JPR INFO .com
Journal of Pharma Research

Journal of Pharma Research
Available online through
www.jprinfo.com

Research Article
ISSN: 2319-5622

ISOLATION OF AN ALKALOPHILIC PROTEASE PRODUCING *BACILLUS* SP. FROM SOIL AND ITS STAIN REMOVAL PROPERTIES

Neethu S. Kumar , Parvathy B.M and Neethu Simon
Post Graduate Department and Research Centre of Botany, Mahatma Gandhi College, Thiruvananthapuram, Kerala, INDIA.

Received on: 09-03-2016; Revised and Accepted on: 23-03-2016

ABSTRACT

Protease enzyme performs proteolysis, which is the catabolism of proteins by the hydrolysis of the peptide bonds. Alkaline proteases have various applications in industrial products and processes such as detergents, food, pharmaceuticals, leather etc. For the present study soil samples were collected from specific niches such as dumping areas of fish, meat, egg, pulses etc and screened for alkaline protease production by skimmed milk agar and gelatin agar plate assays. Out of the 10 bacterial strains screened, the one with maximum protease production has been selected and was coded as PB-3. This strain was identified as Bacillus, on the basis of morphological and biochemical characters using Bergey s Manual of Determinative Bacteriology. The potent strain was cultured for alkaline protease production by submerged fermentation. Alkaline protease activity was screened by protease assay and total protein assay by standard protocols. The partially purified enzyme yielded, 360mg/ml of protein and 155 U/ml of alkaline proteases respectively which was then encapsulated on sodium alginate gel. The encapsulated bacterial proteases (155U/ml) showed encouraging results on degradation of natural pigments and stains.

Keywords: *Isolation, Alkalophilic, Protease, Bacillus Sp., Soil, Stain Removal.*

INTRODUCTION

Proteolytic enzymes are ubiquitous in occurrence, being found in all living organisms, and are essential for cell growth and differentiation. Protease refers to a group of enzymes whose catalytic function is to hydrolyze proteins. They are also called Proteolytic enzymes or proteinases. Proteases, one among the three largest groups of industrial enzymes, account for about 60% of the total worldwide sale of enzymes and is widely used in several industries that include leather processing, meat processing, diary, preparation of organic fertilizer, silk industry and also for the recovery of silver from used X-ray films [1-3].

Alkaline proteases occur widely in plants, animals and microorganisms. The inability of the plant and animal proteases to meet current world demands has led to an increased interest in microbial proteases (Kumar *et al.*, 2008) [4] In addition, proteases from microbial sources are preferred to the enzymes from plant and animal sources, since they possess almost all characteristics desired for their biotechnological applications (Gouda *et al.*, 2006) [5]. Alkaline proteases are produced by a wide range of microorganisms including bacteria, moulds and yeasts. In bacteria, this enzyme is produced mainly by many member belonging to genus *Bacillus* especially, *B. licheniformis; B. horikoshii, B. sphaericus, Bacillus furmis, Bacillus alcalophilus, Bacillus subtilis* (Ellaiah *et al.*,2002) [6]. Currently, a large proportion of commercially available proteases are derived from *Bacillus* strains (Mehrato *et al.*1999) [7]. *Bacillus* proteases are predominantly extracellular and can be concentrated in the fermentation medium. Alkaline proteases are generally produced using submerged fermentation due to its apparent advantages in consistent enzyme production characteristics with defined medium and process conditions and advantages in downstream in spite of the cost-intensiveness for medium components (Prakasham *et al.*,2006) [8].

Since the industrial use of proteases, particularly the alkaline proteases are expected to grow tremendously in the coming decade; microbial proteases producing industries are always in search of new and cheaper methods to enhance the protease production as well as to decrease the market price of this enzyme (Mukherjee *et al.*, 2008) [9]. Each microbial strain is unique in their molecular, biochemical, metabolic and enzyme production properties. This warrants through characterization of isolated individual microbial species to evaluate its potential at commercial level.

***Corresponding author:**
Dr. Neethu S. Kumar
Post Graduate Department and Research Centre of Botany,
Mahatma Gandhi College, Thiruvananthapuram, Kerala, INDIA.
Mob. # : +91 9495133300, 0471 2327300.
**E-Mail: neethu777@gmail.com*

MATERIALS AND METHODS

Isolation and screening for potent isolates for alkaline protease production:

Soil samples were collected from specific niches such as dumping areas of fish, meat, egg, pulses etc. which were not contaminated by detergents and serially diluted as described by Sjodahl et al., (2002) [10]. An aliquot (100µl) of appropriate dilutions were spread plated onto sterilized skimmed milk agar plates at pH 9.0, incubated at 37^{0}C for three days and checked for any zone of clearance due to hydrolysis of skimmed milk.

Screening of the best isolate with reference to their protease activity were carried out with plate assay using gelatin clear zone method in protease specific medium and zones hydrolysis was measured in millimeters as mentioned by Abdel Galil in 1992 [11].

Identification of potent isolate by standard methods:

The isolate was characterized and identified in its finer details by means of morphological, microscopical, biochemical characterization according to Bergy's Manual of Determinative Bacteriology and Mac Conkey agar tests [12].

Screening for alkaline protease activity:

Protease activity and total proteins were estimated as per standard protocols. Protease activity was determined by modified Lowry method and total proteins by Lowry method using bovine serum albumin as standard [13, 14].

Submerged fermentation for alkaline protease production:

The best isolate which showed a marked clear zone was selected for further studies. It was inoculated into 100ml of the basal medium (nutrient agar containing 0.1% casein) at pH 9.0 and incubated at 37^{0}C. Samples were drawn periodically from 24- 144 hours at every 24 hours interval, centrifuged at 10,000 rpm at 4°C

and the crude enzyme supernatant was analysed for pH , total proteins and protease activity [15].

Immobilisation:

The enzyme was partially purified before encapsulation. The sample (10 ml of protease production media) was centrifuged and the supernatant was precipitated out by adding ammonium sulphate at 40% saturation (according to the chart of Gomori (1955) as mentioned by Dixon and Webb (1964) and refrigerated overnight [16, 17]. The enzyme pellet was mixed with 20ml sodium alginate gel and dropped onto cold calcium chloride solution for encapsulation.

Industrial application:

Stain removal:

A clean piece of cloth (2.5cm x 2.5cm) was soaked in blood (obtained from slaughter house) and in other natural stains such as juices of beet root, turmeric etc. The cloth was then dried and soaked in 2% formaldehyde and washed with water to remove the excess formaldehyde. The test sample cloth was incubated with the supernatant obtained by centrifuging the immobilized beads, at 37°C. After 24 hours of incubation, each piece of cloth was washed and dried. Controls were put up without enzyme [18].

RESULT AND DISCUSSION

Isolation of alkaline protease producing microorganism from soil sample:

Protease producing bacteria were obtained through 10^2 serial dilution on 1% skimmed milk agar plate. When screened on SMA with 10% concentration at pH 9, a clear zone of skimmed milk hydrolysis was obtained around the colony within 24 hours indicating alkaline protease production by the microorganism Plate-1. Insitu protease production was demonstrated by the clearing of opaque milk protein in the area surrounding the growing colonies [19]. Gupta *et al*.,in 2005 performed isolation of bacterial strains from environmental samples and screened their capability of protease production using skimmed milk agar [20].

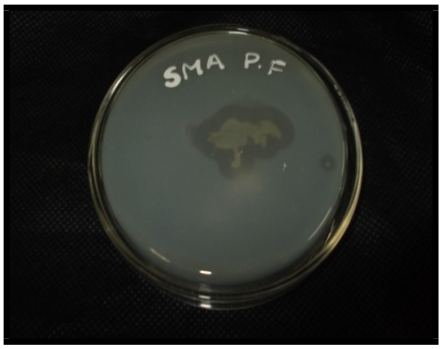

Fig. 1: Plate-1 Zone of hydrolysis of PB-3 on SMA

Screening of potent isolate for alkaline protease production:

The isolate was screened for the alkaline protease activity on protease specific medium containing gelatin. The growth and zone of hydrolysis were monitored over 24 to 48 hours. The isolate coded as PB-3 exhibited a clear zone of diameter 55mm at 48 hours and was selected for further studies. Plate- 2.

Fig. 2: Plate-2 Zone of hydrolysis of PB-3 on Gelatin Agar

Identification of potent isolate by standard methods:

The potent bacteria were identified by morphology, microscopy, Gram's staining, and biochemical characterization. Biochemical characterization was carried out as per Bergy's Manual of Determinative Bacteriology through biochemical analysis. The isolate was Mac Conkey agar negative. From the above standard methods the isolate was found to be a Gram positive, motile, rod shaped, bacterium which formed circular, smooth, slightly convex colonies. Plate-3, 4, 5 & Table 1.

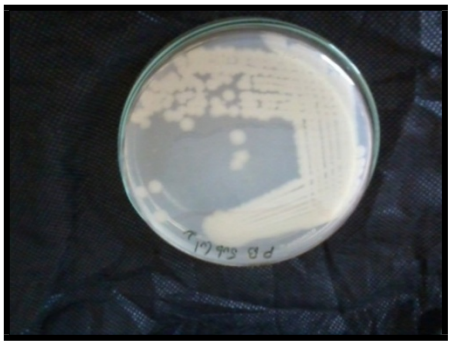

Fig. 3: Plate-3 PB-3 on solid media

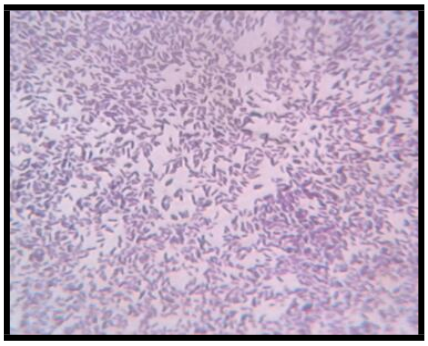

Fig. 4: Plate-4 Gram staining of PB-3

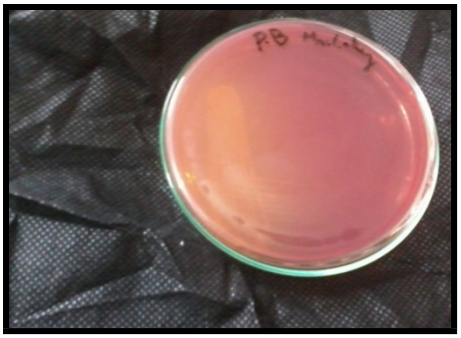

Fig. 5: Plate-5 PB-3 negative on Mac Conkey Agar test MacConkey Agar negative

Table No. 1: Biochemical Test Analysis

SL NO.	BIOCHEMICAL TEST	RESULTS
1	Indole test	Negative
2	MR	Negative
3	VP	Positive
4	Simmons citrate	Positive
5	Catalase	Negative
6	MacConkey agar test	Negative

Submerged fermentation for alkaline protease production:

The isolate grew well on the growth medium. pH of growth medium showed a marked influence. During growth there was an increase in pH to a maximum of 10.0 at 72 hours and then it decreased to 4.0 till 144 hours. Alkaline protease production for *Bacillus polymyxa* was maximum at pH 9.0 [21]. At alkaline conditions, pH 9.0 has been reported as optimum for production of alkaline protease by *Bacillus stearothermophilus* [22]. The protease produced from *Bacillus cereus* was found to be most active at pH10.5 and further increase in pH showed decrease in protease activity [23].

Immobilization:

Submerged fermentation was repeated in basal growth medium with the isolate at pH 9.0 and incubated at 37^{0}C for 72 hours. The medium was centrifuged at 10,000 rpm at 4^{0}C and the culture supernatant was collected. The crude culture supernatant was concentrated by ammonium sulphate precipitation at 40% saturation (according to the chart of Gomori (1955) as mentioned by Dixon and Webb (1964). Fraction obtained at 20% saturation yielded 190mg/ml of protein but showed no enzyme activity whereas fraction obtained at 40% saturation yielded 360mg/ml of protein and 155 U/ml of enzyme respectively. The enzyme pellet was mixed with 20ml sodium alginate gel and dropped on to cold calcium chloride solution to form immobilized enzyme beads. The beads showed good productivity upto a maximum of five cycles with washing and resuspension in sterile saline followed by medium after 72hours. There was a gradual loss of enzyme production as the repeated use of the beads resulted in the disintegration during the 10th batch. The beads were successfully run for 9 batches [24]. Plate-6.

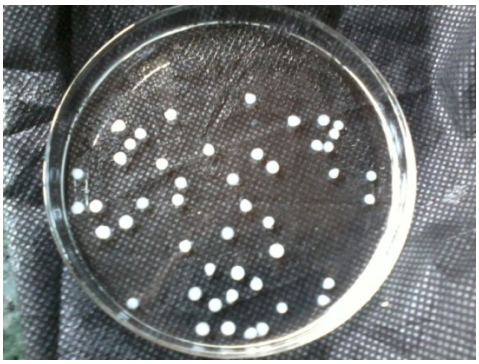

Fig. 6: Plate-6 Alkaline protease encapsulation

Application in industry- Stain removal:

The partially purified bacterial proteases showed encouraging results on degradation of natural pigments and stains. From 8 hours onwards there was haemolysis of blood and lysed upto 75% by 72 hours Plate-7. While natural pigments such as turmeric and beet root showed 25% decolouration at 24 hours and 60% after 48 hours Plate-8 & 9. Thus partially purified enzyme (155U/ml) was found to be effective in removing natural pigments and stains.

Fig. 7: Plate-7 Blood stain

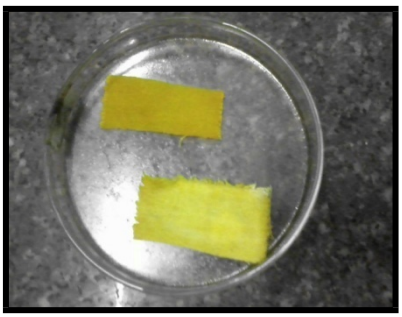

Fig. 8: Plate-8 Turmeric stain

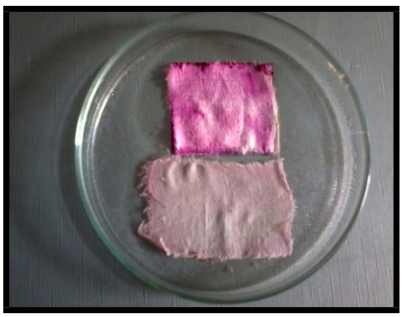

Fig. 9: Plate-9 Beetroot stain

CONCLUSION

The immobilized enzyme beads were used for degradation of natural pigments and stains. Turmeric, beetroot and blood stains were used and destaining was obtained from all the above stains. From 4 hours onwards there was haemolysis of blood and was lyzed up to 75 % by 72 hours. While natural pigments such as turmeric, beet root etc showed 25% decolourisation at 24 hours and after 48 hours showed more than 60% decolourisation. Thus the partially purified enzyme (155 U/l) was found to be effective in removing natural pigments and stains.

REFERENCES:

1. Mala BR, Aparna MT, Mohinis G, Vasanti VD. Molecular and Biotechnological aspect of microbial proteases, Microbiol. Mole. Biol. rev., Sept. **1998**; pp: 597-635.
2. Singh J, Vohra R. and Sahoo D. Alkaline protease from a new obligate alkalophilic isolate of *Bacillus sphaericus*, Biotech. Lett., **1999**; 21: 921-924.
3. Chellappan S, Jasmin C, Basheer SM, Elyas KK, Bhat SG. Chandrasekaran, M. Production, purification and partial characterization of a novel protease from marine *Engyodontium album* BTMFS10 under solid state fermentation, Process Biochemistry, **2006**; 41: 956-961.
4. Kumar PPK, Mathivanan V, Karunakaran M, Renganathan S, Sreenivasan RS. Studies on the effects of pH and incubation

period on protease production by *Bacillus* spp. Using groundnut cake and wheat bran, Indian Journal of Science Technology, **2008**; 1(4): 1-4.
5. Gouda MK. Optimization and purification of alkaline proteases produced by marine *Bacillus* sp. MIG newly isolated from eastern harbor of Alexandria, Polish Journal of Microbiology, **2006**; 55: 119-126.
6. Ellaiah P, Srinivasulu B, Adinarayana K. A review on microbial alkaline proteases, Journal of Science and Industrial Research, **2002**; 61: 690-704.
7. Mehrato, S, Pandey, P,K, Gaur, R, and Darmwa,l, N,S. The production of alkaline protease by a Bacillus species isolate, Bioresour. Technol., **1999**; 67: 201-203.
8. Prakasham, R.S., Subba Rao, C.H. and Sarma, P.N. Green gram husk-an in expensive substrate for alkaline protease production by *Bacillus* sp. In solid state fermentation, *Biores. Technol.,* **2006**; 97: 1449-1454.
9. Mukherjee, K., Adhikari, H. and Rai, S.K. production of alkaline protease by a thermophilic *Bacillus subtilis* under solid state fermentation condition using *Imperrata cylindrical* grass and potato peel as low cost medium: characterization and application of enzyme in detergent formulation, Biochem. Engg. j., **2008**; 39: 353-361.
10. Sjodahl J, Emmer A, Vincent J and Roeraade J. Characterization of proteinases from *Antarctic krill* (Euphausia superba), Protein Expression and Purification, **2002**; 26: 153-161.
11. Abdel Galil O A. Fermation of proteases by *Aspergillus fumigates* and *pencillium* sp., J. king. Saud. Univ., **1992**; 4(2): 127-136.
12. Buchanan RE and Gibbons NE: Bergey's Manual of Determinative Bacteriology, 8 th edition. Baltimore, The Williams and Wilkins Co. **1974**; 15-36.
13. Keay L, and Wildi BS. Proteinases of the genus *Bacillus*. I. Neutral proteinases, Biotechnol. Bioeng., **1970**; 12: 179-212.
14. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ. Protein measurement with Folin phenol reagent, J. Biol. Chem., **1951**; 193: 265-275.
15. Josephine SF, Ramya VS, Neelam D, Suresh BG, Siddalingeshwara KG, Venugopal N and Vishwanatha TJ. Isolation, production and characterization of protease from *Bacillus* Sp isolated from soil sample. Microbiol. Biotech. Res., **2012**; 2(1):163-168.
16. Gomori, G. In Methods in Enzymology. Ed. by Colowick, S. P. & Kaplan, N. 0. New York: Academic Press Inc. **1955**; 1: p. 138.
17. Dixon, M. and Webb, E. G. Enzyme, Academic Press. Inc. New York, 2nd Edn. **1964**.
18. Rai SK, Mukherjee AK. Statistical optimization of production, purification and industrial application of a laundry detergent and organic solvent-stable subtilisin-like serine protease (Alzwiprase) from *Bacillus subtilis* DM-04, Biochem. Eng. J., **2010**; 48: 173–180.
19. K. Krishnaveni, D. J. Mukeshkumar, M. D. Balakumaran, S. Ramesh and P. T. Kalaichelvan,Production and optimization of extracellular Alkaline Protease from *Bacillus subtilis* isolated from dairy effluent, Der Pharmacia Lettre, **2012**; 4(1): 98-109
20. Gupta A, Roy I, Patel RK, Singh SP, Kahre SK, Gupta MN. One-step purification and characterization of an alkaline protease from haloalkaliphilic *Bacillus* sp., J. Chroma A, **2005**; 1–2: 103–108. doi: 10.1016/j.chroma.2005.03.127.
21. Manjeet, K., Dhillon, S., Chandhary, K., Randhir, S. Production, purification and characterization of alkaline protease from *Bacillus polymyxa*, *Indian Journal of Microbiology,* **1998**; 38: 63-67.
22. Dhandapani, R. and R. Vijayaragavan. Production of thermophilic extracellular alkaline protease by *Bacillus stearothermophilus*. Ap-4, *World J. Microbiol. Biotechnol.,* **1994**; 10: 33-35.
23. Banik RM, Prakash M. Laundry detergent compatibility of the alkaline protease from *Bacillus cereus*, Microbiological Research, **2004**; 159: 135-140.
24. Adinarayana K, Jyothi B and Ellaiah P. Production of Alkaline Protease with Immobilized Cells of *Bacillus subtilis,* AAPS Pharm. Sci. Tech., **2005**; 6(3): Article 48.

How to cite this article:

Neethu S. Kumar et al.,: ISOLATION OF AN ALKALOPHILIC PROTEASE PRODUCING *BACILLUS* SP. FROM SOIL AND ITS STAIN REMOVAL PROPERTIES, J. Pharm. Res., 2016; 5(3): 30-33.

Conflict of interest: The authors have declared that no conflict of interest exists.

Source of support: Nil