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

Isolation, screening of alkaline protease producing Bacteria and physio chemical characterization of alkaline Protease

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Abstract

Proteases are a group of enzymes, whose catalytic function is to hydrolyze peptide bonds of proteins and break them down into polypeptides or free amino acids. Large number of bacteria, fungi, Protozoa, Yeast shows proteolytic activities. Soil and waste water sample collected from milk federation effluent area were screened for the presence of alkalophilic bacteria. Isolates were found having proteolytic activity on skimmed milk agar plates. Isolates forming larger zones, as a result of casein hydrolysis were further studied for production of extracellular alkaline protease activity in the shake flask studies. Time course studies indicated that isolate B-5 had the highest protease activity (70.87 µg/ml/min) after 144 h of incubation. The enzyme exhibits maximal activity at 37^0C, and pH 9 of isolate B-5. The enzyme was purified by ammonium sulphate, dialysis and ion-exchange chromatography using DEAE cellulose column. The isolate B-5 was identified on the basis of morphological, cultural and biochemical Characteristics and was found to be *Bacillus subtilis.*

Keywords: Alkaline Protease, alkalophilic*, Bacillus subtilis*

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Introduction

Proteases are one of the most important industrial enzymes accounting for nearly 60% of the total worldwide enzyme production (Anwar.2004, Beg *et.al*.,2003,) Proteases are a group of enzymes, whose catalytic function is to hydrolyze peptide bonds of proteins and break down into polypeptides or free amino acids.

They constitute 59% of the global market of industrial enzymes, which is expected to exceed 2.9 Billion $ by 2012 (Jon,2008). Investigation of proteases is a central issue in enzymology due to both their immense physiological importance and wide application in research and economical activities. Among the various proteases, bacterial proteases are the most significant, compared with animal and fungal proteases (Kotb *et al*., 2023) and among bacteria, *Bacillus* sp are specific producers of extra-cellular proteases (Masi, *et al.*, 2021). These enzymes have wide industrial application, including pharmaceutical industry, leatherindustry, manufacture of protein hydrolysates, food industry and waste processing industry (Pastor et al.,2001). The genus "*Bacillus"* is an important source of industrial alkaline proteases and are probably the only genera being commercialized for alkaline protease production (Ferrari et al., 1993).proteases that have pH optima in the range of 8.0-11.0 are grouped under the category of alkaline proteases. Some of the

important alkaline proteases are solanain, hurain and proteolytic enzymes of *Bacillus* and *Streptomyces* species (Hameed *et al.,* 1996; Lee *et al.,* 2002; Tang *et al*., 2004). The genus *Bacillus* contains a number of industrially important species and approximately half of the present commercial production of bulk enzymes derives from the strains of *Bacillus* sp .These strains are specific producers of extracellular proteases and can be cultivated under extreme temperature and pH conditions to give rise to products that are, in turn, stable in a wide range of harsh environments (Sudipta, 2010)..

The proteolytic system of *Bacillus* contributes to the Liberation of bioactive peptides which could play a significant role in the enzymatic process involved in fermentation. (AlEraky et.al.,2021, Olajuyigbe F M, 2005)

MATERIALS AND METHODS

Sample collection, Isolation and screening of alkaline protease producing bacteria:

Soil and waste water sample was collected from waste dumping area of milk federation, Jalgaon. One gram of soil sample was added to a glass tube containing 09 ml sterilized distilled water. The test tube containing the soil sample was placed in a water bath at 80°C for 15 min. After 15 min they were immediately cooled in ice-cold water. Samples of 100 µL were spread on the Skimmed milk agar medium, (Skimmed milk - 1%., Peptone - 0.1%. NaCl - 0.5%. , Agar - 2 %.) These plates were incubated at 30°C for 48 h. Colonies forming transparent zones, because of partial hydrolysis of milk casein, were selected for further studies. Purified cultures of selected isolates were streaked on NA agar slants and stored at 4°C.

Shake flask studies for screening of isolates

A loop full of the culture from purified cultures of selected isolate was inoculated in 100 mL of autoclaved Tryptone Dextrose Yeast Extract (TDYE) broth (Tryptone-1gm, Dextrose-0.1gm, Yeast Extract-0.5gm, Distilled water-100ml) in a 250 mL conical flask. The flasks were incubated at 30°C for 18 h and agitated at 250 rpm on a rotary shaker. Overnight grown bacterial starter cultures (1%, v/v) were transferred to 50 mL of TDYE medium in 250 mL flasks. The flasks were incubated at 30°C for 48 h and agitated at 180 rpm in a shaker.

After 48 h of incubation, 1 mL of broth culture was transferred to 1.5 mL sterilized tubes and centrifuged at 14,000 rpm for 5 min. Supernatant was used to determine proteolytic activity present in the culture supernatant.

Protease production in liquid culture medium:

Tryptone Dextrose Yeast Extract medium (Tryptone1%, 0.1% yeast extract 0.5%) was used for production of alkaline protease by the isolate B-5.Media was inoculated with 2 days old culture of isolate and incubated at 37^0C for 24 hours. After incubation, the culture medium was filtered and centrifuged at 12000 rpm for 15 min.Thus the cell free supernatant was used as crude enzyme and stored at 4^0C with few drops of toluene (Shumi etal., 2004)

Protease assay

Quantitative analysis of alkaline protease activity in supernatant was determined by modified method of Jayaraman (1981).In this method 0.2 ml of enzyme solution added in 1ml of 1% casein substrate (Himedia laboratories) solution prepared in phosphate buffer pH 9.0 and incubated at 37° C for 1 hr.1 ml of 12% Tri Chloro Acetic acid (TCA) solution was added , cooled the mixture rapidly in ice. Centrifuges at10000 rpm for 5 min and supernatant was collected.

Absorbance of the clear supernatant was measured at 600 nm. The blank was prepared by mixing the buffer instead of enzyme and performing the rest of the steps similar to the experimental samples. One

unit of protease activity was defined as "that quantity of enzyme, which librates amino acids and non precipitated peptides equivalent to 1 µg/mL of tyrosine per minute under assay conditions". (Jayaraman, 1981)

Identification of selected isolate

Morphological and biochemical characters of selected isolate B-5 were investigated according to the methods described in "Bergey's Manual of Systematic Bacteriology 1986".

Physio-chemical characterization of the B-5 crude extracellular protease

Time course studies

Seven flasks containing 100 mL of TDYE medium were inoculated with 1% overnight starter culture of B-5prepared in TDYE broth (5 mL containing 10^8 cells /ml). Quantitative analysis of protease activity present in the culture supernatant was determined till the 168th hr of fermentation at different time intervals.

Determination of optimum pH

The optimum pH for maximum alkaline protease activity was determined by performing protease activity assay in buffers with different pH (pH 5-12). For that purpose, 0.5% casein (substrate) used in the assay was dissolved in the selected suitable medium with selected pH (5,7,9,11,12) .

Determination of optimum temperature

Protease activity was determined by incubating reaction mixtures containing enzyme at different temperatures, that is, 20, 37, 60 °C. Substrate solution was pre warmed at the required temperatures before the addition of enzyme. After incubation, the enzyme activity was assayed.

Purification of alkaline protease

About 1 ml of the enzyme precipitate in 50% saturated from ammonium sulphate salted out was taken out in a dialysis bag (Himedia) for dialysis. The bag was then dipped in 100 ml of 0.025M phosphate buffer and was kept for 24 hrs. for stirring on a magnetic stirrer.Further purification of dialyzed protein was attempted at lab scale by column chromatography using Diethyl amino ethyl (DEAE) column.(Das and Prasad,2010)

RESULTS AND DISCUSSION

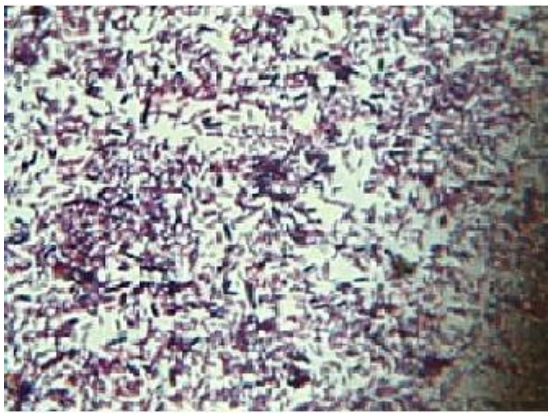


Fig -1: Gram stainig

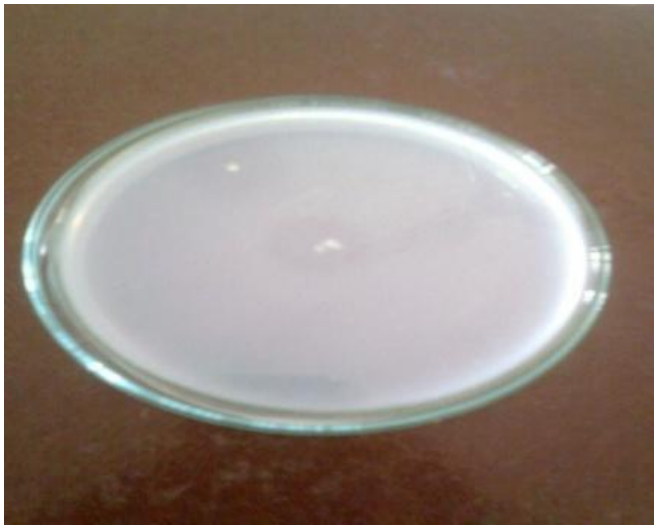


Fig -2: The zone of hydrolysis of *Bacillus subtilis on skimmed milk agar*

Fig 3 : Time course studies of the extracellular protease activity

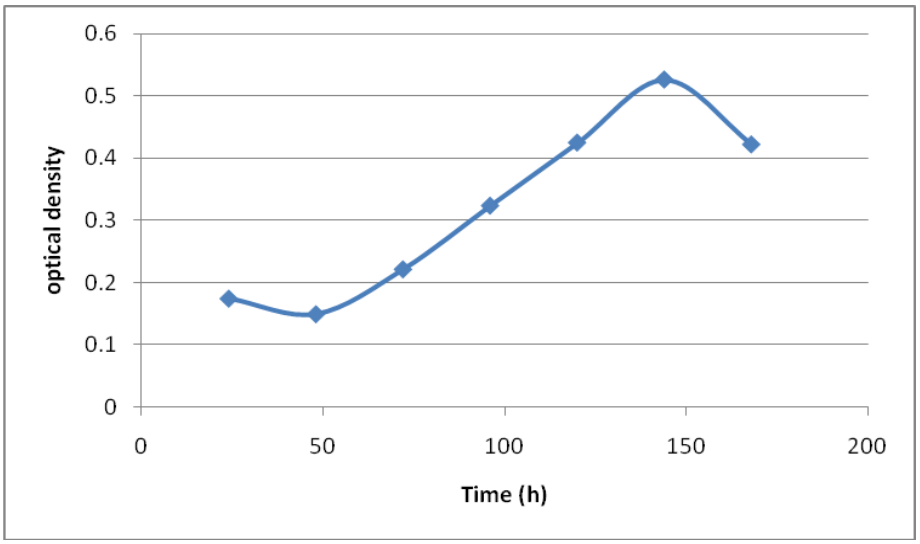
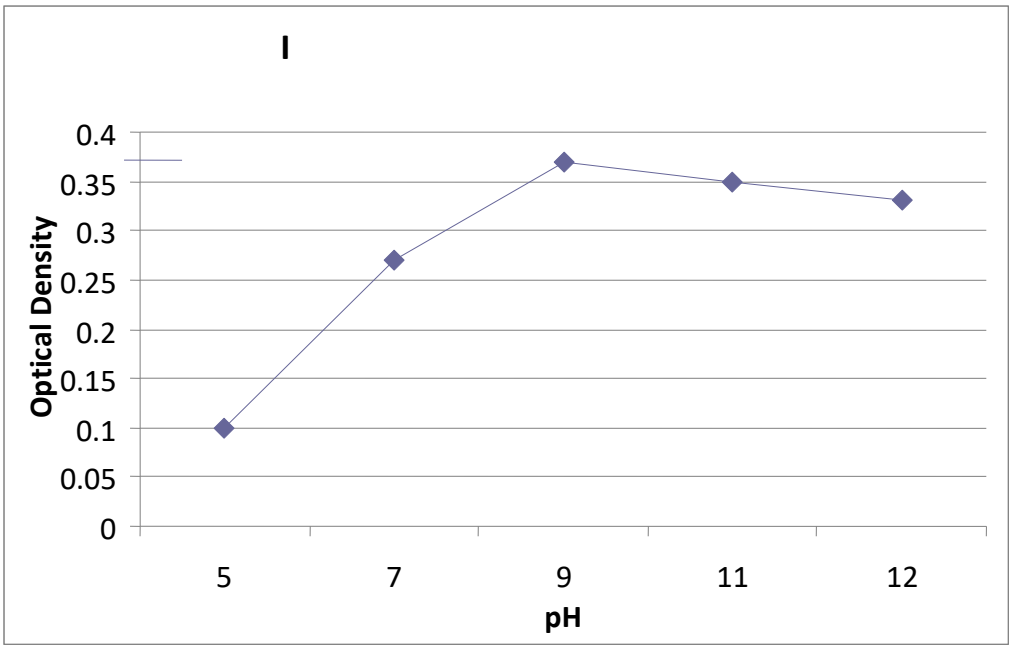


Fig 4 : Production of protease by *Bacillus subtilis* at different pH



The pH value of the soil and waste water in alkaline region from milk processing plant was determined. The highest pH of soil sample 11.0 followed by pH 10.0 of waste water sample was observed. As a result of initial screening of soil and waste water sample, 11 isolates were isolated. The isolates were then preserved and tested for their proteolytic ability using skimmed milk agar medium. In preliminary screening, the isolate B-5 showed better ability to hydrolyze casein as substrate and exhibited as diameter of clear zone in mm (9 mm) (fig 2). Isolate B-5 showing higher proteolytic activity were further screened for the production of alkaline protease in the shake flask containing 100 ml of TDYE medium. The isolate B-5 was Characterized on the basis of their morphological, cultural and biochemical properties. All these properties were then compared with the standard characteristics described in Bergey's manual of determinative bacteriology (8^{th} edition).The bacterial isolate B-5 was belong to the genus *Bacillus spp.*
Maximum variation was reported between the incubation periods for attaining optimal alkaline protease production by different *Bacillu*s spp.,with some *Bacillus* spp. reaching optimal production just after 6 to 8 h of fermentation and other's reaching after 18 to 96 h of incubation (Beg et al., 2002).In this study, maximum activity of 70.87 µg/ml/min was attained at 144 h of fermentation, after which the activity started to decline (Figure 3).Physio-chemical characterization of extracellular alkaline protease present in the culture supernatant of isolate *Bacillus spp.* showed that high pH value of 9.0 was required for its optimal activity (Figure 4), which is suitable characteristic for its industrial acceptability and a common feature of most of commercialized alkaline proteases from *Bacillus* spp. While conducting studies on optimal temperature requirement, maximum activity was observed at 37°C (Figure 5).Same results was obtained by Das and Prasad.,2010. The harvestedprotease was treated with 50 % ammonium sulphate for purification by salt precipitation method.
Then the culture was purified by dialysis.The purified enzyme was loaded onto holes punched on skim milk agar. After 24 hours of incubation it was observed that clear zones were formed successfully. The bacterial isolate named **B5** is almost similar with respect to the control (*Bacillus subtilis*), morphologically

and bio-chemically.So we may conclude that the potential isolate having ability to produce alkaline protease named **B5** may be the *Bacillus subtilis.* Thus at last it can be said that this enzyme has wideapplication in various industrial and medical fields and itcan be produced in large scale from the microorganism *Bacillus subtilis* isolated from soil sample by applying the fermentation technology.

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