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Study of Protease Producing Bacteria and their Enzymatic Activity at Different Parameters

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Abstract - *Bacterial alkaline proteases are among the important hydrolytic enzyme and have been used extensively since the advent of enzymology. Bacterial extracellular alkaline proteases are of great importance due to its wide spectrum applications in detergent industries, bioremediation, food industries, and leather processing and bio-film degradradation. The Optimization of the time of incubation, the effect of temperature, the effect of pH, the effect of different carbon source and effect of different nitrogen source and they were found to be 120hrs, 12, 170Unit/ml for galactose, and 70 Unit/ml for ammonium sulfate respectively for the production of protease and also optimize the parameters on enzyme activity they are temperature, pH, 1% casein substrate, and the different volume of the crude enzymes and the results was 370C,11, 3.5ml, 60 Unit/ml for 2 ml of crude enzymes respectively.*

Keywords: *Alkaline Protease, hydrolytic enzyme, optimization, galactose, and ammonium sulfate*

INTRODUCTION

In recent years, there is renewed interest in the study of proteolytic enzymes, mainly due to the recognition that these enzymes not only play an important role in the cellular metabolic processes but also one of the important enzymes in the industrial community. [1]. Protease is an enzyme produced by microorganism, plant and animal tissues and catalyzes the hydrolysis of peptide bonds in proteins. A protease is an important tool in studying the structure of protein and peptides. Microbial proteases are derived from a wide variety of yeasts, molds, and bacteria like Bacillus subtilis, Aspergillus oryzae Streptomyces cellulase, and Aeromonas hydrophila species.

Most of the commercially available proteases are currently obtained from Bacillus strains. Although the use of fungal proteases is being increasingly realized. Proteases represent one of the three largest groups of industrial enzymes [2]. The extracellular proteases are of commercial value and fined multiple applications in various industrial sectors [1].

Protease is not a single enzyme but consist of proteinases, peptidase, and amidases [3]. A protease is an enzyme that conducts proteolysis through the hydrolysis of the peptides bonds between amino acids in a polypeptide chain. On the basis of their acid-base behavior, proteases have been classified into three categories i.e., acid, neutral and alkaline proteases. Bacterial alkaline protease is characterized by their high activity at alkaline pH. The microorganisms are considered potentially to be the most suitable sources of alkaline protease for industrial application. Alkaline protease is among the important class has been since the advent of enzymology. This enzyme is produced by the bacterium in two forms: intracellular and extracellular. Be purified easily protein degradation various industrial processes.

Most commercial proteases, mainly neutral and alkaline, belonging to the genus Bacillus. Bacillus spp. industrial tools for a variety of reasons, including their high growth rates leading to short fermentation cycle times, their capacity to secrete proteins into media and the as safe (GRAS) the food species such as Bacillus subtilis and Bacillus licheniformis. One of the most important features of many alkalophiles is their ability to adjust their environment. They can convert any neutral or high alkaline medium for their growth[4]. Alkaliphiles are reported to be rich sources of alkaline active enzymes e.g. proteases, amylases, cellulases, and xylanases, etc. Enzymes derived from microorganisms which can survive under extreme pH could be particularly used in commercial applications under high alkaline reaction conditions, e.g. in the production of detergents. Alkaline proteases produced by Bacillus species are of great importance in detergent industries due to their high thermal and pH stability.

Proteases from microbial sources possess almost all the characteristics desired for their biotechnological applications [5]. Production of these enzymes using low-cost substrates would reduce the cost of production. Oilseed cakes, which are a byproduct of the oil extraction

industry, are a potentially useful, low-cost substrate for the production of different enzymes [6, 7].

Recent awareness of the environmental pollution caused based industries has necessitated the development of enzyme-based processes as the currently employed chemical processes. Protease finds widespread applications in food processing, pharmaceuticals, leather processing, bio-film degradation, silver film, and various industrial sectors. The potential application of protease is that it can be partially or totally replaces currently employed toxic chemical processes. It plays a major role in Bioremediation. Bio detergents is also known as green chemicals, account for about 30% of the total worldwide enzyme production. Used in detergents to remove protein-based stains by hydrolyzing them into small peptides which are readily dispersed in the washing liquor.

Application of the protease enzyme-

Proteases are used in various industries such as detergent industry, textile industry, pharmaceutical industry, and food industry.

1. In detergent Industry, it can be used in an additive because the presence of typical detergent ingredients such as surfactants and Laundry detergent they help removing protein-based stains from clothing [8].
2. In the pharmaceutical industry, it can be used as contact lens cleaners and enzymatic deriders and also used as a digestive aid [9].
3. In the food Industry, it can be used in canned meats and soups and also making cheese [10].

MATERIALS AND METHODS

Collection of sample: -

A soil sample was collected from the fish market at Ahmednagar, Maharashtra.

Enrichment:-

Enrichment was performed using 250ml Erlenmeyer flask containing 100 ml of nutrient casein broth medium. The medium was sterilized by autoclaving. 1gm of soil sample was inoculated in nutrient casein medium and incubated for 7 days at 37 0C in shaking incubator.

Screening and isolation for protease producing bacteria:-

After incubation spot inoculation was done onto an alkaline skim milk agar plate. Then these plates were incubated at 370C for 48hrs. To visualize the clear zone of proteolytic activity. To indicate the protease activity of the organisms, diameters of the clear zone around colonies were measured. The culture with a large zone of clearance was selected for further studies.

Characterization of isolate:-

Isolated bacterial culture was characterized by morphologically and biochemically as per Bergey's Manual of Determinative Bacteriology 9th edition. Gram's staining and different biochemical tests were performed, which include catalase and oxidase. The fresh culture was used for all the tests.

Development of Inoculums and production medium -:

100 ml nutrient casein broth in 250ml Erlenmeyer flasks was prepared and inoculated with a single colony of protease producing bacteria then incubate the flask for 24 hrs. at 37ºC 100ml production medium broth in 250ml Erlenmeyer flask containing casein was inoculated with 10ml of inoculums and incubated at 37ºC for 5days in shaking incubator at 150 rpm.

Preparation of crude enzyme solution -:

The organism was grown in a liquid nutrient casein medium. After 3 days of incubation with continuous shaking, cell-free filtrates were prepared by centrifugation at 10,000 rpm for 10 min. The supernatant was collected and used as stock enzyme solution. This supernatant was used for the analysis of enzyme activities.

Preparation of substrates:-

1% casein was used as substrate. The substrate was prepared by adding1%casein into 100mL of 0.1M sodium phosphate buffer (pH 8).

Enzyme assay:-

The amino acid released from casein per ml per min. at 750nm was determined by Folin-Lowry method. Total protease activity was determined by measuring the amount of amino acid formed from casein by incubating 0.1mL of Crude enzyme with 0.9mL of 1% casein in0.1M sodium phosphate buffer (pH 8) and incubated at room temperature for 10 min. After incubation, the reaction was stopped by the addition of 1ml of 10% TCA reagent. Amino acid liberated was estimated by measuring absorbance spectrophotometrically at 750 nm. Protease production was estimated by using tyrosine as standard. One unit (U) of enzyme activity is expressed as the quantity of enzyme, which is required to release 1µmol of tyrosine per minute under standard assay conditions. One unit of enzymatic activity is defined as the amount of enzyme that releases 1

μmol amino acid (measured as tyrosine) per mL per minute.

Optimization of the different parameters on protease production

Five main parameters were selected for the optimization of Protease production: time of incubation, the effect of temperature, the effect of pH, the effect of different carbon source and effect of different nitrogen source. Each variable was optimized by varying only a single parameter at a time, that is, time of incubation alone (24hrs, 48hrs, 72hrs, 96hrs, 96hrs,120hrs and 144hrs), temperature (300C, 370C, 520C, 600C), pH (5.0, 7.0, 9.0, 11.0, 12.0 and 14.0), 1% of carbon source (glucose, sucrose, fructose, galactose) and 1% Nitrogen source (Ammonium sulphate, Ammonium nitrate, Ammonium chloride, and tryptone) The absorbance of resultant samples was measured within the range of 750nm.

Effect of different parameters on enzyme activity

Effect of temperature

The bacteria were inoculated into the 250 ml Erlenmeyer flasks containing the 100 ml nutrient casein broth of pH 12 and incubated at 370C for 120 hrs. Then broth was centrifuged at 8000 rpm for 15 min. and the supernatant was collected. The supernatant was used as a crude enzyme. Then 0.1 ml of the enzyme was taken in a test tube and 0.9 ml of 1% substrate was added. The effect of temperature on enzyme activity was determined by incubating the reaction mixture for 10 minutes at different temperature 300C, 370C, 520C, and 600C. Then the enzyme assay was performed and readings were recorded at 750 nm.

Effect of pH

The bacteria were inoculated into the 250 ml of different Erlenmeyer flasks containing the 100 ml nutrient casein broth incubated at 370C for 120 hrs.at pH 12 .. Then broth was centrifuged at 8000 rpm for 15 min. and the supernatant was collected. The supernatant was used as a crude enzyme. Then 0.1 ml of the enzyme was taken in a test tube and 0.9 ml of 1% substrate was added. The supernatant used as a crude enzyme. The casein was dissolved into the phosphate buffer having different pH5, 7, 9, 11, 12, and 14 used as a substrate. Then take 0.1 ml of enzyme and 0.9 ml of 1% substrate. The reaction mixture was incubated for 10 minutes. The enzyme assay was performed and readings were recorded at 750 nm.

Effect of different volume

The bacteria were inoculated into the 250 ml Erlenmeyer flasks containing the 100 ml nutrient casein broth pH 12 and incubated at 370C for 120 hrs. Then broth was centrifuged at 8000 rpm for 15 min. and the supernatant was collected. The supernatant was used as a crude enzyme. Then 0.1 ml of the enzyme was taken in a test tube and 0.9 ml of 1% substrate was added. The supernatant used as a crude enzyme. The effect of different volume of a crude enzyme on alkaline protease activity was determined by incubating the reaction mixture for 10 minutes with a various volume of crude enzyme i. e. 0.5ml, 1ml, 1.5ml, 2ml, 2.5ml 3ml, and 3.5ml. The activities of protease were measured at 750 nm.

Effect of 1% casein substrate on alkaline protease activity-:

The bacteria were inoculated into the 250 ml Erlenmeyer flasks containing the 100 ml nutrient casein broth pH 12 and incubated at 370C for 120 hrs. Then broth was centrifuged at 8000 rpm for 15 min. and the supernatant was collected. The supernatant was used as a crude enzyme. Then 0.1 ml of the enzyme was taken in a test tube and 0.9 ml of 1% substrate was added. Alkaline protease activity was determined by incubating the reaction mixture for 10 minutes with the various volume of 1% casein as a substrate i.e. 0.5ml, 1ml, 1.5ml, 2ml, 2.5ml 3ml, and 3.5ml. Then the enzyme assay was performed. The activity of protease was measured at 750 nm.

RESULTS

Isolation and screening of protease producing bacteria -:

The screening was carried out by using the skimmed milk agar. After incubation at 370C for 72hrs.The zone of proteolytic activity was seen on a skimmed milk agar plate. Three isolates showed a clear zone of proteolytic activity. Isolate which shows 3.5 cm i.e. the highest zone of proteolytic activity was selected for further studies.

Figure 01- Skim milk agar plate showing zone of proteolytic activity

Morphological and biochemical characteristics of isolates-:

Morphological characters of isolate-:

After incubation at 370C for 48 hrs.on nutrient casein agar plate the following colony characters were observed.

Table no-1 characterization of an isolate

Characterization of isolate		
Sr.No.	**Characters**	**Observation**
1	Size	1 mm
2	Shape	Circular
3	Color	White
4	Margin	Entire
5	Consistency	Sticky
6	Elevation	Convex
7	Opacity	Translucent
8	Gram character	Gram positive short rod
9	Motility	Motile

Biochemical Characters of isolates-:

The isolates were catalase and oxidase positive

Table No: 02 Biochemical characteristics of isolates

Sr.No.	Test	Result
1	Oxidase	+
2	Catalase	+

Optimization of the different parameters on protease production

Figure 02 shows that the effect of incubation time on protease production by the isolate was studied. It was found that the concentration of protease production increases with an increase in incubation time. The maximum protease production was obtained after 120 hrs. which was 210 Unit/ml. When incubation time was further increased, the concentration of protease was reduced. The effect of temperature on protease production by the isolate was studied. It was found that the concentration of protease increase with the increase in temperature. The maximum protease production was found at 370C which was 150Unit/ml, but when the temperature was further increased, the concentration of protease was reduced. This indicates that the optimum temperature for protease production by isolate was 370C (Figure03). The effect of pH(from 5 to 14) on protease production by the isolate was studied. It was found that the concentration of protease production increases with increase in pH. Maximum protease production observed at pH 12 after 120 hrs which was 270Unit/ml. But when the pH was further increased the concentration of protease was reduced. This indicates that the optimum pH for protease production by the isolate was 12. The influence of pH on enzyme production was found to be an important parameter (Figure 04), the effect of different carbon sources on protease production was studied. The maximum protease production was obtained when galactose used as carbon source which was 170Unit/ml followed by sucrose was 40Unit/ml. The lowest protease production by isolate was obtained when glucose used as a carbon source (Figure 05) and figure 06 shows the effect of different Nitrogen sources on protease production was studied. The maximum protease production was obtained when ammonium sulfate used as a nitrogen source which was 70Unit/ml followed by ammonium chloride was 50Unit/ml. The lowest protease production by isolate was obtained when ammonium nitrate used as a nitrogen source.

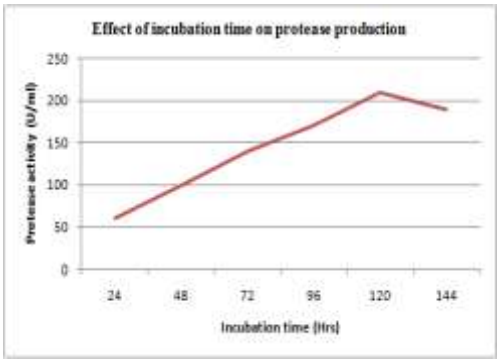

Figure no: 02 Effect of incubation time on protease production

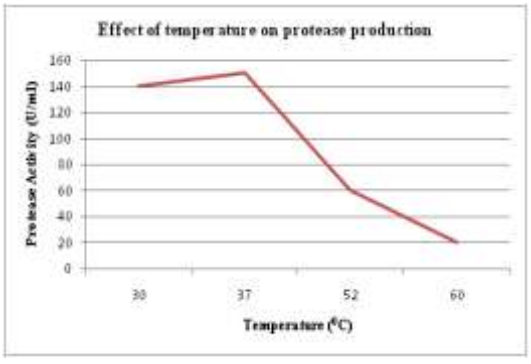

Figure no: 03 Effect of temperature on protease production

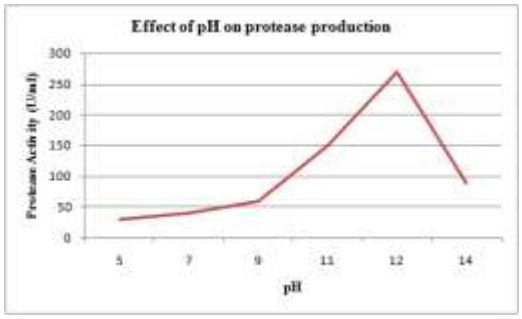

Figure no: 04 Effect of pH on protease production

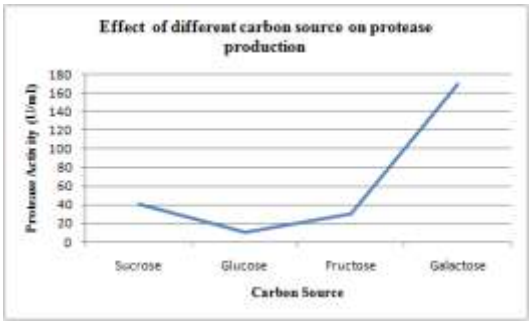

Figure no: 05 Effect of carbon source on protease production

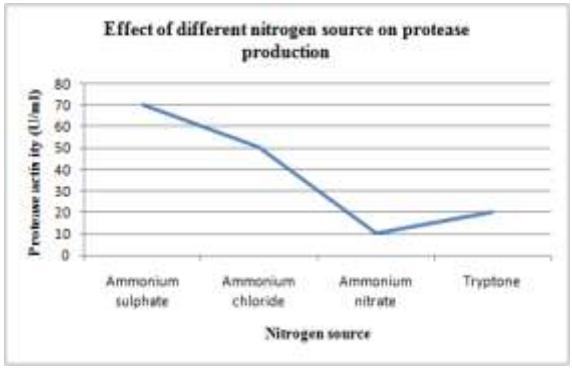

Figure no: 06 Effect of nitrogen source on protease production

Effect of the different parameter on enzyme activity

Effect of temperature on enzyme activity

The effect of temperature on enzyme activity was studied. The maximum enzyme activity was found at 370C which was 70Unit/ml, but when the temperature was further increased, enzyme activity was reduced. This indicates that the optimum temperature for enzyme activity by isolate was 370C.

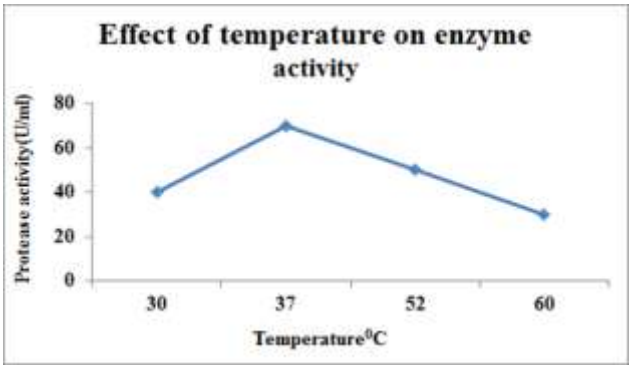

Figure no: 07 Effect of temperature on enzyme activity

Effect of pH on enzyme activity

The effect of pH(5 to 14) on enzyme activity was studied. It was found that the concentration of protease production increases with increase in pH. Maximum protease production observed at pH 11 which was 90Unit/ml. But when the pH was further increased enzyme activity was reduced. This indicates that the optimum pH for enzyme activity was pH 11.

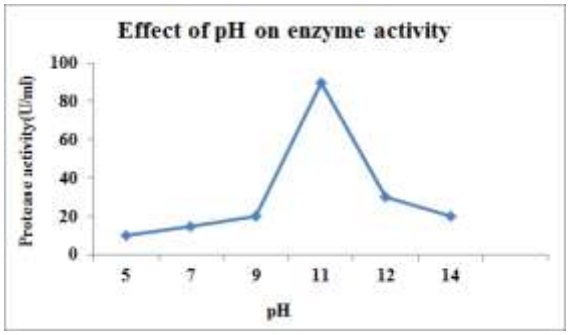

Figure no: 08 Effect of pH on enzyme activity

Effect of 1% casein substrate on enzyme activity

The effect of substrate concentration on enzyme activity was studied. It was found that the concentration of protease production increases with an increase in substrate concentration. Maximum protease production observed when substrate concentration was 3.5 ml which was 140Unit/ml. This indicates that the maximum substrate concentration is 3.5 ml.

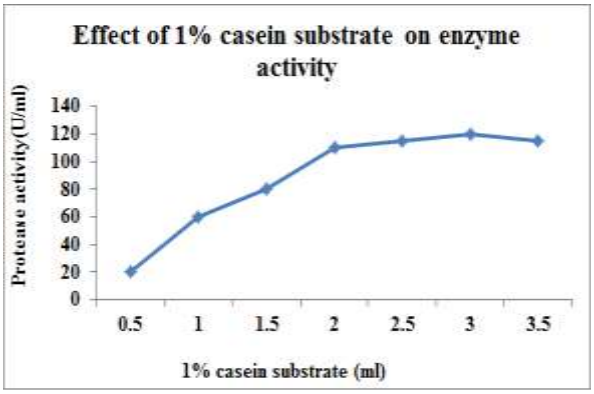

Figure no: 09 Effect of 1% casein substrate on enzyme activity

Effect of different volume of the crude enzyme on enzyme activity

The effect of different volume of the crude enzyme on enzyme activity was studied. It was found that the concentration of protease production increases with an increase in enzyme concentration. Maximum protease production observed when enzyme concentration was 2 ml which was 60 Unit/ml. But when the volume of the crude enzyme was further increased enzyme activity was reduced. This indicates that the maximum enzyme activity observed when enzyme concentration is 2 ml.

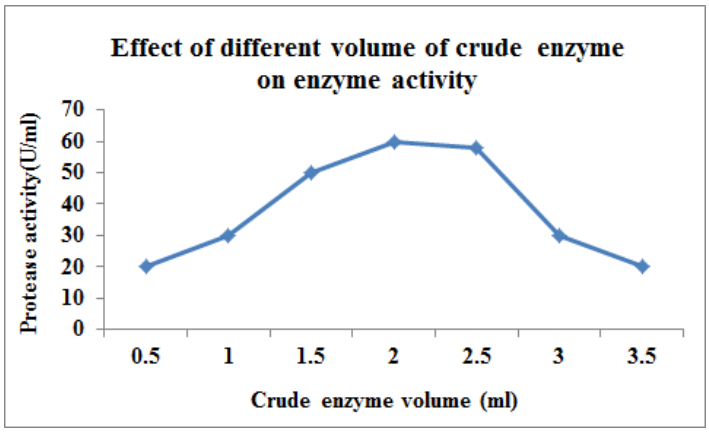

Figure no: 10 Effect of different volume of the crude enzyme on enzyme activity

CONCLUSION

Protease producing bacteria was isolated from a soil sample, collected from the fish market at Mukundnagar. The morphological and biochemical characterization indicated that isolate was Gram-positive, rod-shaped aerobic, non-endospore forming bacteria. The optimum protease production was observed after 120 hrs. pH 12, the temperature of 37ºC, and maximum production observed with galactose as a carbon source and ammonium sulfate as a nitrogen source. The optimum enzyme activity was observed at pH 7, temperature 370C and maximum activity observed when3ml of 1% casein substrate was used and 2 ml of crude enzyme. The present research indicated that isolate effectively produced protease and may be utilized for the biotechnological industry.

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