International Journal for Multidisciplinary Research (IJFMR)
E-ISSN: 2582-2160 ● Website: www.ijfmr.com ● Email: editor@ijfmr.com

Screening and Characterization of Protease Producing Bacteria from Raw Milk Sample

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Abstract:
The purpose of the current research was to screen, characterize and isolate protease producing bacteria from raw milk sample. Initially, the raw milk sample was spread on nutrient agar, at 35°C for 20 hrs. Four bacterial colonies of different morphology for further study. Among these, M-4 colony were selected and isolate produced highest protease activity and was identified as Proteus mirabilis by morphological and biochemical tests. Moreover, various physiological characters were also studied such as pH, temperature, carbon source and nitrogen sources. The bacteria showed maximum activity at pH 7, temperature requirement is 35°C, lactose as a carbon source and potassium nitrate as a nitrogen source respectively. At the end, enzyme activity assay was done by using well diffusion method. Microbial proteases which are environmentally friendly are interestingly used for their commercial importance.

Keywords: Proteolytic enzymes; Proteases; Screening and Characterization. morphological identification; Enzyme activity.

1. INTRODUCTION

Proteolytic enzymes are found in all kinds of organisms‟ i.e., viruses to animals (Rao et al., 1998). Microbial proteases which are environmentally friendly are interestingly used for their commercial importance (Mienda et al., 2014). They have potential for application in different industries including detergent, leather, food industry (Raveendran et al., 2018), dairy, baking, beverages, pharmaceutical industries (Craik et al., 2011) and agriculture ventures. Enzymes are biological compounds (Robinson, 2015) used to bring about specific biochemical reaction generally forming parts of the metabolic processes of cells. They are protein in nature {exception- RNA acting as ribozyme}, colloidal and thermolabile in character, and specific in their action. Protease or Proteolytic enzyme catalyze the hydrolyses of the peptide bonds between amino acid residues of proteins (Ward, 2011). They are often referred to as proteases or peptidases. Proteases have been used for a long term for the benefits of humans (Lopez-Otin et al., 2008). Most proteases are produced as a zymogen, the inactive form whereas they are activated by environmental cues or upon encountering their specific substrate, to bring conformational change (Khan et al., 1998).

At organism level, proteases are recruited by viruses and pathogenic bacteria as well as insect pests to enter their target host. Proteolytic enzymes are present in bacteria, archaea, certain types of algae, some viruses, and plants; they are most abundant, however, in animals (Razzaq et al., 2019).

Based on the sites at which they catalyze the cleavage of proteins, there are two major groups exopeptidases (target the terminal ends of protein) and the endopeptidases (target the sites within proteins)

(Oda, 2011). Endopeptidases employ various catalytic mechanisms; within this group are the aspartic endopeptidases, glutamic endopeptidases, metallo- endopeptidases, serine endopeptidases, and threonine endopeptidase (Oda, 2011). The Proteolytic enzymes are classified according to the mechanism of action based on the catalytic amino acid residue involved in four categories as serine, aspartate, cysteine, metalloproteases. Therefore, new class of threonine proteases was described in 2010.

Proteolytic enzymes are produced by all life forms- plants (Martinej et al., 2019), animals and micro-organisms. However, to meet huge demand of industries, microorganisms have been the mainstay of the source of proteolytic enzymes (Razzaq et al., 2019). A high yield of Proteolytic enzyme can be obtained by culturing microorganisms which grow in short time and require less space as compared to plant and animal sources.

Taking Proteolytic enzymes supplements has been linked to several health benefits (Aladdin et al., 2017). May improve digestion, decrease inflammation, promote healing, and speed recovery, help irritable bowel syndrome and inflammatory bowel disease, reduce muscle Soreness and certain proteolytic enzymes may have cancer-fighting properties (Chakraborti et al., 2017).

In present study, raw sample was collected from nearby dairy for screening of protease producing bacteria and effects of myriads of physiological character were also examined.

2. MATERIAL AND METHOD

2.1 Collection milk sample:

The bacteria used for study was isolated from the cow milk sample collected from the nearby dairy. The milk sample was collected in a plastic bottle and transported to the laboratory.

2.2 Isolation of protease producing bacteria

Predominantly, prepared and sterilized the nutrient agar media at 121°C at 15psi for 15 min. Initially, spreading technique was done for the isolation of bacteria. Further, four morphologically different colonies were selected. Then, prepared the protease media. Before, sterilized the media by autoclaving and streaked to form mother plate of further study selected bacteria and incubated for 18-20 hrs. at 35-37°C. To preserved the pure line of bacteria, prepared a quadrant streak plate by using the skimmed milk (SM) agar media and incubated it as mentioned above. Similarly, prepared a nutrient broth also of the selected bacteria colony. And the colony was grown on SM agar plate repeatedly. Based on the morphological and biochemical tests the isolated bacteria were identified and physiological characters were also studied for more information about favorable conditions required for maximum growth of the protease bacteria (Sony et al., 2017).

3. IDENTIFICATION OF BACTERIA: The identification of protease bacteria was commenced by morphological tests that include gram staining and motility test. Despite of this, many biochemical tests were also performed i.e. amylase test, catalase test, carbohydrate utilization test, MR and VP test, urease production test, citrate test, nitrate reduction test, H_2S production test (Happy et al., 2018).

Moreover, different physiological factors such as pH, temperature, nitrogen and the caron sources were also included to characterized the isolated protease bacteria (Dalal, 2015).

3.1 Effect of temperature on protease activity: To examine the optimum temperature at which an enzyme shows its maximum activity, the substrate with crude enzyme was exposed to different temperature range i.e., 4°C, 25°C, 35°C and 45°C. Observed the growth of bacteria after 24 hrs. and read

absorbance at 600nm using spectrophotometer (Pokhrel et al., 2014). Plotted the graph between temperature and absorbance.

3.2 Effect of pH on protease activity: To study the effect of pH, four different range of pH likewise 3, 5, 7, 9 was adjusted of the culture media containing 100µl bacteria in four separated test tubes. Incubated the test tube to the 35°C (maximum growth at this temp.) for 18-20hrs. and the absorbance was read at 600nm.

3.3 Effect of Nitrogen on protease activity: To determine the effect of different nitrogen sources such ammonium chloride, ammonium sulphate, sodium nitrate, potassium nitrite. Each nitrogen source with 1% concentration was used. Autoclaved it and 100µl bacteria was inoculated in each test tube. Finally, the test tubes were incubated at 35°C (maximum growth at this temp.) for 18-20hrs and the absorbance was read at as same.

3.4 Effect of Carbon source on protease activity: Prepared the nutrient broth and transfer the media with 1% of each carbon source that were glucose, sucrose, D-mannitol, lactose in different test tube. Incubated the test tubes at 35°C for 18-20 hrs. Read sOD at 600 nm using spectrophotometer (Pokhrel et al., 2014).

3.5 Crude Enzyme Preparation: The protease producing bacteria was inoculated in fermented media and incubated at 121°C at 15psi for 15 min. Incubated period was of 2 days for fermentation process. Using Whatman No. 1 filter paper, the culture medium was filtered aseptically in laminar air flow. The filtered culture medium was directed to centrifugation at 10,00rpm for 10 minutes to remove the undesired particles. The supernatant was used as crude enzyme preparation for future research (Padmapriya et al., 2012).

3.6 Enzyme Activity Assay: To examine the proteolytic activity, the above-mentioned supernatant was used as protease enzyme source. The technique used was known as well diffusion method (Vijayaraghavan et al., 2013), considering all necessary physiological factors for obtaining maximum results, where the protease media plate with 25µl centrifuged fermentation medium poured in all four wells was incubated at 35°C for 18-20 mins. Zone of inhibition around the wells would indicate the activity of protease enzyme.

4 RESULT AND DISCUSSION

4.1 Screening of proteolytic activity: Different isolates were screened and characterized for enzyme activity on protease media plates. Protease enzyme activity was observed through the zone of hydrolysis on nutrient agar media as displayed in figure 1. For further research the strain showing largest zone of hydrolysis was considered and coded as M-4 and was maintained by sub- culturing.

4.2 Assay of protease enzyme activity:

4.2.1 Effect of temperature on protease activity: Increase in temperature will increase the activity of enzyme, but if the temperature rises, enzyme activity will diminish and the protein will denature. Finally, the maximum activity of protease was found at 35^{o}C and minimum at 4^{o}C.

4.2.2 Effect of pH on protease activity: To study the effect of pH, four different range of pH such as 3, 5, 7, 9 was adjusted in four separated test tubes containing culture media with 100µl of selected bacteria. Incubate the test tube at the 35°C for 18-20hrs and the maximum activity of protease was at pH 7.

4.2.3 Effect of Nitrogen on protease activity: To determine the effect of different nitrogen sources such ammonium chloride, ammonium sulphate, sodium nitrate, potassium nitrite. Each nitrogen source with 1% concentration was used. In order to grow, bacteria with maximum enzyme activity were in potassium nitrate medium and minimum in ammonium chloride medium.

4.2.4 Effect of Carbon source on protease activity: To study the effect of carbon sources media was prepared with 1% of four distinct carbon sources such as glucose, sucrose, D-mannitol, and lactose in different test tubes, later test tubes were incubated at 35°C for 18-20 hrs. From all, only lactose medium showed the maximum enzyme activity whereas minimum by the D-mannitol medium.

4.2.5 Effect of fermented media on protease activity: Zone of inhibition represented the amount of protein degradation. In which C well represented the characterized bacteria that showed the maximum zone of inhibition whereas the D well depicted the light-colored inhibition that was of uncharacterized culture (Vijayaraghavan et al., 2013).

Table 1. Results of staining of M-4

S.no.	Staining	Results
1.	Gram Staining	Negative
2.	Motility Test	Motile

Table.2 Biochemical Characterization:

S.no.	Test	Results
1.	Amylase Test	Negative
2.	Catalase Test	Positive
3.	Methyl Red	Positive
4.	Voges-Proskauer Test	Negative
5.	Glucose Test	Positive
6.	Lactose Test	Negative
7.	D-mannitol Test	Negative
8.	Sucrose Test	Negative
9.	Nitrate Test	Positive
10.	Citrate Test	Positive
11.	Urease Test	Positive
12.	H_2S Test	Positive
Bacteria Identified		***Proteus mirabilis***

Figure.1 Primary screening of protease bacteria

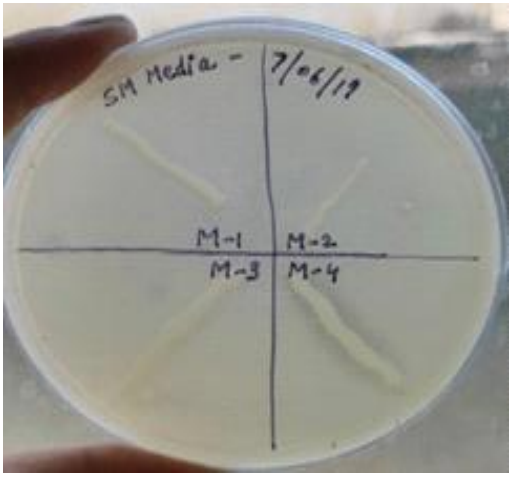


Figure.2 Zone of Inhibition on Nutrient Agar

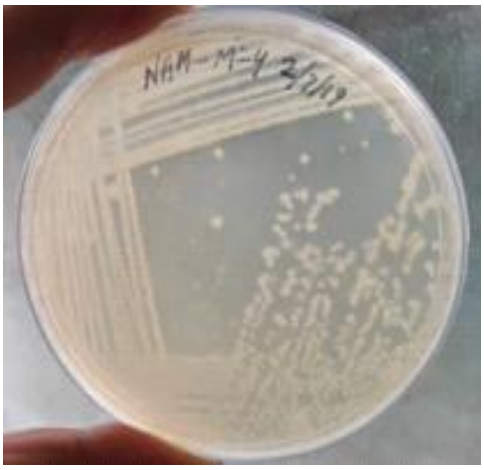


Figure 3. Isolated colony on nutrient agar

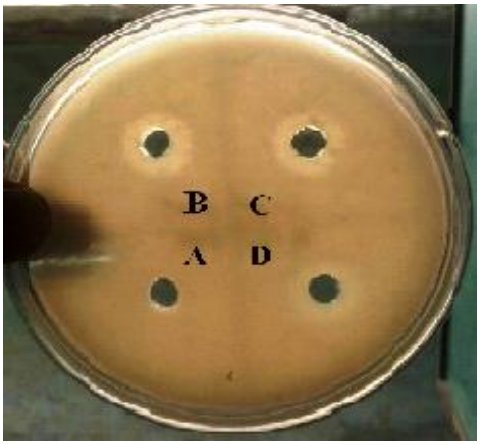
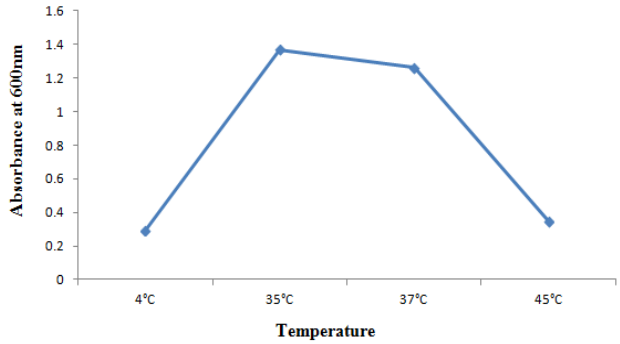
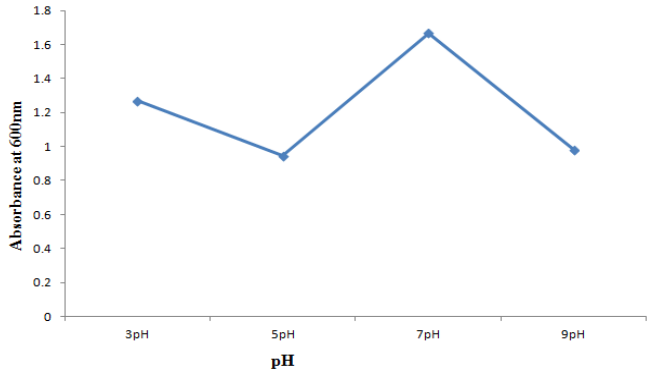


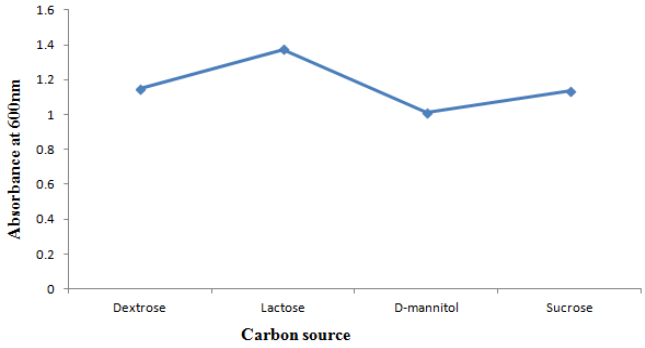
Figure.4 Protease activity- Well Diffusion Method



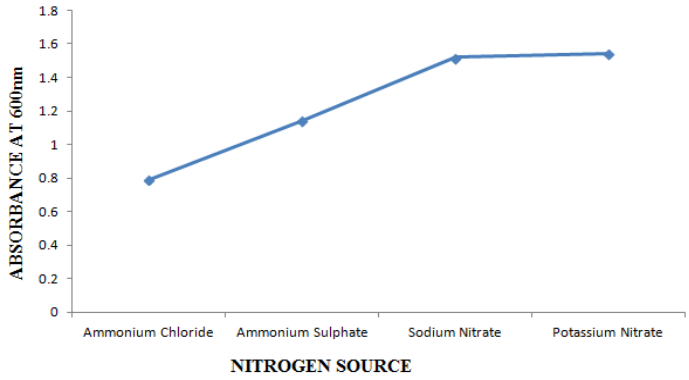
Graph.1 Effect of Temperature On protease activity:



Graph.2 Effect of pH on protease activity:



Graph.3 Effect of Carbon Sources on protease activity.



Graph.8 Effect of Nitrogen on protease activity:

5 Conclusion

By doing this project we were able to screen protease producing bacteria from non-producing bacteria from raw milk. In this report, Proteus Mirabilis was the protease producing bacteria that was isolated and characterized the bacteria from the raw milk sample to understand the optimum growth (Vijayaraghavan et al., 2013). The demand of protease enzyme is elevating day by day at commercial, industrial, pharmaceuticals, analytical, diagnostic sectors. They found ubiquitously in animals, plants, and microorganisms (Solanki et al., 2021). As enzymes obtained from plants and animals are not only much effective but also, they are costly, so the enzymes produced through micro-organisms are mostly preferred due to their rapid growth and with limit space requirement for cultivation. In future protease will be used in novel protein engineering strategies and techniques will continue to expand the commercial protease markets (Dyer et al.2022), activity in diseased tissues for site specific drug targeting (Drag et al., 2010) and tumor imaging (Yang et al., 2009).

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