DOI: https://doi.org/10.54393/mjz.v3i1.46

MARKHOR
THE JOURNAL OF ZOOLOGY
https://www.markhorjournal.com/index.php/mjz
Volume 3, Issue 1 (Jan-Jun 2022)

Original Article

Production of Milk Clotting Aspartic Protease from Bacterial Species Isolated from Dumping Site of Mehmood Booti, Lahore

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ARTICLE INFO

Key Words:
Aspartic Protease, Casein, Milk Clotting, Isolation

How to cite:
Ghafoor, K. ., & Majeed, T. . (2022). Production of Milk Clotting Aspartic Protease from Bacterial Species Isolated from Dumping Site of Mehmood Booti, Lahore: Production of Aspartic Protease from Bacterial Species. MARKHOR (The Journal of Zoology), 3(1). https://doi.org/10.54393/mjz.v3i1.46

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Received Date: 21st May, 2022
Acceptance Date: 7th June, 2022
Published Date: 30th June, 2022

ABSTRACT

Food and dairy industries play a very important role in the economy of every country**.** Aspartic proteases are important enzyme of dairy industry and is used in cheese making. Previously main sources of protease enzyme were plants, animal or fungi, but due to increased demand globally they are now mostly isolated from bacteria. **Objectives:** To isolate the milk clotting bacteria from the soil collected from dumping site of Mehmood Booti and produce aspartic protease from them. **Methods:** Soil sample was collected from Mahmood Booti dumping site near ring road, Lahore. After serial dilutions, sample was inoculated on nutrient agar plates. After 24 hours at 37°C temperature, opaque, round and cream-colored colonies were observed which were sub cultured in LB agar. From there colonies were grown on selective medium made of K_2HPO_4, $(NH_4)_2HPO_4$, casein, $MgCl_2$, yeast extract and agar. After incubation, a colony with clear zone was selected and grown in LB broth for enzyme production. After incubation, broth was centrifuged and supernatant was isolated. While performing protease assay, 3 mL of 5% TCA was added in the mixture. **Results:** The mixture remained clear which depicted the hydrolysis of casein by protease. While the test tube containing water as blank showed precipitation of casein after the addition of TCA because in this enzyme was not present. **Conclusions:** This shows that the isolated bacteria had the ability to produce protease which was evident from the protease activity assay and that such bacteria are abundant in dumping site.

INTRODUCTION

Enzymes are group of proteins that are produced in living systems of microorganisms, plants and animals. Enzymes take part in various biochemical reactions involved in metabolism of these living organisms. Enzyme act as stimulator or as reaction catalyzing agents. Without these catalytic agents there will be no metabolism. So, revival of any life will be impossible without enzymes. Protease belong to the hydrolases and peptidases group of the enzymes. They are also called as peptide hydrolases as they can dissolve or hydrolyze the peptide bond between two amino acids [1]. Aspartic proteases represent almost 60% of the global market enzymes production and sale [2]. These are group of enzymes which have proteolytic activity and are produced by many microorganisms [3]. These are used in many industries including food and dairy, leather industry and pharmaceutical industry [4, 5]. Proteases can play their role in improving the taste, texture and appearance of the product. Due to their higher demand in the industries, the market for their production is increasing day by day [6, 7]. Among all the living organisms, microorganisms are the best source of protease production because they can be used to produce enzyme in bulk amounts. The plant or animal protease cannot meet the global demand of this enzyme. So, microbes are the best option because they have all the attributes that are required by industrial and food biotechnological applications [8]. Each reaction of milk clotting involves two phase which are affected by any change in the chemicals involving this reaction. The first phase of reaction is the cleavage of the casein chain [9]. The second phase is a non-enzymatic phase in which casein start to aggregate due to the influence of the calcium ions. These two steps

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meet each other even before the enzymatic process stop [10]. Due to few studies conducted on bacteria it is generally believed that bacteria usually do not produce aspartic protease in large amounts but a study has been done that shows that Escherichia coli and Haemophilus influenzae, produce a recombinant protein resulting from the expression of each of recombinant DNA are active aspartic proteinases [11]. Bacillus subtilis has also been reported to produce acidic proteases which is GRAS (genetically regarded as safe) and with passing time is more used in cheese making as compare to chymosin [12-14]. Aspartic protease produced from B. subtilis var. natto has also been reported to exhibit milk clotting to a significant extent [15]. Aspartic protease from bacterial origin is best option because they have all the desired characteristics which are key requirement of any proteolytic reaction and beside it is easy and economical to produce enzyme from the bacteria as they can produce enzyme in bulk amounts which can later be sold at commercial level [16]. In this study milk clotting bacteria from the soil of dairy industry were isolated. The strains were tested and identified based on their colony morphology and biochemical behavior. Aspartic protease produced from the strain was checked for its ability to produce cheese and its efficiency in milk clotting.

METHODS

Soil Sample was collected from slum area of Mehmood Booti dumping site near Ring Road, Lahore and kept in falcon tube in refrigerator at 4 °C. Stock solution of soil sample was prepared by adding 1 gram of soil into 100 mL of distilled water in 100mL flask. From this stock solution serial dilutions were done up to 10 times. Nutrient agar medium was made by adding 2 gram of nutrient agar and 1 gram of casein and dissolving in 100 mL of distilled water. The flask was then sealed with tight cotton plug and covered with aluminum foil to avoid the entry of contaminations. The medium was autoclaved at 120 °C for 15 minutes. 5 petri plates were autoclaved. The autoclaved nutrient agar medium was poured into all five plates near flame and under biosafety cabinet. When agar was solidified, the last five serially diluted samples were inoculated into five plates separately with the help of micropipette and blue tips and was spread with glass spreader near flame and in biosafety cabinet. The 5 plates were sealed with paraffin tape and labeled as 1 to 5 and kept in incubator at 37°C for 24 hours. After 24 hours, plates were observed. The colonies that showed clearance zone around them were further selected. 10 such colonies were selected. The selected 10 colonies were picked and inoculated in 2 plates. One plate was divided into six and other one was divided into five sections. Sections were labeled as A to J. The LB agar medium was made by mixing 1.75 gram agar, 1 gram casein, 1 gram NaCl, 1 gram Tryptone and 2 gram of yeast extract in 100 mL of distilled water. The medium was autoclaved before pouring it into the plates. The plates were kept in incubator at 37°C for 24 hours. After 24 hours, all 2 plates were observed and colonies with clearance zone around them were selected. 6 such colonies were selected. Selective medium was made adding 0.1 gram of K2HPO4, 0.1 gram of. (NH4)2HPO4, 0.5 gram of MgCl2, 1 gram of yeast extract, 1 gram of casein, 1.75 gram of agar in 100mL of distilled water. The selected colonies were inoculated in a sterile petri plate with 6 sections containing this media. The sections were labeled as 1,2,3,4 and 5. The plate was placed in incubator at 37°C for 24 hours. Out of five colonies, one colony with prominent clearance zone was selected and inoculated in LB broth in flask. The flask was placed in incubator at 37 °C for 24 hours. After 24 hours, broth was poured into five Eppendorf's tubes which were centrifuged at 10,000 RPM for 5 minutes. The supernatant was collected in a test tube labeled as enzyme. The next step was to check the protease activity of the enzyme. For this 1% casein solution was made. 1mM of potassium phosphate buffer was made by adding 17.4 gram of K2HPO4 in 100mL of distilled water. 0.1 mL of this buffer solution was added into 100mL of distilled water to make 1mM potassium phosphate buffer. Then 0.1 gram of casein was added into 10mL of 1mM potassium phosphate buffer to make 1 % casein. To make 5% TCA solution, 5 mL of trichloroacetic acid was added in 95 mL of distilled water. The assay for proteases activity was done by mixing1mL of enzyme and 1mL of 1% casein solution in a test tube. A blank was made by mixing 1mL of distilled water and 1 mL of 1% casein solution in a test tube. Both test tubes were placed in water bath set on 37 °C for 10 minutes. After 10 minutes, 3 mL of 5% TCA was added in both test tubes and test tubes were left for 10 minutes at room temperature. After 10 minutes, both blank and enzyme containing test tubes were centrifuged at 10,000 RPM for 5 minutes.

RESULTS

The soil sample collected from slum area of Mehmood Booti contained the protease producing bacteria as was expected. The bacteria in the soil sample were alive and active after they were stored in refrigerator at 4 °C and showed a good growth on nutrient agar plates. The stock solution was made by adding 1 gram of soil into 100mL of distilled water. After sample inoculation, the plates were placed in incubator. After 24 hours of incubation, all of the five plates showed growth and colonies with clear zone around them. Ten of such colonies were selected for sub culturing. Selected ten colonies were transferred onto two

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petri plates. One plate was divided into 4 the other was divided into six sections. For sub culturing of selected colonies, LB agar medium was used for growth. After 24 hours, sub cultured plates also showed growth and colonies of bacteria with clear zones around them which was the sign of protease production (Figure 1).

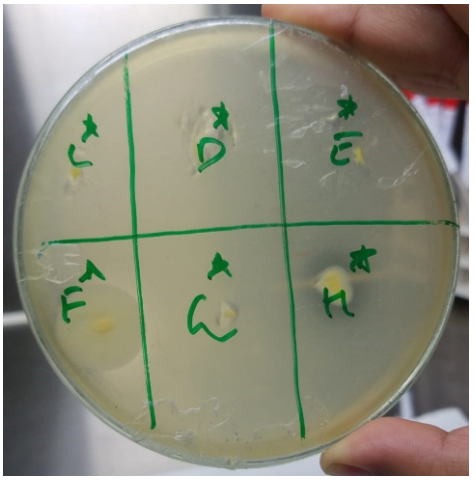

Figure 1: Colonies with clear zone around them

Selective media was prepared using potassium hydrogen phosphate, ammonium hydrogen phosphate, magnesium chloride and casein. One plate of this media was made with six sections labeled as 1 to 6. This media helped in the growth of protease producing bacteria. Colonies C, D, E, F, G, and H from sub cultured plates were selected and inoculated on this plate. After 24 hours, the plate showed growth and colonies with clear zone around them (Figure 2).

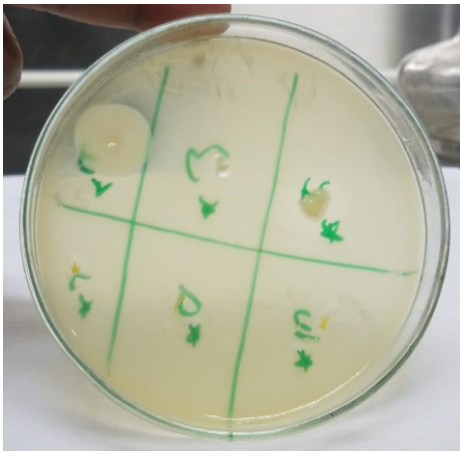

Figure 2: Growth on selective media. Colonies are showing clear zone which is the sign of protease activity

One colony was selected for growth in LB broth. The colony was transferred into broth flask and kept in incubator at 37°C for 24 hours. After 24 hours, the clear medium turned turbid showing the growth. For testing the presence of enzyme, 5 Eppendorf's were filled with media and centrifuged at 10,000 RPM for five minutes. After five minutes, the supernatant was collected in test tube and labeled as enzyme. For determining the protease activity 1 % casein solution made in 1mM phosphate buffer was used along with 5% TCA solution. The test was performed in duplicate. One test was run on mixture of 1mL enzyme, 1mL of 1 % casein and 3 mL of 5 % TCA solution. The other test was blank and run on 1mL of distilled water, 1 mL of 1% casein and 3mL of 5% TCA solution. The contents of both test tubes were clear before the addition of TCA solution. When TCA solution was added the tube containing water turned milky and there were white colored precipitates which was showing the presence of casein (Figure 3).

Figure 3: The contents of tube turned milky after the addition of TCA

On the other hand, the tube containing enzyme was clear even after the addition of TCA solution as compare to the fist tube. This tube contained small amount of precipitates. Which suggest that only a little amount of casein is still present while the rest was hydrolyzed by the enzyme which is proteases (Figure 4).

Figure 4: The contents of tube containing enzyme were cleared after the addition of TCA solution.

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DISCUSSION

The soil was collected from slum area Mahmood Booti. The slum area was selected for soil sample collection based on literature review which showed that chances of protease producing microbe to present in bacteria are high as described in a study done by Patil et al., in which they collected 24 soil samples from different dairy industries of Aurangabad (India). In this study they the isolated bacteria from the soil we're able to produce protease enzyme which was confirmed after testing the protease activity [12]. Another study describes the production of protease enzymes from bacteria isolated from soil sample. The Bacillus species usually are found in soil and can survive in soil. In this study, twenty samples of soil we're collected from different industrial areas of Lahore. After growth of bacteria on medium it was observed that they had the ability to produce protease enzyme [15]. In a study done by Prita et al., she collected soil samples from milk processing plant and from drainage of slaughter house Nandeed, India. She selected 42 isolates. For enzyme production selected colony was grown in present study in LB broth while in study of Prita et al., she carried out production of protease in a medium that was containing glucose, peptone, salt solution, K2HP04 and FeS04 and keep them in shaking incubator at 37°C for 48 hours. While in this study the medium was kept in incubator at 37°C for 24 hours [17]. For protease assay Prita et al., used the same method as done in the present study i.e., using casein solution, carbonate buffer and trichloroacetic acid [17]. In a study done by Das et al, they collected their soil samples from lake of Basawa Nagar and from a community garbage in Vignan Nagar and from compost pit in Indra Nagar in Bangalore [14]. In a study done by students of Ahmadu Bello University, Madika et al., isolated fifteen samples from different locations of their campus. They used nutrient agar medium as done in present study, for the growth of microorganisms [18]. In a study done in Chennai by Pant et al., soil sample were collected from road side near Armats Biotek Institute. They made serial dilution up to five times while in present study serial dilution of stock solution was done up to ten times [19]. In study done by Marathe et al., Marathe and other, the sample was collected from sea and it was undiluted. While in present case the sample was soil which was collected from dump site. For determining the protease activity, Marathe et al., used Sigma's nonspecific protease assay. The casein solution was mixed with enzyme and after incubation at 37 °C for 10 minutes in water bath, 3 mL of 5 % TCA were added in both test tubes in blank as well as in enzyme containing test tube. The blank one got milky while the other one remained clear with the exception that there was very little amount of precipitation. This showed that the enzyme belonged to the protease family and that it can hydrolyze substrate that is casein [20].

CONCLUSIONS

Bacillus subtilis is commonly found in soil and show good growth in nutrient agar and in LB agar medium. The optimum conditions for its growth are 37°C and 24 hours of incubation. The morphology of its colony is such that it exhibits round or in some cases irregular shape. The colonies are opaque and are cream colored. For enzyme production, it can be growing in LB broth. The enzyme isolated from the broth shows proteolytic activity such that the enzyme containing test tube remained cleared even after the addition of TCA which shows they presence of protease enzyme that hydrolyzed the substrate casein. While the test tube containing water as blank showed precipitation of casein after the addition of TCA because in this enzyme was not present.

REFERENCES

[1] Yegin S and Dekker P. Progress in the field of aspartic proteinases in cheese manufacturing: structures, functions, catalytic mechanism, inhibition, and engineering. Dairy Science and Technology. 2013 Nov; 93(6):565-94. doi: 10.1007/s13594-013-01372.

[2] Szecsi PB. The aspartic proteases. Scandinavian Journal of Clinical and Laboratory Investigation. 1992 Jan; 52(sup210):5-22. doi: 10.1080/00365519209104650.

[3] Sandhya C, Sumantha A, Szakacs G, Pandey A. Comparative evaluation of neutral protease production by Aspergillus oryzae in submerged and solid-state fermentation. Process Biochemistry. 2005 Jul; 40(8):2689-94. doi: 10.1016/j.procbio.2004.12.001.

[4] Claverie-MartÌn F and Vega-Hernàndez MC. Aspartic proteases used in cheese making. Industrial enzymes. 2007:207-219. Springer, Dordrecht. doi: 10.1007/1-4020-5377-0_13

[5] Sumantha A, Larroche C, Pandey A. Microbiology and industrial biotechnology of food-grade proteases: a perspective. Food Technology and Biotechnology. 2006 Mar; 44(2):211.

[6] Dubal SA, Tilkari YP, Momin SA, Borkar IV. Biotechnological routes in flavour industries. Review Literature and Arts of the Americas. 2008 Mar; 14:15.

[7] Widsten P and Kandelbauer A. Laccase applications in the forest products industry: a review. Enzyme and microbial technology. 2008 Mar; 42(4):293-307. doi: 10.1016/j.enzmictec.2007.12.003.

[8] Dunn BM. Introduction to the aspartic proteinase family. Aspartic Acid Proteases as Therapeutic Targets. 2010 Aug:1-21. doi: 10.1002/9783527630943.ch1.

DOI: https://doi.org/10.54393/mjz.v3i1.46

[9] Yegin S, Fernandez-Lahore M, Salgado AJ, Guvenc U, Goksungur Y, Tari C. Aspartic proteinases from Mucor spp. in cheese manufacturing. Applied microbiology and biotechnology. 2011 Feb; 89(4):949-60. doi: 10.1007/s00253-010-3020-6.

[10] Crabbe MJ. Rennets: general and molecular aspects. Cheese: Chemistry, physics and microbiology. 2004 Jan; 1:19-45.

[11] Mamo J and Assefa F. The role of microbial aspartic protease enzyme in food and beverage industries. Journal of Food Quality. 2018 Jan; 2018:1-15. doi: 10.1155/2018/7957269.

[12] Patil RC and Bhaskar. Isolation and Characterization of Protease Producing Bacillus Species from Soil of Dairy Industry. International Journal of Current Microbiology and Applied Sciences. 2017 Jun; 6(6):853-860. doi: 10.20546/ijcmas.2017.606.100.

[13] Yegin S, Fernández-Lahore M. Production of extracellular aspartic protease in submerged fermentation with Mucor mucedo DSM 809. African Journal of Biotechnology. 2010 Oct; 9(38):6380-86.

[14] Das G and Parasad MP. Isolation, purification and mass production of protease enzyme from B.subtilus . International Research Journals of microbiology. 2010 Apr; 1(2):026-031.

[15] Malik H, Hadi S, Shahzad MI, Irfan M, Ali S, Muhammad A. Isolation and identification of protease enzyme producing Bacillus subtilis from soil. PSM Microbiology. 2017 Mar; 2(1):15-9.

[16] Razzaq A, Shamsi S, Ali A, Ali Q, Sajjad M, Malik A, et al. Microbial proteases applications. Frontiers in bioengineering and biotechnology. 2019 Jun; 7:110. doi: 10.3389/fbioe.2019.00110

[17] Prita B. Protease production Bacillus subtilis. The Pharma Innovation Journal. 2018 Apr; 7(5):622-626.

[18] Madika A, Ameh JB, Machido DA. Isolation and Screening of Bacillus subtilis from Soil for Amylase Production. UJMR 2017 Dec; 2(2):82-86.

[19] Pant G, Prakash A, Pavani JV, Bera S, Deviram GV, Kumar A, et al. Production, optimization and partial purification of protease from Bacillus subtilis. Journal of Taibah University for Science. 2015 Jan; 1(9):50-5. doi: 10.1016/j.jtusci.2014.04.010.

[11] Marathe SK, Vashistht MA, Prashanth A, Parveen N, Chakraborty S, Nair SS. Isolation, partial purification, biochemical characterization and detergent compatibility of alkaline protease produced by Bacillus subtilis, Alcaligenes faecalis and Pseudomonas aeruginosa obtained from sea water samples. Journal of Genetic Engineering and Biotechnology. 2018 Jun; 16(1):39-46. doi: 10.1016/j.jgeb.2017.10.001.