ISSN (Online): 2091-2609
DOI Prefix: 10.3126/ijasbt

International Journal of Applied Sciences and Biotechnology (IJASBT)

Research Article

Molecular Characterization of Alkaline Protease Gene Isolated from *Aeromonas hydrophila strain AH10*

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Article Information

Received: 22 April 2020
Revised version received: 15 June 2020
Accepted: 17 June 2020
Published: 25 June 2020

Cite this article as:
N.G.R. Babu and A.M. Aji (2020) Int. J. Appl. Sci. Biotechnol. Vol 8(2): 154-160.
DOI: 10.3126/ijasbt.v8i2.29583

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Peer reviewed under authority of IJASBT

Abstract

Alkaline protease enzymes are enzymes which can catalyze the process of proteolysis between the pH ranges from 8 to 12. Extracellular alkaline proteases are used as additives in detergent powders. In the present study, source of the organism was from a detergent contaminated area. The study has been carried out in *Aeromonas hydrophila AH10* strain that produces protease enzyme with an alkaline pH optimum. The organism was a gram-negative rod with a protease enzyme activity of 0.385 ml/min. purification of the protease enzyme from the bacteria was carried out. This protease is suitable for use in alkaline detergent powders as well as in silver recovery process. The *Aeromonas hydrophila strain AH10* gene encoding this high-alkaline protease was cloned and characterized.

Keywords: Aeromonas hydrophila; Alkaline protease; Gene cloning

Introduction

Enzymes are biological catalysts that significantly speed up the rate of chemical reactions that are carried out within the cells. Protease enzymes can catalyze the process of proteolysis, the breakdown of proteins into smaller polypeptides. Proteases refer to a mixture of enzymes including proteinases, peptidases and amidases (Kumar *et al.*, 2011). They are one among three largest industrial enzymes. Proteases are classified according to their active pH range into acidic, neutral and alkaline proteases. The enzymes in the class of alkaline proteases include the enzymes that are active at alkaline pH with optimum pH range between 9 and 11. As compared to animals and plants, microbes represent a better source of alkaline proteases, as they can be cultured in large quantities within a short time. They can also produce regular and abundant supply of products. Among the bacterial species, bacillus species are prolific producers of extracellular proteases with a wide range of applications, particularly in the detergent, food, pharmaceutical, leather and chemical industries (Abbasi-Hosseini *et al.*, 2011).

Since 1960's bacterial proteases are an integral part of detergents (Abbasi-Hosseini *et al*., 2011). With the introduction of new frontiers in biotechnology, the spectrum of protease application has expanded into many new fields, such as medicinal, clinical analytical chemistry. To meet the current expanded demand, studies on cost effective production of industrially important enzymes have become the need of the day (Tiwari *et al*., 2015). The enzymes used by laundry industry are, proteases, amylases, lipases and cellulases. Out of these, alkaline proteases are the most favorable one because it is the only enzyme which can remove mostly all stains in a cloth. They can remove the proteinaceous stains of blood, milk, egg etc. by hydrolyzing them. However, the most difficult challenge faced by the biotechnologists is making the protease stable inside the detergent with the other ingredients. This is because, proteases can be easily denature or hamper by the basic ingredients of detergents such as bleaching agents, surfactants, bleach activators, fabric softeners etc. However, biotechnologists are modifying the proteases via mutation to make it more stable inside the detergent.

Materials and Methods

Collection of Samples

The source of the sample was detergent contaminated soil. Three different soil samples were collected from the local habitat of Ernakulam, Kerala, India.

Isolation of Bacterial Strains

Samples were serially diluted using distilled water up to 10^{-5} dilutions in fresh test tubes and 0.1 ml of such dilutions was spread on the surface of nutrient agar plates. After incubation at 37°C for 24 h colonies were isolated according to the morphology types and purified by further sub culturing. Isolated and purified strains were cultured on skim milk agar plates, for the confirmation and determination of proteolysis by various bacteria. In the present study, milk powder has been provided as the only nutrient source for the bacteria to identify the bacteria with the protease genes. In order to proceed with the bacterium of highest activity; well diffusion method was used with skim milk agar. Proteolytic activity was estimated by the extent of the clear zone around the colony (Vazquez *et al*., 1995). Gram staining was performed for the bacterial strain which showed highest activity in well diffusion method (Claus, 1992)

Biochemical Assays

Different biochemical tests were performed to check and differentiate the bacteria. IMViC assay was carried out in the present study. Indole test, Methyl red test, Voges Proskauer test and Citrate utilization tests were included in this (Vashist *et al.*, 2013; Hemraj *et al.*, 2013).

Enzyme Purification

The enzyme production media was prepared by autoclaving. The selected isolate was inoculated with fermentation media and incubated in shaker for 3-4 days. After incubation, this was centrifuged at 12,000 rpm to for collecting the supernatant. This was used for further processes (Kalaiarasi *et al*., 2009).

Protease assay was done for purified sample to estimate the amount of protease enzyme present in it. L-tyrosine was used as the standard. The centrifuged broth was estimated for the protease activity as per the standard methods using casein as the substrate. It is a major milk protein, a macromolecule of amino acid linked by peptide bond. The tyrosine (peptide) liberated during the proteolytic digestion was measured at 660 nm in UV-Vis spectrophotometer (Kumar *et al*., 2011; Vadlamani *et al*., 2011).

The amount of ammonium sulphate needed for the precipitation was calculated using ammonium precipitate calculator and found to be 85.05 grams. The broth was collected in 2 ml eppendoff tubes and centrifuged at 6000 rpm for 6 minutes. Supernatant was collected in a beaker containing a stir bar and place on a magnetic stirrer. While the sample was being stirred, 5 grams of saturated ammonium sulphate was added slowly every time to bring the final concentration to 80 % saturation. The precipitate was transferred to the refrigerator at 4 ℃ overnight. The sample was transferred to centrifuge tubes and centrifuged at 6000 rpm for 10 minutes. Supernatant was discarded. The eppendoff tubes were inverted and drained well. Pellets were suspended in 20 ml of 1XPBS. The sample solution was transferred into a beaker and stored in the refrigerator (Schreier *et al.*, 1997).

The pretreatment of the dialysis bag was done by treating the membrane with distilled water at 65℃ for one minute in a water bath. This helps to remove the glycol in the membrane. Then, the bag was soaked in 10 mM Na_2ED

TA following the treatment with 10mM sodium carbonate. The membrane was washed with distilled water and the ends were clipped. One third volume of the bag was filled with ammonium sulphate precipitate and immersed in PBS buffer for dialysis. The buffer solution was changed every hour overnight (Pant *et al*., 2015).

Estimation of Protein – Lowry's Method

Protein estimation of purified sample was carried out using bovine serum albumin as working sample. Lowry's method was done to estimate the amount of protein in the culture broth and crude sample (Waterborg *et al*., 1994). The method is based on two chemical reactions; first is the reduction of copper ions under alkaline conditions, which forms a complex with peptide bonds. And the second is the reduction of Folin-Ciocalteu reagent by the copper- peptide bond complex which leads to a colour change of the solution into blue.

Table 1: Polymerase chain reaction thermal profile

Cycle	Step	Process	Temperature	Time
1	**I**	Initial denaturation	94ºC	3 minutes
1	**II**	Denaturation	94ºC	30 seconds
1	**III**	Annealing	59ºC	30 seconds
1	**IV**	Primer extension	72ºC	30 seconds
	Go to step 2 for 29 times			
30	**V**	Final extension	72ºC	7 minutes
1	End or 4ºC forever.			

Molecular Characterization

The isolation of bacterial DNA was performed by following the procedure (Yeates *et al*., 1998)

Polymerase Chain Reaction

The procedure and thermal profile of the polymerase chain reaction followed is given in the Table 1.

The PCR reaction mixture (20 µl) contained 10 µl master mix ($MgSO_4$, taq polymerase, dNTPs and dye), 1 µl of each oligonucleotide primers and 7 µl of deoinized water. 16srDNA region of the bacterium was amplified. This gene is the universal target for bacterial identification (Baldus Patel *et al*., 2001; Mignard *et al.*, 2006).

Forward primer: 5'- GAGTTTGATCCTGGCTCAG -3'

Reverse primer: 5' - GAATTACCGCGGCGGCTG – 3'

8 % agarose (0.24g in 30ml 1X TBE) solution was prepared. Ethidium Bromide (0.5ml EtBr for 10 ml TBE) was added to the gel. 10µl of DNA with 4µl loading dye was loaded in agarose gel. The gel was run at constant voltage of 100V. The gel was viewed on UV transilluminator.

Gel electrophoresis of the PCR sample was carried out to view the amplified gene. Amplified gene sequence was then used for similarity check using BLAST (Basic Local Alignment Search Tool) programme in the NCBI GenBank (wwsw.ncbi.nlm.nih.gov) DNA database for identifying the sample.

Gene Cloning

Luria Bertani (LB) broth was prepared as per standards and inoculated with the competent cell DH5alpha under aseptic conditions. DH5alpha are *E. coli* cells mutated to maximize the transformation efficiency. The inoculated broth was then kept inside the incubator for hours at 37°C (Sadeghi *et al.*, 2010; Wang *et al.*, 2010). The DNA sample was isolated from the bacteria and PCR was carried out. 1 µl of this fresh PCR product was used for TOPO cloning and incubated at room temperature for 5 minutes. The components and quantity of TOPO cloning mixture is given in Table 2.

Table 2: TOPO cloning mixture

Fresh PCR product	0.5 – 4 µl
Salt solution	1 µl
Deionised Water	5 µl (add to a total volume of 5 µl)
TOPO vector	1 µl
Final volume	6 µl

The TOPO cloned sample was then added to the Luria Bertani broth and incubated for 20 minutes. Luria Bertani agar was prepared as per standard and the above broth was inoculated to the plate for white-blue screening. It is a rapid and efficient technique for the screening of recombinant genes. The plate was kept inside the incubator for a day.

Blue-white screening of bacterial colonies is a rapid and effective tool often used to detect recombinant bacteria in cloning experiments. LB agar was prepared according to the standards and sterilized in an autoclave. To the sterilized medium, 5µg/ ml of ampicillin was added. The agar was poured into the plates and 10-100 µl of transformed cells were spread onto the plates. The plates were incubated at 37°C for 48 hours (Brown *et al*., 2011). Later these colonies were proceeded to PCR with ApR gene sequence which resulted the cloned sequence.

Amino Acid Sequencing

The nucleotide sequence of the cloned protease gene and its flanking DNA regions was determined using ExPASy translate tool in the Bioinformatics Resource Portal (https://web.expasy.org/translate/).

Applications

Removal of blood stain

This experiment was done to evaluate how protease enzyme works as a bio-cleaner by testing their washing performance on blood. A clean cloth was cut into 4 equal pieces and stained with blood, incubated about 30 minutes and washed

with water, detergent and crude protease. After an incubation period of 30 minutes as compared to the detergent and water, crude bacterial protease removed blood stain on fabric completely (Lakshmi *et al.*, 2015).

Extraction of silver from X-ray film

Waste x-ray film was washed with ethanol and wiped with cotton, dried at 40℃ and cut into small pieces and dipped into a beaker containing the solution of Tris-HCl and crude protease in 1:1 ratio (50ml total volume) and kept in water bath at 50℃ with continuous stirring until the gelatin layer was completely ripped off, once the gelatin layer is ripped off, the turbidity of the medium was changed and it was visible, the solution was further tested for the presence of silver in it.

To detect the silver in the solution, 10 drops of solution, add 6 M HCl drop wise, with shaking, until precipitation is completed. And then centrifuged and decanted. Discard the centrifugate. Suspend the silver chloride precipitate in 1 ml of water and add 6 M NH3 (aq) drop wise until the precipitate dissolves. Acidify the solution with 6 M HNO3 and the white precipitate should reappear (Al-Abdalall *et al*., 2016)

Results

Protease producing bacteria were isolated from detergent contaminated soil. Based on the morphological and biochemical characteristics, the isolates were identified as *Aeromonas hydrophila strain AH10.* The spread plate method performed resulted in the growth of different types of colonies on each plate, later organisms on these plates were separated on the basis of morphology and plated on nutrient agar media. The identification of bacteria with proteolytic activity was carried out by plating the bacteria on skim milk agar plates. The bacteria with protease activity used the milk protein provided in the plates.

This is followed by the qualitative analysis of bacteria by well diffusion method. And the sample 2 showed the highest zone of clearance among all the samples. This strain was chosen for further experiments.

The biochemical test was carried out and it is listed in Table 3.

Table 3: Results of the biochemical assays

Test	Results
Indole production	Positive (indole conversion)
Methyl red	Positive (formation of any acid product)
Vogesproskauer	Negative (absence of acetyl-methyl carbinol)
Citrate utilization	Negative (bacteria didn't utilize citrate as an energy source)

Fig. 1: Protease activity of bacteria on skim milk agar plate

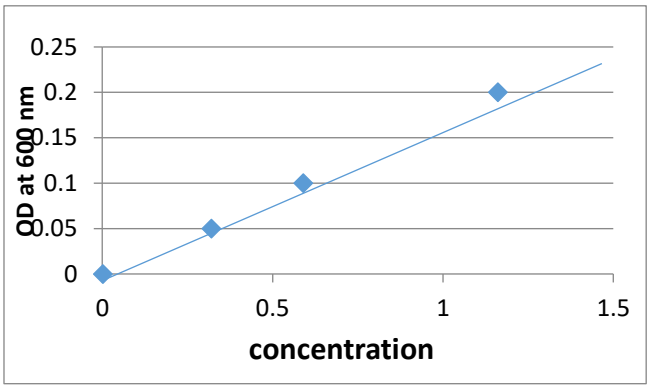

Fig. 2: Graph showing the tyrosine standard curve

The activity of enzyme was measured by tyrosine standard curve.

Activity of purified enzyme = 0.385 ml/ min

Activity of supernatant = 0.225 ml/min

The enzyme production media broth was prepared and inoculated with the selected bacteria. The media was kept in incubator shaker for 3 days. Protein estimation and protease assay were done for the culture broth. Ammonium sulphate precipitation was performed on the broth to purify protein from culture broth. Dialysis was carried out on the precipitated sample and the protein was stored in the refrigerator for further use.

Protein estimation of purified sample was carried out. Bovine serum albumin was used as working standard. Estimation of protein was carried out by Lowry's protein estimation method represented in the follows.

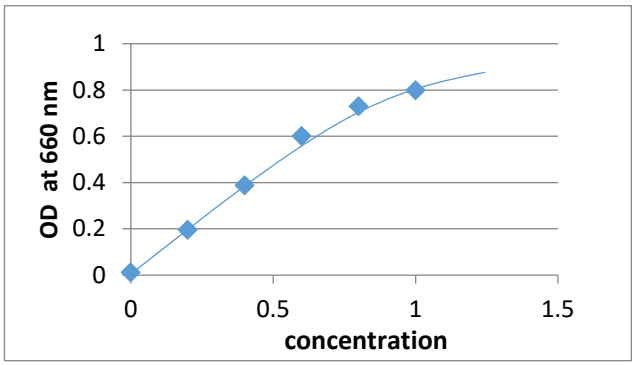

Fig. 3: Graph showing the protein estimation of purified enzyme

DNA was isolated from the sample bacteria and polymerase chain reaction was carried out using 16sDNA region of DNA. Gel electrophoresis of the PCR sample was carried out to view the amplified gene. Amplified gene sequence were then used for similarity check using BLAST (Basic Local Alignment Search Tool) programme in the NCBI GenBank (www.ncbi.nlm.nih.gov) DNA database for identifying the sample.

The PCR amplification resulted in following

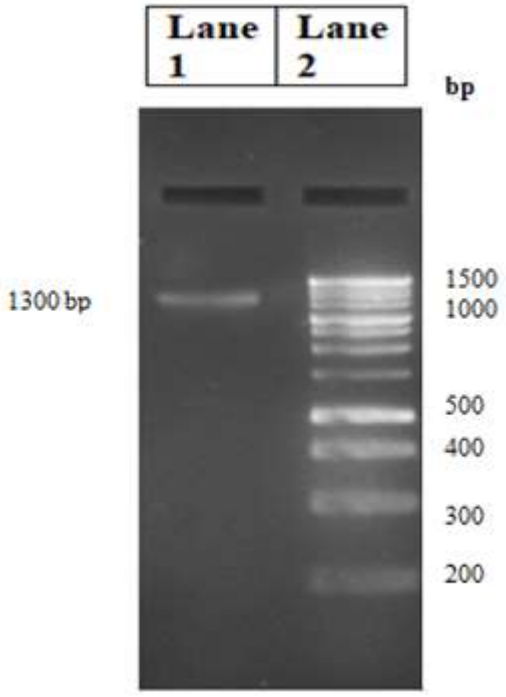

Fig. 4: Agarose gel electrophoresis photograph of Polymerase chain reaction; Lane 1- PCR product (1300 bp); Lane 2- 16s DNA ladder

DNA Sequencing

1042 base pairs were presented in the sequence and the strain showed 98.64 % similarity with *Aeromonas hydrophila* strain AH10, complete genome.

GenBank accession number - SUB7603541 seq_10836 MT605959

Cloned DNA Sequence

The blue white screening result gives the presence of blue and white colonies. Thus the cells were transformed. The cloned sequence was identified.

The nucleotide sequence of the cloned protease gene and its flanking DNA regions was determined using ExPASy translate software in the Bioinformatics Resource Portal (https://web.expasy.org/translate/).

The gelatin layer of X-ray sheets were completely ripped off and change in the turbidity of the solution was observed. Further, the presence of silver was confirmed by the presence of white precipitate.

After washing the blood-stained clothes by water, detergent and protease sample, it was observed that the protease enzyme, as compared to water and detergent was able to remove proteinacious blood stain completely without leaving any secondary stains behind.

Discussion

Bacteria are one among the widest spreading microorganisms in the environment. They are pathogenic as well as useful at the same time. The source of sample in this study was detergent contaminated soil. The selection of organism was based on the zone of clearance that it showed on the skim milk agar plate. The protease enzyme produced by the organism showed a clear zone around the colony as the protein near the colonies was utilized. These colonies were gram negative rods. Comparison has been made on the protease concentration in the supernatant of the culture broth and crude protein that has been produced through dialysis.

The alkaline protease producer was found to be *Aeromonas hydrophila.* They are heterotrophic, gram negative bacterium found in areas with a warm climate. This organism can be found in brackish or fresh water. And they can digest materials such gelatin and hemoglobin which directly shows its protease activity. The alkaline protease producing gene was ligated to TOPO vector and cloned to DH5alpha competent cells. It is observed that the cloned gene has open reading frames. When expressed in E. coli, active *A. hydrophila* alkaline protease was produced. This protease was entirely found in the culture supernatants of E. coli. This secretion process was specific and appeared to be similar to that existing in the natural host organism.

The proteases from *Aeromonas hydrophila* can be used in various industries such as food, chemical, detergent and leather industries as it shows high rates of enzyme production.

The potential of removing blood stains was tested by *A.hydrophila*. It was observed that it removed stains completely as compared to water and detergent without hampering the texture of the cloth and this potential of

Aeromonas hydrophila makes it ideal to be used as biocleaner or in detergent manufacture.

There is a scarce literature regarding the potential of *A.hydrophila* in recovering silver from waste x-ray films. However, in this study *Aeromonas hydrophila* was tested for same potential in the laboratory and the presence of silver was detected in the solution at the end of the experiment where it can be used as an agent for recovering silver from waste X-ray films.

Bacterial alkaline proteases are important participants of feather degradation, detergent industry and experiments are ongoing for the invention of new sources of alkaline protease. Existing knowledge on bacterial alkaline proteases is increasing its depth day by day due to the innovative findings on the same. Members of the genus *Aeromonas* have a number of interesting characteristics which are of major interest to biologists. Many of the species have emerged as pathogenic causing disease in cold- and warm-blooded animals including animals. *Aeromonas* secrete a wide range of extracellular enzymes which play a vital role in the ecology, survival and pathogenicity of these microorganisms.

In this present study, detergent contaminated soil of Ernakulam city showed presence of protease producers. The bacteria were screened and tentatively identified as *Aeromonas hydrophila* by morphological and microscopical methods. These bacteria can be very useful in treating cloth that is stained with proteinacious substances such as blood. Further, experiment regarding the recovery of silver from waste X-ray sheet was performed. The results showed that it can be used for the recovery of silver. Therefore, these findings on alkaline protease producing *Aeromonas hydrophila* are definitely helpful for industrial purposes.

Author s' Contribution

Anupa Mary Aji performed the experiments, Literature survey, Final manuscript preparation, Correspondence for publication. N.G. Ramesh Babu designed the research planning, worked in manuscript editing, Literature survey, referencing of article. Final form of manuscript was approved by both of authors.

Conflict of Interest Statement

The authors declare that there is no conflict of interest on this project.

Acknowledgement

We are indebted to Dr. K.S. Rishad and Dr. Bivya Gopalan, UniBiosys Biotech Research Labs, South Kalamassery, Kochi, Kerala for their valuable guidance without which this project work would not have been so nicely accomplished.

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