ISOLATION AND IDENTIFICATION OF PROTEASE ENZYME FROM MARINE BACTERIUM

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ABSTRACT

Protease enzymes are produced in the marine environment as a bioactive compound. Different parameters such as temperature, pressure, salinity and density control the synthesis of these bioactive substances. Protease producing bacteria was isolated from marine soil sample, Laccadive Sea shore, Kanyakumari, Tamil Nadu. Twelve bacterial stains were isolated and identified from the marine environment. Most of the isolated were found to be grams positive and rod shaped microbes. Bacillius species were the most predominant microbes present in the total isolates. Regarding to bacillus species, twenty five bacterial isolates were obtained from soil samples of which nine isolates were identified as the bacillus species.

Key words: Protease enzyme, protease activity, Bacteria, Marine Soil Sample

I. INTRODUCTION

Enzymes are the biocatalysts used for enhancing metabolic rate of reactions. A large number of enzymes are produced in vivo having importance in industries. Protease is the most important enzyme produced industrially. Protease enzyme is naturally present in all organisms and it corresponds to 1-5% of total protein content [1]. Protease is the third largest group of industrial enzymes and has a worldwide sale of 60% [2]. Proteases can hydrolyse peptide bonds in proteins and they are also called peptidase or proteinase or proteolytic enzymes [3]. Proteases are classified into three groups based on their acid base behavior as acid, neutral and alkaline proteases [4]. Protease enzymes are majorly used in various industries such as in dry cleaning, detergents, meat processing, cheese making, silver recovery from photographic film, production of digestives and for certain medical treatments of inflammation and virulent wounds [5]. Bacterial protease is used for accessing genetic manipulations.The protease producing bacterial strains are Bacillus subtilis, Bacillus licheniformis and Bacillus thuringiensis [6].The current study aims to isolate protease producing bacterial stains from the marine soil sample.

II. MATERIALS AND METHODS

i. Collection of Sample

The marine soil and water samples were collected in a sterile container from Laccadive Sea shore, Kanyakumari, Tamil Nadu.

ii. Isolation of Bacteria From Marine Source

The bacteria were isolated by serial dilution method. One gram of the sample was mixed with 10 ml of sterile distilled water. The tube was vigorously vortexes for 3 minutes to obtain uniform suspension of microorganisms. A series of test tubes were labelled as 1,2,3,4,5,6,7,8,9,10 and filled with 9 ml of sterile distilled water. From the diluted sample, 1 ml was transferred to the tube marked 1 and vigorously vortexed. From this tube 1 ml was transferred to the tube marked 2 and vigorously vortexed. This process was continued up to 10 water sample. The serial dilution method was followed for water sample. Spread plate method was followed for the isolation of marine bacteria in the nutrient agar medium with 2% NaCl.

iii. Composition of Nutrient Agar Along With 2% NaCl

The composition includes 5gPeptone with 1.5gBeef extract, 1.5gYeast extract, 25gSodium chloride, 20gAgar and 1000ml Distilled water with7.2+ 0.2pH. The 0.1ml of sample was poured on the medium and spread by sterile L-rod. The plates were incubated at 37ºC for 24 h. After the incubation time, single colonies were isolated and streaked on fresh nutrition medium with 2% NaCl. The isolated single colonies were maintained on the slant culture. Finally they were subjected to the genus identification by Gram's staining and biochemical analysis.

iv. Gram's Staining

A loop full of overnight grown culture was taken and uniform smear was made on a clear glass slides. They were heat- fixed. Crystal violet solution was added on theslides and kept for a minute. The slides were then washed with water and flooded with Gram's iodine solution. After a minute, the slide was washed and de-colorized with 95% ethanol. The washed slides were counter-stained with safranin and observed under light microscope. The Gram-positive organisms appeared violet and Gram-negative organisms in pink.

III. RESULTS: BIOCHEMICAL CHARACTERIZATION

1. Catalase Test

Culture tubes were taken and 2% hydrogen peroxide solution was added directly. Appearance of brisk effervescence indicates positive result and absence of brisk effervescence indicates negative result.

2. Citrate Utilization Test

A loop full of overnight grown culture was streaked on Simmon's citrate agar medium and the tubes were incubated at room temperature for 24 h development of blue colouration indicates positive reaction. No colour changes indicate the negative result.

3. Oxidase Test

A loop full of overnight grown culture was placed on moistened oxidase disc. Appearance of purple colour within a minute indicates that the microbe is capable of producing oxidase. No colour indicates negative result.

4. Methyl Red Test

A loop full of microbe was inoculated into the test tube containing methyl red medium. These test tubes were incubated at 37°C for 24 h. After the incubation time, 5 drops of methyl red reagent was added to the test tubes. Change in the colour of the medium to red indicates the positive result and no colour change indicates negative result.

5. Vogus Proskaver

A loop full of organism was inoculated into the test tube containing Vogus Proskauer medium. These test tubes were incubated at 37°C for 24 h. After incubation 40% Potassium Chloride (KOH) was added to the test tubes and shake well for the availability of atmospheric oxygen. Then add ethonal and α-napthol. Change in the colour of the medium to red indicates the positive result and no colour change indicates negative result.

6. Screening for Protease Producing Bacteria

The isolated bacteria were screened for their protease producing capability using skim milk agar medium along with 2% NaCl. The bacteria were grown on the media.

Table1: Components for agar medium

Components	g/l
Cascin	5
Yeast extract	2.5
Dextrose	1
Skim milk powder	28
Agar	20
Distilled water	1000 ml
pH	7.0-7.2

The protease producing ability of the microbe was determined by the zone of inhibition in the skim milk agar medium.

IDENTIFICATION OF BACTERIUM USING 16S RRNA ANALYSIS

7. Isolation of Genomic DNA from Bacteria

Take 5 ml of exponentially growing bacterial cells were harvested by centrifugation and washed twice with wash buffer. The washed cells were resuspended in 200µl of solution I followed by the addition of 2 mg of lysozyme and incubated at 37ºC for 1 hour. The SDS was added to a final concentration of 2% and mixed vigorously by inversion. The 100µl of 5M NaCl was added and the contents were mixed by inversion and left at 20ºC for 10 minutes. The mixture was centrifuged at 12,000 rpm for 5 minutes and to supernatant equal volume of phenol:Chloroform was carried out and the supernatant was precipitated with 2.5 volumes of chilled 95% ethanol. The pellet was dried and dissolved in 25µl of STE buffer. The RNAse (µg) was added to the tube and placed at 37ºC for 10 minutes. The solution was made up to100µl with sterile deionised water. Equal volume of phenol:chloroform mixture (1:1,v/v) was added, mixed well and centrifuged for 5 minutes at 12,000 rpm. The upper aqueous phase was transferred to a fresh microfuge tube, 2.5 volumes of chilled 95% ethanol was added and incubated at -20°C over night for precipitation. The DNA was pelleted by centrifugation at 12,000 rpm for 5 minutes and after washing with 70% ethanol, the pellet was dried DNA was dissolved in 0.1X TE16Buffer. The DNA was analyzed by electrophoresis on 0.8% agarose gel in 0.5x TEBuffer.

8. Polymerase Chain Reaction

The PCR reactions were carried out in 0.2 ml tubes in Eppendorf Personal Mastercycler. Identification of the strain was done by amplification of the partial 16SrRNA gene using 16S F 5' AGAGTTTGATCCCTGGCTCAG 3' and 16S R 5'GTACGGCTACCTTGTTACGAC 3'. The programme used for the amplification was 95ºC for 5 minutes for initial denaturation and 34ºC cycles of 95ºC for 1 minute, 56ºC for 2 minutes and 72ºC for 1 minute and 72ºC for 10 minutes for final extension.

Table 2: Components of Reaction Mixture

S.No	Reagents	Concentration	Volume (µL)
1	Sterile water	-	34.5µL
2	10X Taq buffer	10X	5µL
3	2mm Dntp MIX	0.2mM	5µL
4	Primer I(M13 forward)	4µM	2µL
5	Primer II(MI3 reverse)	4µM	2µL
6	Template DNA	˜50ng	1µl
7	Taq DNA Polymerase	5U/µL	0.5µL
TOTAL			50µL

9. Agarose Gel Electrophoresis of DNA

The isolated pure DNA was run in agarose gel for confirmation of the presence.

a. Preparation of 1% Agarose Gel

1) Seal the gel casting tray with cellophane tape on both sides, check leakage and place the comb in a slot.
2) Prepare 1% Agarose in 0.5X TEB.
3) Weigh 250 mg of Agarose and dissolved in a small conical flask containing 25ml of 0.5XTEB.
4) Boil the Agarose either using microwave oven or water bath until Agarose appears as a clear solution.
5) Cool the agarose solution around 40°C.
6) Add 1µl of Ethidium Bromide (5mg/ml) when the agarose gel temperature around 40°C.
7) Assemble the gel casting tray.
8) Pour Agarose gel on tray.
9) Do not disturb the tray until complete solidification.
10) Pour 0.5X TEB buffer in horizontal electrophoresis reservoir tank. (It should appear milky white).
11) Carefully remove the tape and placed in the electrophoresis tank.
12) Buffer level should be maintained just above the gel tray (5mm of buffer over the gel).
13) Carefully pull out the comb without damaging the wells.

b. Preparation of Samples, Loading and Running Electrophoresis

1) Place 3µl of loading dye onto square of parafilm.
2) Pipette 10µl of DNA sample to each onto this 3µl spot of loading dye slowly.
3) Pipette the mixture up and down to mix it.
4) Reset the micropipette to 10µl and carefully pipette the DNA sample/loadingdye mixture into wells of the gel.
5) Load 5µl of DNA marker in a separate well.
6) Connect the positive and negative electrodes into the appropriate connection on the power supply.
7) Turn the power supply "ON" a pre-set the voltage to 50 volts.

c. Gel Electrophoresis of Amplified DNA

The amplified DNA has been electrophoresed in the agarose gel and the fragments as the bands are eluted and purified by gel purification process

d. Elution And Purification Of Amplified DNA

After visualizing the amplified DNA fragments in a UV Trans illuminator, the bands has been eluted and purified by gel purification process. The bands of the DNA fragments have been

cut out from the gel carefully with a sterile blade without disturbing the bands. Then the clean-up kit solution was added which will separate the DNA from the gel. Then the isopropyl alcohol was added to precipitate the DNA from other contaminates and centrifuged at 12,000 rpm for 2-3 minutes. Then added the wash buffer to remove the alkali in the pellets and then again centrifuged. The pellet contains the purified amplified DNA fragments which are now ready for cloning.

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Table 3: Identification of marine bacteria

Isolated code	Gram reaction	Catalase test	Oxidase test	Methyl red test	Voge'spros kaver test	Citrate utilization	Cellular morphology	Probable identity
1	+	+	+	-	-	-	cocci	Micrococcus species
2	-	+	+	-	-	-	Rods	Flavobactrerium speices
3	+	-	-	+	+	-	Rods	Bacillus species
4	+	+	+	+	-	-	Rods	Bacillus species
5	-	+	-	-	+	+	Rods	Enterobacter species
6	+	+	-	-	+	+	Rods	Bacillus species
7	+	+	+	-	+	+	Rods	Bacillus species
8	+	+	-	-	-	-	Rods	Bacillus species
9	-	+	-	-	-	+	Rods	Acinetobacterspecies
10	+	+	+	-	+	-	Rods	Bacillus species
11	-	+	-	-	-	+	Rods	Acinetobacter species
12	+	+	+	-	+	-	Rods	Bacillus species
13	+	+	+	-	+	+	Rods	Bacillius species
14	+	+	-	-	+	+	Rods	Bacillus species
15	-	+	+	-	-	+	Rods	Choromobacter species
16	+	+	+	-	-	-	Cocci	Micrococcus species
17	-	-	+	-	+	-	Rods	Bacillus species
18	-	+	+	-	-	+	Rods	Pseudommonas species

19	+	+	-	-	-	-	Rods	Corynebaterium species
20	+	+	+	-	-	+	Rods	Bacillus species

IV. CONCLUSION

The 12 bacterial stains were isolated and identified from the marine environment. Most of the isolated were found to be grams-positive and rod shaped microbes. Bacillius species were the most predominant microbes present in the total isolates. Regarding to bacillus species 25 bacterial isolates was obtained from soil samples of which 9 isolates were identified as the bacillus species. The identification was carried out by the reference of Bergy's manual of determinative bacteriology. Further, the genetic and protein engineering can play a big role for the large scale production as well as for the alteration of different properties of proteases, keeping in view the harsh conditions during industrial processes.

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