# Characterization, Partial Purification of Alkaline Protease from Intestinal Waste of Scomberomorus Guttatus and Production of Laundry Detergent with Alkaline Protease Additive

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## ABSTRACT

**Background:** Fish industry wastes are an important environmental contamination source. A way of minimizing the environmental problems generated by the high amount of fish wastes is its transformation to a commercially useful ingredient. Alkaline proteases, one of the most important industrial enzymes are extracted especially from the Intestine of fish. They are widely used in laundry detergents for the degradation of protein. **Methods:** The protease extracted from Seer fish (*Scomberomorus guttatus*) was precipitated with ammonium sulphate (70%). The enzyme was more stable over a wide range of pH (8-12) and its optimum temperature was around 60°C.The enzyme activity was also analyzed by Zymogram with casein. The enzyme was partially purified using Sephadex G-75. The compatibility of the Enzyme with various commercial detergents was studied. **Results:** Enzyme showed activity in detergent even after 1 hour of incubation. De-staining activity of the enzyme with various stains such as Blood stain, Food spices, Beetroot stain, raw banana stain etc was studied. Enzyme showed de-staining activity. Thus proving its applications in Laundry industry. **Conclusion:** By virtue of our findings, it could infer that alkaline protease was successfully produced from the intestinal waste of seer fish. Also, a new laundry detergent with enzyme additive was produced and the de-staining activity of the newly prepared detergent with enzyme additive was promising and thus can be used in places such as hospitals, food industry and households. It can be concluded that not only a valuable enzyme can be produced from a cost free sample but also dumping of the waste be rectified.

**Key words:** Protease, Seer Fish, Scomberomorus guttatus, Alkaline proteases, Zymogram, commercial detergents, Laundry detergent.

## INTRODUCTION

The fish industry developed around fishing ports at a time when landing were plentiful and there was a little concern about environmental impacts.[1] The fish industry generates a significant amount of waste which has been estimated.[1] Fish waste from the nearby market is dumped into the ocean at the end of each day.[1,2] The contamination caused by the residues from fish processing has created an imperative challenge that needs

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to be efficiently dealt.[1,2] So the fishing opportunities are reduced and waste generation disposal are increasingly penalized. There is a considerable potential for gaining more volume from fish waste.[3] It is rich in valuable minerals, enzyme, pigment and flavours that are required by many industries including food, agriculture, aqua culture and pharmaceutical.[4] Fish industry wastes are an important environmental contamination source.[5] Research has been carried out in order to develop methods to convert these wastes into useful products.[6] A way of minimizing the environmental problems generated by the high amount of fish wastes in its transformation to a commercially useful ingredient.[7]

Alkaline proteases are the enzymes extracted especially from the Intestine of fish.[8,9] They are widely used in laundry detergents for the degradation of protein. Proteases are found in animals, plants, bacteria and viruses.[8,9] A number of eukaryotic and prokaryotic organisms are reported to produce proteolytic enzymes.[10] Proteolytic enzymes are ubiquitous in occurrence, being found in all living organisms and are essential for cell growth and differentiation.[10] The extra cellular proteases are of commercial value and find multiple applications in various industrial sectors. Proteases constitute one of the most important groups of industrial enzyme in the total global production scale.[11]

Alkaline proteases are present in intestinal waste of Seer fish (*Scomberomorus guttatus*). Since it has long thermal stability it has long shelf life and high activity over a wide range of pH levels. Alkaline protease from bacteria, fungi and insect origin can be exploited commercially.[12] Bacterial alkaline proteases have long been used in detergent but their main drawback is that it requires cost-intensive technologies to obtain a micro organism free enzyme preparation.[13] However to obtain high, commercially viable yield of proteases, it is essential to optimize the fermentation medium for the growth and production of proteases.[14] Moreover, the enzyme selected for detergent composition is not ideal enzyme for detergent due to their short shelf life.[15] It is relevant to search for protease from different sources presenting properties such high thermal stability and alkaline activity that are more compatible with washing system.[16] Although, there have been number of studies on the proteases production by fish waste, little information on kinetic analysis of the proteases production process is available.

The aim of the present study was to utilize the intestinal waste of seer fish for the production and characterization of alkaline protease. This study describes the extraction, specific activity, partial purification, characterization of alkaline protease from fish intestinal waste as well as their compatibility with commercial laundry detergent.

## MATERIALS AND METHODS

Seer fish (Scomberomorus guttatus) intestinal waste from fish market situated in Vanagaram, a rural area in Chennai, Tamilnadu, India for free of cost. All reagents used in enzymatic assays were purchased from Sigma Chemicals.

**Extraction of enzyme:** 10 g of fish intestinal waste (Scomberomorus guttatus) was homogenized with 0.9% [w\v] NaCl in an Ice cold condition. The resulting crude was centrifuged at 10,000 rpm for 10 mins at 10°C. The supernatant was collected and stored at -20°C.

### Concentrating the enzyme

**Ammonium sulphate precipitation of Enzyme (Alkaline protease):** 70% ammonium sulphate is added to the supernatant obtained from Enzyme extraction was to precipitate the total protein at 4°C. After 1 hour the precipitate was recovered by centrifugation at 10,000 rpm for 10 minutes. The pellet obtained was dissolved in tris-HCl buffer (pH 8.0).[17-19]

**Dialysis:** Ammonium sulphate precipitated enzyme solution was packed in treated Dialysis bag. It was placed in a beaker containing 0.05 M tris-HCl buffer (pH 8.0) and stirred with magnetic stirrer for 2 hours after this period, the buffer was renewed; and dialysis was repeated for further 2 hours. Finally, after a 3$^{rd}$ renewal of buffer, dialysis was allowed to proceed overnight at 4°C. The enzyme solution was collected and stored at-20°C.[17-19]

**Estimation of total protein by Lowry's method:** The amount of total proteins present in the sample was estimated by the method described by Lowry *et al.*[20]

**Determination of activity and specific activity of alkaline protease:** The activity of the enzyme and the specific activity of the enzyme were determined.

**Determination of molecular weight by Sodium Dodecyl Sulphate-Poly Acryl amide Gel Electrophoresis (SDS-PAGE):** The SDS PAGE was used as a conformation test for visualization and determination of molecular weight of the enzyme alkaline protease. The SDS plate assembly was set with the help of glass plates, spacers and clips. The separating gel (pH 8.8) mix and stacking gel mix (pH 6.8) was poured and allowed to set with comb inserted. After the stacking gel was polymerized the comb was removed and the assembly was placed in the buffer tank. Buffer (pH 8.2) was poured in the tank and sample was loaded in the wells along with the marker protein. This is followed by stacking the gel in 10% Trichloroacetic acid for 1 hour. Then the gel is put in staining solution for 1 hour and then in destaining solution for overnight. The bands are viewed under white light illuminator.[18,19,21]

### Kinetic properties

**Determination of optimum pH of Enzyme (Alkaline protease):** 5 sets of clean test tube were taken and labeled as test and control. 1.0 ml of tris-HCl buffer and glycine NaOH buffers of pH 7.0, 8.0, 9.0, 10.0 and 11.0 were pipetted out into respective tubes. 0.5 ml of 1% casein was added. All the tubes were pre-incubated at 30°C for 10 min. 0.5 ml of enzyme was pipetted out into tubes labeled with test alone. All the tubes were incubated for 30 min at room temperature. To all the tubes 0.5 ml of TCA was added and vortexed 0.5 ml of Enzyme was added to all the control tubes. All the tubes were centrifuged and 0.5 ml of filtrate was pipetted out to next set of tubes which were labeled as T1 to T5 and C1 to C5. The volumes in all the tubes were made up to 2 ml with distilled water. To all the tubes alkaline copper reagent and Folin's phenol was added, Incubated and OD was taken at 640 nm against a reagent blank.[18,19,22]

**Determination of optimum Temperature of Enzyme:** 5 sets of clean test tube were taken and labeled as test and control. 1.0 ml of tris-HCl buffer and glycine NaOH buffers of pH 7.0, 8.0, 9.0, 10.0 and 11.0 were pipetted out into respective tubes. 0.5 ml of 1% casein was added. All the tubes were pre-incubated at 30°C for 10 min. 0.5 ml of enzyme was pipetted out into tubes labeled with test alone. All the tubes were incubated for 30 min at room temperature. To all the tubes 0.5 ml of TCA was added and vortexed 0.5 ml of Enzyme was added to all the control tubes. All the tubes were centrifuged and 0.5 ml of filtrate was pipetted out to next set of tubes which were labeled as T1 to T5 and C1 to C5. The volumes in all the tubes were made up to 2 ml with distilled water. To all the tubes alkaline copper reagent and Folin's phenol was added, Incubated and OD was taken at 640 nm against a reagent blank.[18,19,23,24]

**Compatibility assay for enzyme incubated with respective detergent:** The alkaline protease extracted from fish waste was incubated with various detergent like (Surf excel, Wheel, Ujala, Arial) and its activity in the presence of Detergent was studied as a compatibility assay.

**Partial purification of enzyme by Gel Filtration Chromatography:** Partial purification of the enzyme has been carried out by Gel filtration chromatography of concentrated Enzyme extract using Sephadex-G75 column. Sephadex is beaded gel prepared by cross-linking dextrin. It is widely used technique for the separation of low and high molecular weight protein molecules with high activity recoveries with very small amount of the sample. These crossed linked polymers swell readily in water forming a gel of 3D network of pores. The sizes of the pore determined by the degree of cross linking of the polymeric chains.[18,19]

### Criteria of purity

**Qualitative analysis of protein in the eluted fraction at 280 nm:** The presence of Protein was estimated qualitatively at 280 nm using UV spectrophotometer.[18,19]

**Determination of alkaline protease activity in Chromatography elutant:** Alkaline protease enzyme activity was determined in the elutant obtained in the chromatography.

**By Sodium Dodecyl Sulphate-Poly Acryl amide Gel Electrophoresis (SDS-PAGE):** The SDS PAGE was used as a confirmation test for visualization and determination of molecular weight of the enzyme Alkaline Protease. Fraction No: (5, 6, 7, 8, 9) which showed the presence of alkaline protease was loaded in to SDS PAGE and gel profile was evaluated for the presence of the enzyme in the eluted fraction.[19,25]

**Zymogram:** Zymogram of Alkaline protease was prepared by adding 1% casein to stacking gel. To the substrate loaded gel crude enzyme was added and native page was developed (Figure 11).

**Production of detergent with enzyme (alkaline protease) additive:** The detergent was produced by using the alkaline protease enzyme as additive by Lyophilization.

**De-staining activity of the detergent with enzyme (alkaline protease) additive:** Dirty cloth pieces were soaked and washed with commercial detergent, water and newly prepared detergent with enzyme additive. Destaining efficiency of the enzyme was tested on various stained cloths like Blood, Spicy food, Beetroot juice, Raw banana dirt etc.

## RESULTS

Seer fish (Scomberomorus guttatus) intestinal waste was procured from fish market situated in Vanagaram, a rural area in Chennai, Tamilnadu, India for free of cost (Figure 1). The enzyme from the intestinal waste has been obtained by homogenization (Figure 2). Concentrating of the enzyme was achieved by precipitation of the enzyme by ammonium sulphate (Figure 3) and by dialysis (Figure 4). Total protein concentration was estimated


**Figure 1: It shows the intestinal waste of Seer fish obtained from Vanagaram fish market**


**Figure 2: It shows the homogenization of the fresh intestinal waste of Seer fish obtained from Vanagaram fish market**

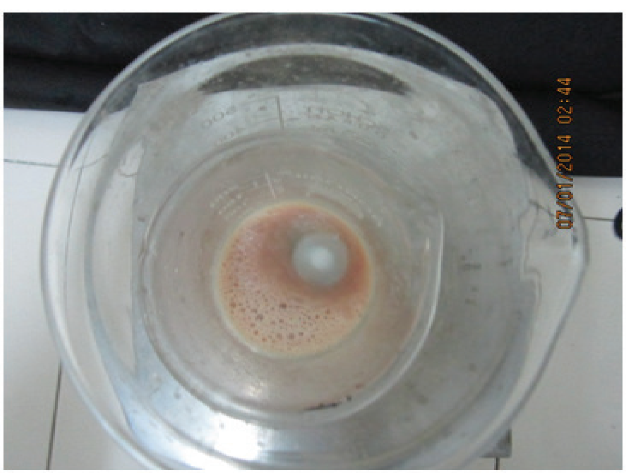

**Figure 3: It shows the concentrating of the enzyme by ammonium sulphate precipitation**


**Figure 4: It shows the concentrating of the enzyme by dialysis**

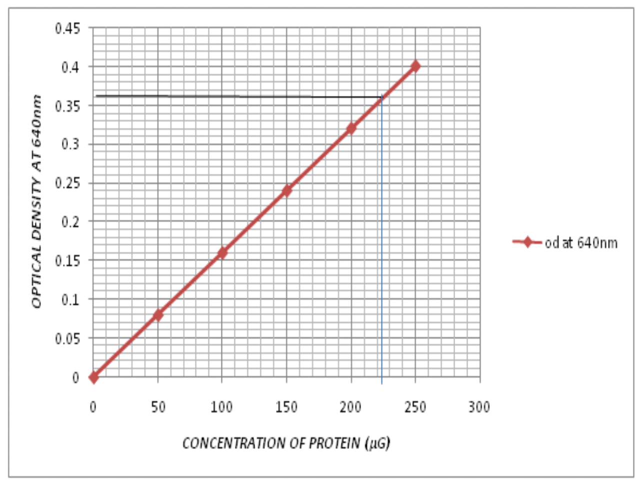


**Figure 5: It shows the estimation of total protein by Lowry's method**

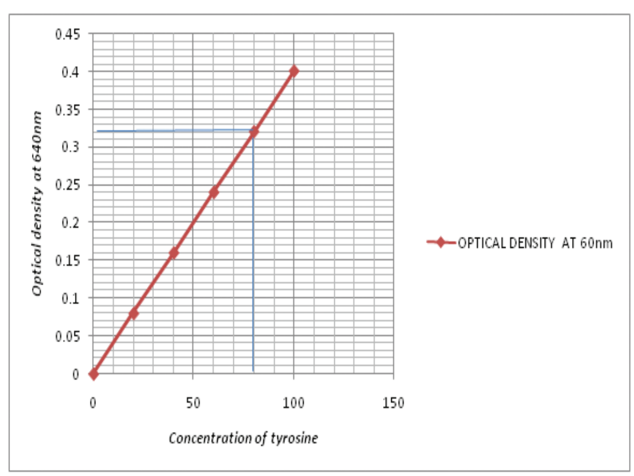


**Figure 6: It shows the determination of activity and specific activity of alkaline protease**

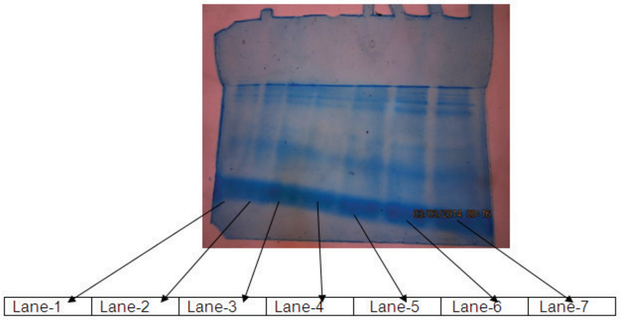


**Figure 7A: It shows the determination of molecular weight of alkaline protease by SDS-PAGE: SDS PAGE loaded with crude sample.**

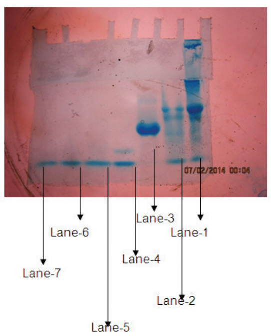


**Figure 7B: It shows the determination of molecular weight of alkaline protease by SDS-PAGE: SDS PAGE loaded with Chromatographic elutant**

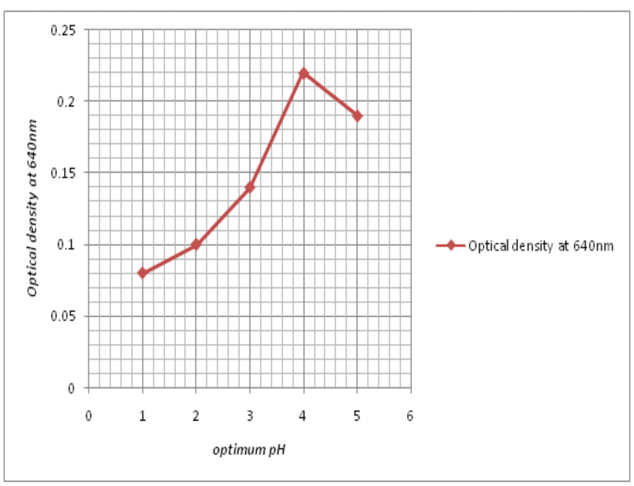


**Figure 8A: It shows the optimum pH of alkaline protease**

by Lowry's method and the total amount of protein in crude was found to be 10 mg/ml (Figure 5). The specific activity of the enzyme was measured and was found to be 0.01655 µmoles/ml/mg of protein (Figure 6). Molecular weight of the protein was calculated by using Sodium Dodecyl Sulphate-Poly Acryl amide Gel Electrophoresis (SDS-PAGE) and the molecular weight of the

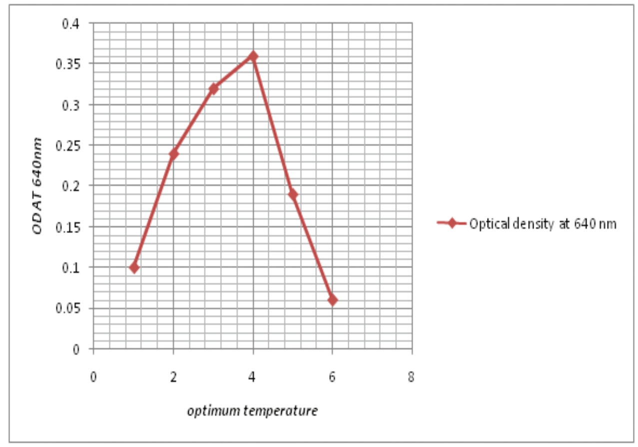


**Figure 8B: It shows the optimum temperature of alkaline protease**

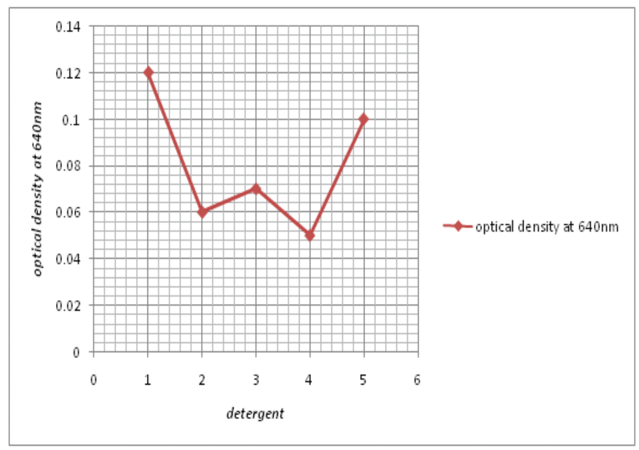


**Figure 9A: It shows the compatibility assay for enzyme incubated with respective detergent for 60 min**

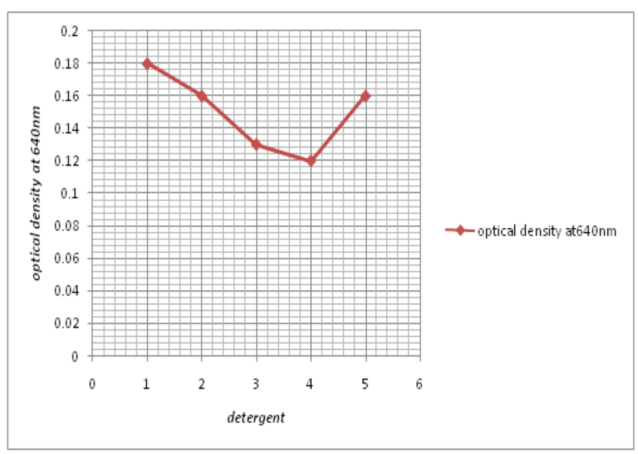


**Figure 9B: It shows the compatibility assay for enzyme incubated with respective detergent for 30 min**

sample is corresponds to Lysozyme. Thus, the eluted sample contains purified alkaline protease (14.4kd) (Figure 7a & Figure 7b). The optimum pH of enzyme (Alkaline protease) and was found to be 10 (Figure 8a).

**Figure 10: It shows the partial purification of alkaline protease by gel filtration chromatography using Sephadex G-75**

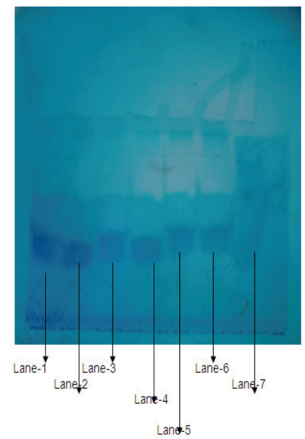


**Figure 11: It shows the Zymogram**

**Figure 12: It shows the production of detergent with enzyme (alkaline protease) additive**

The optimum temperature of enzyme (Alkaline protease) and was found to be 60$^0$C (Figure 8b). So the enzyme was stable at the optimum temperature of 60$^0$C. The compatibility assay for enzyme incubated with

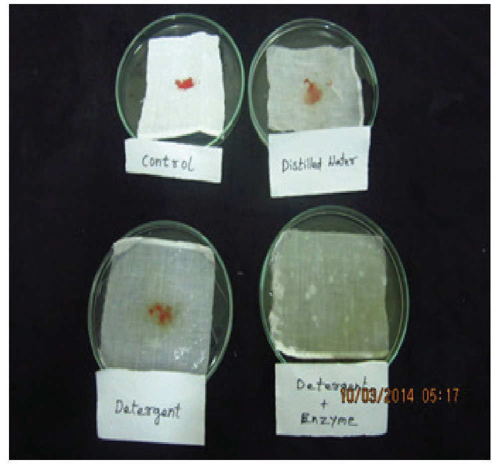


**Figure 13A: It shows the de-staining activity of the detergent with enzyme (alkaline protease) additive on blood stains**

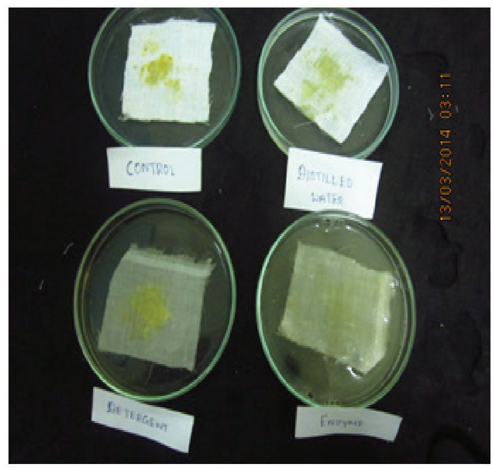


**Figure-13B: It shows the de-staining activity of the detergent with enzyme (alkaline protease) additive on stains caused by spicy food material**

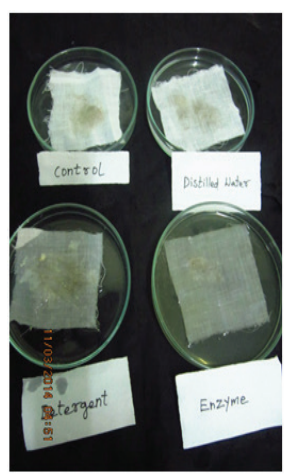


**Figure-13C: It shows the de-staining activity of the detergent with enzyme (alkaline protease) additive on stains caused by raw banana**

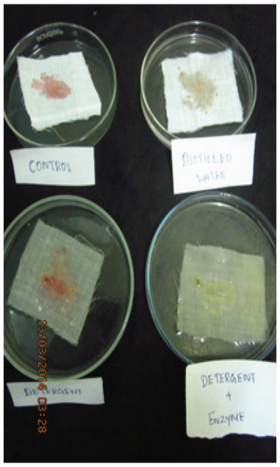


**Figure-13D: It shows the de-staining activity of the detergent with enzyme (alkaline protease) additive on stains caused by beetroot stain**

various detergents (Surf excel, Wheel, Ujala, Arial) for 60 min (Figure 9a) and 30 min (Figure 9b). The alkaline protease enzyme was partially purified by Gel filtration chromatography using Sephadex G-75 (Figure 10). The Zymogram of Alkaline protease was prepared by adding 1% casein to stacking gel (Figure 11). The detergent was successfully produced by adding the alkaline protease enzyme as additive (Figure 12). De-staining efficiency of the enzyme was tested on various stained cloths like Blood, Spicy food, Beetroot juice, Raw banana dirt etc (Figure 13A-D).

## DISCUSSION

Alkaline protease enzyme was extracted from intestinal waste of Seer fish (*Scomberomorus guttatus*). The enzyme was characterized, partially purified using Sephadex G75.[18] The optimum pH of the enzyme was found to be pH 10 and optimum temperature was around 60°C. Zymogram was also done to prove the activity of enzyme.[19] The extracted enzyme showed activity even in the presence of various commercial detergents. The enzyme was lyophilized and added to the detergent produced in the laboratory. Thus a laundry detergent with enzyme additive was produced and its destaining activity was tested on various stains.[26] The de-staining activity of the newly prepared detergent with enzyme additive was promising and thus can be used in places such as hospitals, food industry and households.[26]

In future, the newly prepared detergent with enzyme additive can be manufactured in industrial scale with a commercial name tag and sold for the public use. The enzyme can be completely purified so that it can be used in pharmaceutical and clinical sectors.

## CONCLUSION

A new laundry detergent with enzyme additive was produced and its de-staining activity was tested on various stains. The de-staining activity of the newly prepared detergent with enzyme additive was promising and thus can be used in places such as hospitals, food industry and households. In future, the newly prepared detergent with enzyme additive can be manufactured in industrial scale with a commercial name tag and sold for the public use. The enzyme can be completely purified so that it can be used in pharmaceutical and clinical sectors.

## ACKNOWLEDGEMENT

None.

## CONFLICTS OF INTEREST

The author declare no confict of interest.

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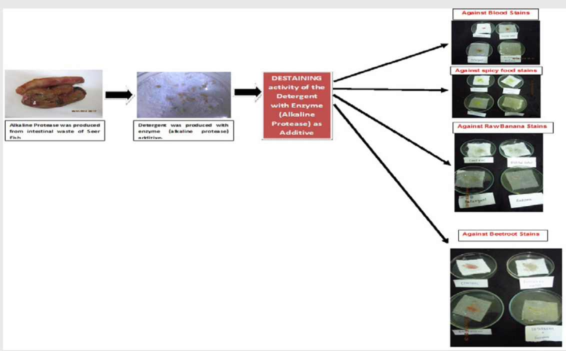
## PICTORIAL ABSTRACT




## ABBREVIATIONS USED

**SDS:** Sodium Dodecyl Sulphate; **SDS-PAGE:** Sodium Dodecyl Sulphate-Poly Acryl amide Gel Electrophoresis; **TCA:** Tri carboxylic acid; **Tris-HCl:** Tris-Hydro Choric Acid; **NaOH:** Sodium Hydroxide; **NaCl:** Sodium Chloride.

## SUMMARY


1. Alkaline protease enzyme was extracted from intestinal waste of Seer fish (*Scomberomorus guttatus*). The enzyme was characterized, partially purified using Sephadex G75.
2. The optimum pH of the enzyme was found to be pH 10 and optimum temperature was around 60°C. Zymogram was also done to prove the activity of enzyme. The extracted enzyme showed activity even in the presence of various commercial detergents.
3. The enzyme was lyophilized and added to the detergent produced in the laboratory. Thus a laundry detergent with enzyme additive was produced and its destaining activity was tested on various stains.
4. The destaining activity of the newly prepared detergent with enzyme additive was promising and thus can be used in places such as hospitals, food industry and households.
5. In future, the newly prepared detergent with enzyme additive can be manufactured in industrial scale with a commercial name tag and sold for the public use.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**Ms. Daisy Mary J:** Is a post graduate in biotechnology. Has interest in research. Her field of interest is plant biotechnology.

**Dr. Vishnu Priya V:** Holds PhD in Biochemistry and her thesis was highly commendable. She published around 25 research papers in various national and international, indexed journals to her credit. She was the recipient of IFCC Fellowship in Brazil, DNA Topology fellowship in Japan. She has won "Young achievers award", best research paper award in national conferences and best teacher award. She is guiding many students for their research projects. She completed TPRM from university of McMaster. She attended many national and international conferences and chaired various session in international arena.

**Dr. Surapaneni Krishna Mohan:** Holds PhD (Medical) in Biochemistry and presently working as Professor of Biochemistry and Vice Principal in Saveetha Medical College & Hospital, Saveetha University, Chennai, India. His research interests include biochemical studies in Non Alcoholic Steatohepatitis (NASH) and other liver diseases, Operations research in population health, public health informatics and research in health professions education. He was awarded the prestigious "Chartered Scientist (CSci)" qualification, which represents a single chartered mark for all scientists, recognizing high levels of professionalism and competence in science, by "The Science Council", UK and "The Royal Society of Chemistry (RSC), UK. Also, he is a "Certified Clinical Chemist" by National Registry of Certified Chemists (NRCC), USA. He published several scientific research papers in reputed national and international indexed journals to his credit. He is the life member of many professional national and international scientific societies / bodies. He has received several international travel grants and fellowships to present his work in several international conferences and workshops. He has received many awards like "Young Investigator Award" & "Young Scientist" award, to name a few, for his research work on international platform.