producing *Bacillus clausii*

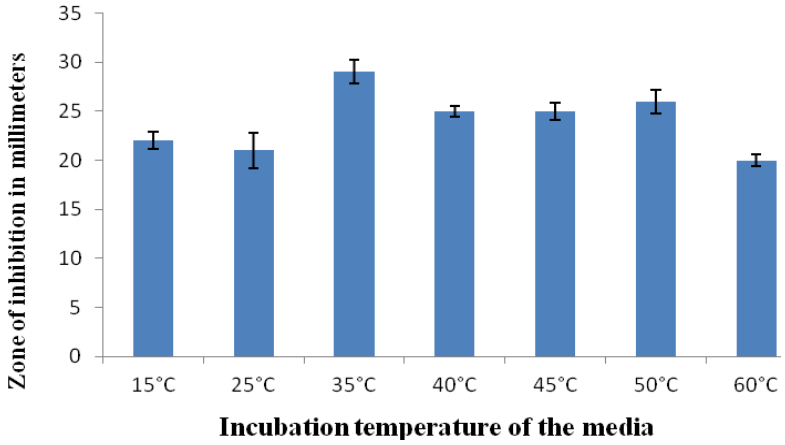
**Optimization of pH and temperature for protease production**

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 (Graph. 1).




**Graph. 1. Effect of pH on alkaline protease production by *B. Clausii***

The growth and protease production influenced by different incubation temperature. 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 (Graph.2) which significantly higher than other temperatures of incubation. Besides 35°C, other incubation temperatures showed approximately equivalent to enzyme production.




**Graph. 2. Effect of temperature on alkaline protease production by *B. clausii***

Similar research on 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 condition with 2.5 X $10^9$ cells/ml as an initial inoculum density for 36 h of incubation. The result confirms that isolates from same raw source (fish juice) exhibits maximum protease yield when compared to the reference standard *Bacillus subtilis* 14410. Also the purified protease enzyme from *Bacillus* sp., exhibited maximum activity at 35°C temperature (160.37±0.18 $Uml^{-1}$) and beyond this temperature, the enzyme activity decreased (Kumaran et al. 2013). 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 industrial fermenter, ease of purification, production within short period, wide range of physical and chemical characteristics, 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 biocatalysts for various industrial applications (Jabalia, et al. 2014).

**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 yield of 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 (Fig.3 & 4; Table 1). When organism cultured in fish waste media, maximum enzyme produced consumed for hydrolysis of protein present in the media, as rest of the enzyme recovered, comparatively less amount of enzyme from the fish waste media recovered. Though 40% less yield achieved from fish waste media compared to production cost in artificial media, fish waste media considered as efficient.

**3.A. Nutrient broth; 3.B. Production media; 3.C. Fish homogenate media; 3.D. Combined media**


**Fig. 3. Microbial production of alkaline protease**

**4. A. Dialysis in ammonium sulphate solution** **4. B. Immobilization on Calcium alginate**

**Fig. 4. Purification and immobilization of alkaline protease**

**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 (Graph 3). The enzymatic range is approximately closer to the maximum activity. Comparatively the all purified enzymes showed maximum activity in alkaline environment as the composition and purity same and experiments conducted under standard conditions.

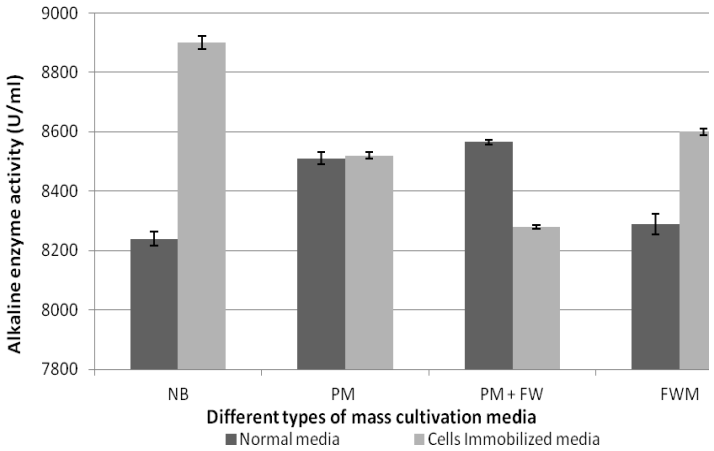
Non-immobilized enzyme showed enzyme activity between 8200 U/ml to 8350 U/ml, whereas immobilized enzyme activity ranged between 8280 U/ml to 8900 U/ml. About 1% increase in proteolytic

activity of alkaline protease recorded from the study. Even 1% increase in enzyme activity also recognized as cost effectively at industrial scale. Immobilization helps to improve stability of 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 duration of their use (Geethanjali and Anitha Subash, 2013). 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, advent of immobilized enzyme technology has led to increasing efforts to replace conventional enzymatic processes with immobilized preparations (Kotwal and Shankar, 2009).

**Table 1. Alkaline protease enzyme recovered by dialysis**

| S. No. | Culture media | Yield of enzyme in g/ml | |
|---|---|---|---|
| | | Normal cells | Immobilized cells |
| 1 | Nutrient Broth | 0.110 | 0.230 |
| 2 | Production Media | 0.300 | 0.350 |
| 3 | Production media + Fish waste homogenate | 0.240 | 0.160 |
| 4 | Homogenate of fish waste | 0.100 | 0.190 |

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. A research on optimization of 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




**Graph 3. Assay of alkaline protease activity purified from different substrates**

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 (Tanksale et al. 2001). 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 (Geethanjali and Subash, 2013). 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 decrease 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 (Kumar et al. 2006).

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 (Ellaiah et al. 2002). As alkaline protease has wider applications, the study suggests to use alkaline protease from fish waste media for the commercial usage in industrial level.

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

The visual observation of results revealed that destaining of blood stain from cotton fabric under standardized condition (Fig. 4). Blood stains 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 non-immobilized enzyme (Fig. 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.

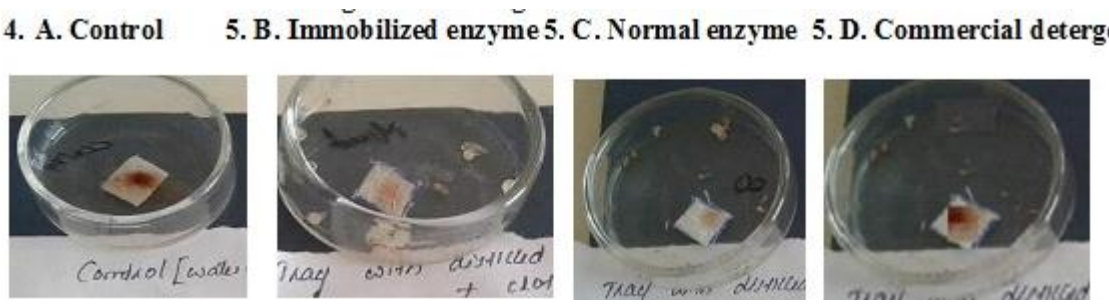



**Fig. 5. Destaining of blood stain on fabric**

The similar research on the role of immobilized alkaline protease produced by *B. amyloliqefaciens* as detergent studied. Rapid bloodstain removal noticed with supplementation of commercially available detergents (Ariel) with immobilized alkaline in less than 25 min. However, individual treatment of distilled water, detergent (1 % Ariel) and immobilized enzyme was not able to remove stains (Guleria et al. 2016). There are few reports showed the use immobilized protease as detergent additive; however, similar results noticed with protease of *B. alveayuensis* CAS (Annamalai et al. 2011). During the removal of blood stains from cloth recorded that the protease enables to remove blood stain very easily without addition of any detergent. The novel alkaline protease showed 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 usefulness of protease from *Spilosoma obliqua* for removal of blood stains from cotton cloth in the presence and absence of detergents but the purified protease is more effective.

## CONCLUSION

Fish and fish waste are available throughout the year and fish waste is abundantly disposed 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 rich source of proteins and nutrients required for the growth of bacteria, an experiment to exploit it as substrate carried out. Indigenous alkaline protease producing, thermo tolerant strain isolated and identified by molecular techniques as *Bacillus clausii. B. clausii* has the potential of producing alkaline protease from fish waste as substrate in an eco-friendly way. Alkaline protease mass produced in fish waste media and average yield of enzyme recorded, when it purified and immobilized on calcium alginate beads, their stability increased. Immobilized enzyme 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 blood stain and proved as potential candidate for industrial application. Also the current study suggests the usage of microbial alkaline protease as house hold detergent to replace chemical detergent and save the environment from chemical pollutants.

## ACKNOWLEDGEMENT

The authors are thankful to the Principal and the Management of Sree Narayana Guru College and Rathnavel Subramaniam College of Arts and Science, Coimbatore for providing facilities.

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