Original Article

Production of Probiotics from Environment

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Abstract

Bacillus sp. are widely used for isolating these protease enzymes. In this project, organisms were isolated from the soil sample was identified by morphological and biochemical characterization as *Bacillus sp.* The bacterial isolates were sub-cultured in Casein agar plates by quadrant streaking. Large amount of the enzymes was produced using fermentation process.Enzyme crude extract was prepared by centrifuging the fermented broth at 4500 rpm for 15 minutes at 4ºC. The precipitation method using ammonium sulphate was used to complete the crude enzyme partial purification. Partial purified enzyme showed maximum zones., when compared to the crude activity of the enzyme**.**The protein content was estimated by Bradford assay and was found to be 7.0µg/ml and 13.0µg/ml for crude and partially purified enzyme respectively.

In this review reported that bacteria from soil environment, bacillus sps. Which acted as a source of protease.

Keywords: Proteases, *Bacillus sp.*, Casein agar plate, Partial purification, Mass production

I. INTRODUCTION

Using Modified biotechnological methods, various industrially important enzymes are extracted from economically important microbes. Proteases cleaved the large protein molecules into smaller fragments, In food and leather industry, these enzymes as signal molecues,use to process various dairy products. The availability of economically and industry useful microbes and its diversity are rich in soil environment.

In our research study, we identified the soil source to characterization of the protease producing bacteria near madipakam region. Milk agar plate technique employed to screen the bacteria using standard protocol.Azocasein substrate used to detect the Proteolytic activity of protease extract of the bacteria .we observed two colourcolonies(white and yellow) to determine the activity of bacteria. During this process, we maintained the pH 8.5 ,37-degree temperature for yellow colonies and 60 degree temperature for white colonies to check the proteaseactivity. The proteaseactivity was observed at the ofPH 8.5 for both bacteria, but minimum temperature was 37°C for yellow and 60°C for white.

II. AIM AND OBJECTIVE

In this research, we aimed to isolate, identification of the bacteria, protease enzyme and protein estimation from bacillus *sps*.

III. METHODOLOGY

3.1 Collection and Preparation of the Sample:

Various soil samples were collected from various places using air tight container bags and brought to the lab for further research analysis.The samples were labelled, such as Sample-1 from madipakkamSample-2 from keelkattalii, Sample-3 from ponmarand Sample-4 from gudavancheriwere collected and soaked in distilled water overnight. The samples were filtered using filter paper.

Serial dilutions were made from 10^{-1} to $10^{-5}$of each soil sample was prepared in sterilized distilled water and 1ml of that diluted sample was spread on Casein agar medium

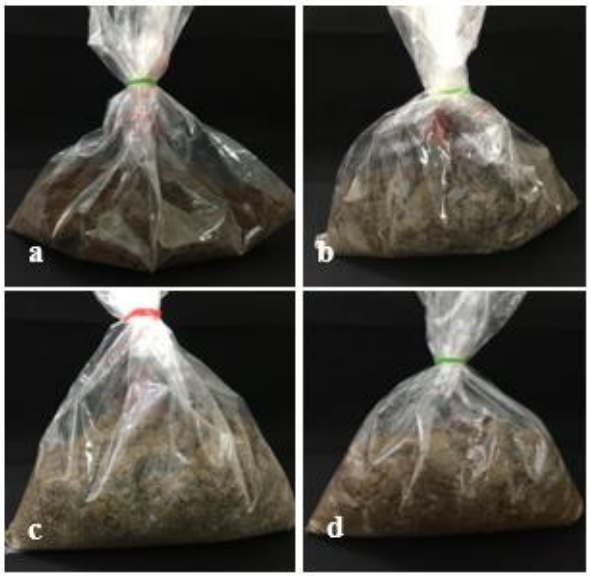

Figure: 1 Forest soils from (a) Puliyanthoppu, (b) Gumidipoondi, (c) Avadi and (d) Maraimalai Nagar

Figure 2. Filtrates of the Samples

3.2. Isolation, identification and Screening of proteolytic bacteria:

Based on the morphology with respect to the clear zone of proteolytic from bacterial isolates, confirmed the isolates as proteolytic bacteria. Higher zones of colonies showed that bacterial pure isolate species. Bacterial sps isolation, identification was identified by morphological characters, grams staining and biochemical test. The colonies was purified and preserved at 44 degrees to detect the production of the protease enzyme.

.Media Preparation:

The casein agar media was prepared by dissolving 24.5 g of nutrient agar, 2.91 g of 0.5% casein and 6.55 g of agar agar in 1000ml of distilled water and autoclaved.

Gram staining Test:

A bacterial smear was prepared and heat fixed. Then crystal violet was added and allowed to stand for 1 minute and the smear was washed in an indirect steam of tap water. The slide was flooded with iodine mordant and washed in tap water (indirect steam). Smear was blotted with absorbed paper and it was immersed in 95% ethanol solution for 30 seconds. It was then washed in tap water and blotted with absorbent paper. Saffranin was added to the smear and allowed to stand for ½ min and the slide was washed with tap water. The slide was air dried and examined in a microscope. Appearance of blue or violet color indicates gram positive bacterium and appearance of red color indicates gram negative bacterium.

Biochemical characterization:

Thebiochemicalcharacterizationofbacteriawasdonebasedontheergy'smanualofdeterminativeBacteriology. Basedonthebiochemicalcharacters,theybacteriawasidentifiedtobe*Bacillussp.*

Submerged fermentation process (Mass Production):

To produce mass production of protease enzyme, in our research we used bacillus *sps* identified as proteolytic*sps.*

Preparation of Crude enzyme:

After the fermentation period, the broth was centrifuged using centrifuge at 1600 rpm for 20 min at 4°C to remove the unwanted material. After centrifuge m the supernatant used as crude extract source of enzyme source from the bacteria.

Method of protease enzyme partial purification:

The 100ml of crude enzyme was mixed by 93g of ammonium sulphate. The mixture was kept in a magnetic stirrer. When all the ammonium sulphate was dissolved, the mixture allowed to stand for 30 minutes to 1 hour, mixture was centrifuged at 10000 rpm for 30 minutes. The partially purified enzyme was dialyzed against the phosphate buffer.

Procedure to detect the crude and partial purified enzyme activity:

To evaluate the difference among enzymatic activity of crude and partially purified enzyme used casein agar plate**.** Hydrolysis zones observed and compared around the two wells which contain the partial and crude purified enzyme samples.

Estimation:

The protein estimation was done by using Coomassie brilliant blue strain.500µl of CBB strain was added to the supernatant, the quantity of protein in a sample was measured directly by the absorption at 595nm.

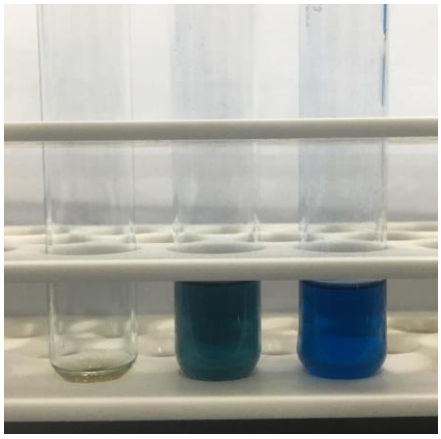

Figure: Protein Estimation

IV. RESULTS AND DISCUSSION

Isolation and Screening:

The 3 out of 5 serial dilutions of each of the 4 samples were selected; which are 10^{-2}, 10^{-3} and $10^{-4,}$ and were spread plated in two plates each. Single colonies that showed maximum proteolytic zones as shown in fig: 13.1 were identified and isolated.

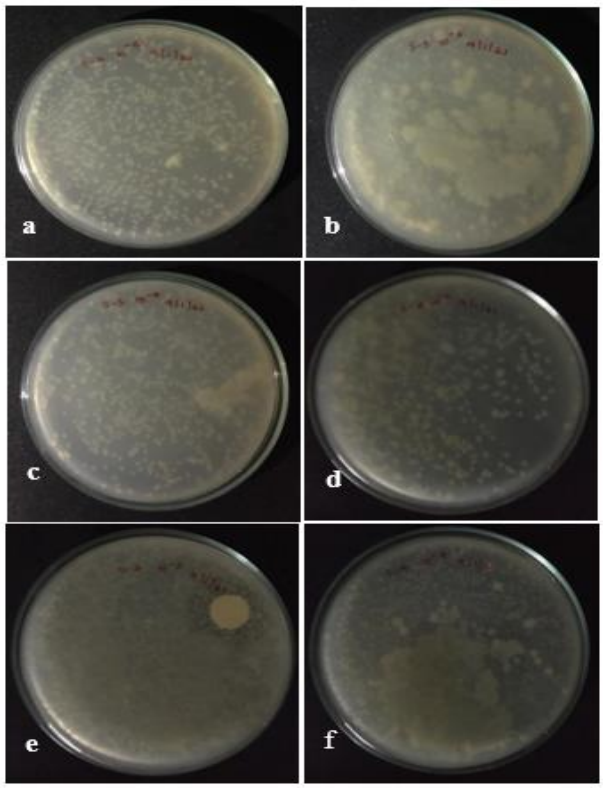

Figure :(a) sample 2 10^{-4}, (b) sample 3 10^{-3}, (c) sample 3 10^{-4}, (d) sample 4 10^{-2}, (e) sample 4 10^{-3} and (f) sample 4 10^{-4}

The bacterial isolates were sub-cultured in separate respective casein agar plates as shown below:

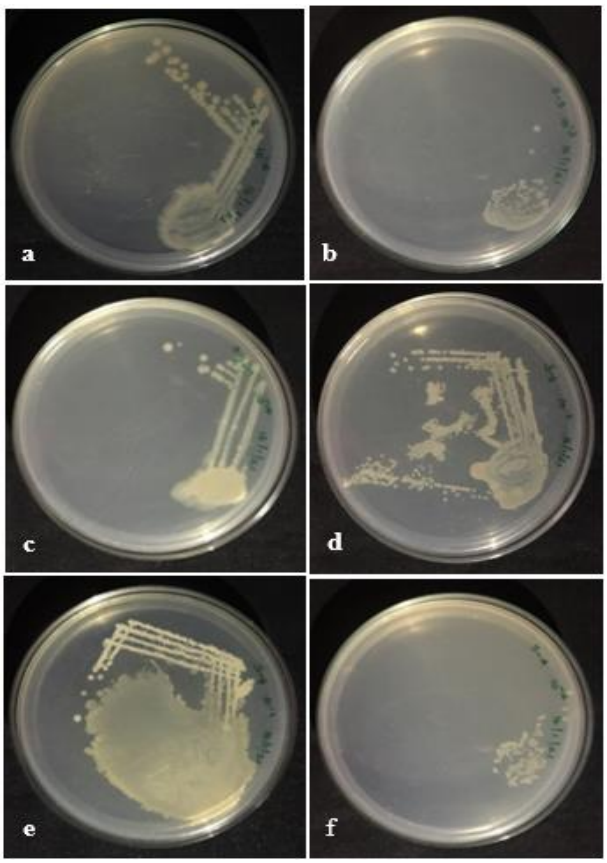

Figure: Respective Subcultures (quadrant streaks) of the selected plates mentioned in Figure 13.1

Submerged Fermentation Process:

The single colonies that were screened (sample 4 10^{-4}) for proteolytic bacteria from the sub-cultures were inoculated in casein broth for fermentation. After fermentation the resultant broth was plated in casein agar plates and observed for zone hydrolysis as shown fig: 14.2.

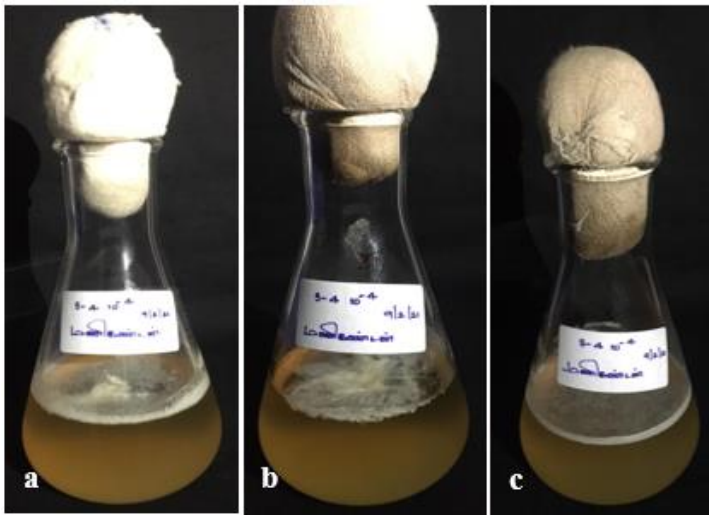

Figure 14.1 a, b and c are the fermented broth

Table: Proteolytic Zone Hydrolysis

S.no:	Plate 1	Plate 2
1.	0.7mm	0.7mm
2.	0.8mm	0.9mm
3.	1.0mm	1.1mm

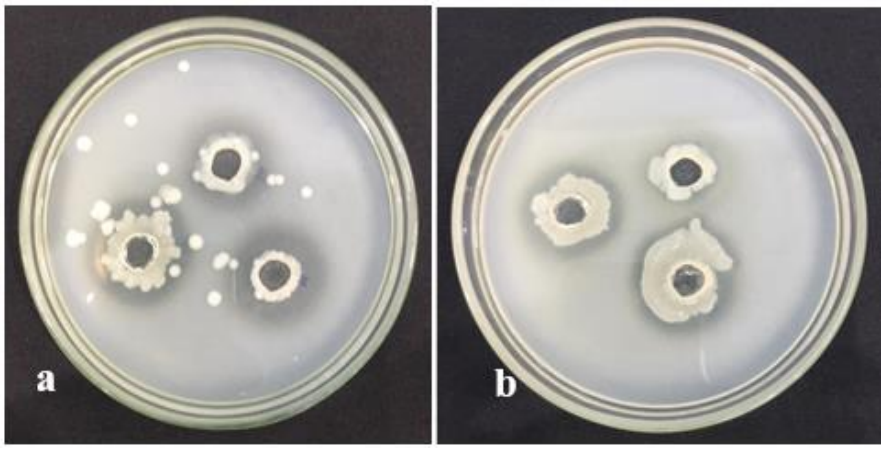

Figure: ProteolyticZone Hydrolysis of (a) Plate 1 and (b) Plate 2

Partial purification of Protease enzyme:

The fermented broth was centrifuged 4500 rpm for 15 minutes at 4ºC. The 100ml centrifuged broth was shifted to a conical flask and 93 g of ammonium sulphate was added and centrifuged 10,000 rpm 10 minutes 4ºC as shown in fig: 14.3.

Figure: Protease enzyme Partially purified by ammonium sulphate precipitation

Comparison of the Activity of the Crude Enzyme and Partially Purified Enzyme:

Among the crude and partially purified enzyme, the partially purified enzyme was found to have the maximum proteolyticzone hydrolysis as shown in fig: 15.

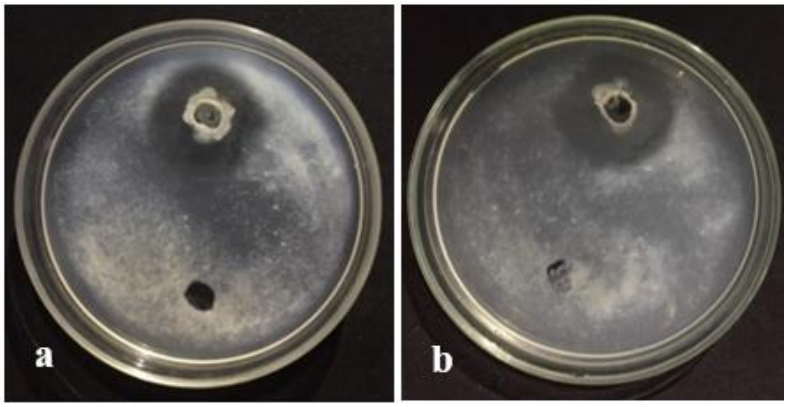

Figure: Comparison of the Proteolytic Activity of the Crude Enzyme and Partially Purified Enzyme (a) Plate 1 crude enzyme and (b) Plate 2 partially purified enzyme

Estimation of Proteins:

The estimated proteins of the crude and the partially purified enzyme 7.0µg/ml and 13.0µg/ml, among which the partially purified enzyme had the higher protein content.

Table 2 Amount of proteins estimated by Bradford assay

S.no:	Sample	OD at 595nm	Concentration of protein µg/ml
1.	Culture Filtrate	0.932	7.0
2.	Ammonium Sulphate precipitate	1.757	13.0

V. CONCLUSION

The 3 out of 5 serial dilutions of each of the 4 samples were selected; which are 10^{-2}, 10^{-3} and 10^{-4},and were spread plated in two plates each. Single colonies of plate sample 2-10^{-4}, sample 3-10^{-3}, sample 3-10^{-4}, sample 4-10^{-2}, sample 4-10^{-2} and sample 4-10^{-4} that showed maximum proteolytic zones as shown in fig: 13.1 were identified and isolated. The bacterial isolates were sub-cultured in separate respective casein agar plates as shown in fig: 13.2. The single colonies that were screened (sample 4 10^{-4}) for proteolytic bacteria from the sub-cultures were inoculated in casein broth for fermentation. After fermentation the resultant broth was plated in casein agar plates and observed for zone hydrolysis as shown fig: 14.2. The fermented broth was centrifuged 4500 rpm for 15 minutes at 4ºC. The 100ml centrifuged broth was shifted to a conical flask and 93 g of ammonium sulphate was added and centrifuged 10,000 rpm 10 minutes 4ºC as shown in fig: 14.3. Among the crude and partially purified enzyme, the partially purified enzyme was found to have the maximum proteolytic zone hydrolysis as shown in fig: 15. The estimated proteins of the crude and the partially purified enzyme 7.0µg/ml and 13.0µg/ml, among which the partially purified enzyme had the higher protein content.Our results proved the bacteria isolated from soil ,bacillus sps, have protease enzyme. This enzyme has economical important in various industries.

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