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RESEARCH ARTICLE

AN APPROACH ON ISOLATION, SCREENING AND PRODUCTION OF PROTEASE FROM *ASPERGILLUS ORYZAE*

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ABSTRACT

This study investigates the production of extracellular protease synthesis were carried out by using *Aspergillus oryzae* was evaluated under different fermentation parameters by employing submerged fermentation method. The protease producers detected by the clear zone (casein hydrolysis) around the colony by simple plate assay method. *Aspergillus oryzae*KS 5 is the potential strain among the fungal isolates. The Protease synthesis was increased their yield after the optimization of fermentation parameters. The optimum pH 6.0, temperature 35^0C and inoculum size 1.0 ml and it showed 1.7 IU.

Key words: Protease, Plate assay, Optimization and *Aspergillus oryzae*

INTRODUCTION

Protease constitutes one of the most important groups of industrial enzymes, accounting for about 60% of the total enzyme market [1]. Proteases are of commercial value and find multiple applications in various industrial sectors. Proteases are widely used in detergent, food and leather tanning industries [2-4]. Several alkaline proteases were reported for hydrolysis of fibrous proteins of horn, feather and hair and their application for various value added byproducts [5,6]. Other potential industrial applications of alkaline protease include the utilization in peptide synthesis, in the resolution of the racemic mixture of amino acids, in the hydrolysis of gelatin layers of X-ray films and in the recovery of silver [7-8].

Proteases are essential constituents of all forms of life on earth including prokaryotes, fungi, plants and animals. Proteases are highly exploited enzymes in food, leather, detergent, pharmaceutical, diagnostics, waste management, and silver recovery. The protease enzyme constitutes two thirds of total enzymes used in various industries and this dominance in the industrial market is expected to increases.

A large number of microbes belonging to bacteria, fungi, yeast and actinomycetes are known to produce alkaline proteases of the serine type [3].Proteolytic enzymes play an important part in the metabolism of almost all organisms (plants, animals, fungi, bacteria, and viruses). Investigation of proteases is a central issue in enzymology due to both their immense physiological importance and wide application in research and economical activities.

The aim of the present study is to isolate the *Aspergillus oryzae* strains from different soil samples, screen the isolates for protease production by plate assay and fermentation kinetics for the biosynthesis of protease from the potential *Aspergillus oryzae*.

MATERIALS AND METHODS

Isolation of Fungal strains

The *Aspergillus oryzae* strains were isolated from different soils samples. Totally thirty strains of *Aspergillus oryzae*. The Soils are taken from different regions from Tirupattur, Tamil nadu. Tentatively identified in the laboratory and further the strains were identified at Agarkar research Institute (ARI), Pune.

Screening of protease producers by plate assay

Aspergillus oryzae strains were screened for their protease production by plate assay and among the thirty isolates, *Aspergillus oryzae* were used for further studies.The screening medium is as follows. Glucose,2; skim milk,1; KH2PO4,1.52; KCL,0.52; MgSO4.7H2O,0.52; CuNO3.3H20, trace; ZnSo4.7H20, trace FeSO4, trace; agar, 20.0 and pH-5.0 (g/L distilled water) (Plate-1).

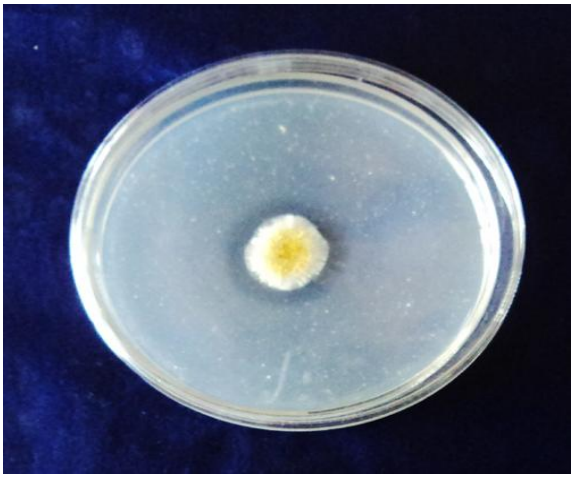

Plate-1: Screening of *Aspergillus oryzae* stains for protease by plate assay

Fermentation Medium

The selected *Aspergillus oryzae* KS5were cultured on production medium. The production medium consists,

- Peptone - 0.1 g
- Trisodium Citrate - 1.0 g
- Di potassium hydrogen PO4 - 0.2 g -
- NH4NO3 . 1.0 g
- KCl - 0.03
- Distilled Water - 100 ml
- pH - 5.0

Optimization Studies for biosynthesis of protease:

The 250 ml Erlenmeyer flasks containing 100 ml of production medium were prepared by mixed with acid/alkali solution to obtain required pH. The pH was adjusted in the range of 3-7 with increments of 1.0. Thus prepared flasks were cotton plugged and autoclaved at 121 ^{0}C for 15 min. The flasks were inoculated and incubated. The 100ml of the production medium was separately taken in 250 ml Erlenmeyer flasks and prepared for submerged fermentation. Thus prepared flasks were incubated at different temperatures like 25-40 ^{0}C with in increments of 5 ^{0}C. The inoculum was prepared separately by reviving the 168h old culture of *Aspergillus oryzae* KS 5at different levels i.e., 0.25, 0.50, 0.75, 1.0 and 1.25 ml and then fermentation studies were carried out.

Extraction of protease from production medium:

The samples were withdrawn periodically at 24 hrs in aseptic condition. The extract was filtered through Whatman filter No.1. The clear extract was centrifuged at 2000-3000 rpm for 15 min, supernatant were used as enzyme preparation. Thus prepared crude enzyme was used for assay of protease.

Assay of Protease

The protease activity was determined by the method proposed by Keay et al.,[9]. 0.5 ml of suitably diluted enzyme was added to 1.0 ml of 1% casein and 0.5 ml of glycine-NaOH buffer (25 mM, pH 10.0) whole mixture was incubated at 75^{0}C for 10 min. The reaction was terminated by the addition of 3 ml of 10% TCA solution. The solution was allowed to stand for 10 min in cool and was filtered. To the clear filtrate, 5 ml 0.4 M Na_2CO_3 and 0.5 ml of FolinCiocalteau reagent (FCR) was added, mixed thoroughly and incubated at 37^{0}C for 30 min, in dark. The absorbance was measured at 660 nm.

International units (IU)

One protease unit was defined as the amount of enzyme that released 1 µg of tyrosine per ml per minute under the above assay conditions.

RESULTS AND DISCUSSION:

Fungal isolates were identified as *Aspergillus oryzae* in Agrakar Research Institute, Pune. All thirty strains of *Aspergillus oryzae* produced clear zone around colony in casein plate medium; those were selected from the soil sample. Of the thirty isolates *Aspergillus oryzae* KS5was considered to be the best and high protease producing strain. It showed 1.15cm of cleared zone around the colony. The data obtained in the present study on the effect of pH and temperature on submerged fermentation is shown in (Fig. 1 and 2) which reveals that the production of protease increased with the increase in the pH of the medium up to pH 6.0 temperatures 35 ^{0}C and thereafter the decrease of protease was observed.

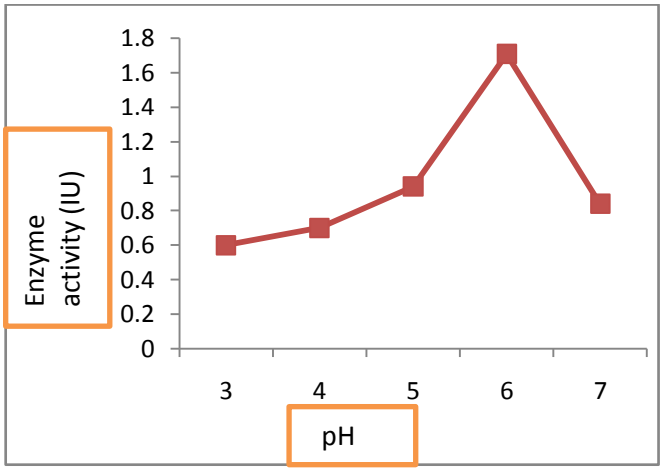

Fig-1: Effect of pH on protease production

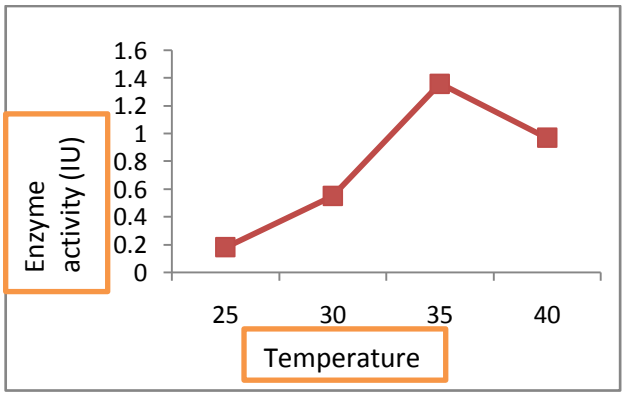

Fig-2: Effect of temperature on protease production

The maximum production of protease is 1.17 IU was obtained at pH 6.0 and the minimum production of protease 0.312 IU was observed at pH 7.0. The production of protease increased significantly with the increase in fermentation temperature from 25-35 ^{0}C and decreased above 35 ^{0}C. The maximum protease production obtained at 35 ^{0}C was 1.36 IU and the least production was observed at 25 ^{0}C resulted only 0.18 IU of protease at 72 hrs of fermentation period. Any temperature beyond the optimum range is found to have some adverse effect on the metabolic activities of the

microorganisms and it is also reported by various scientists that the metabolic activities of the microbes become slow at lower or highertemperature[10].

The pH of the medium is one of the most critical environmental parameter affecting the mycelial growth, enzyme production and the transport of various components across the cell membrane[11].

In our study, the data revealed that the pH of 6.0 was found as suitable for maximum production of protease with *Aspergillus oryzae* KS2strain under submerged fermentation. Fungal strains are noted for their best performance in the range of 3.5-7.0 and also low pH avoids the contamination by other microbes[12]. Francis et al.,[13] reported that the best protease production was observed when the initial pH was 6.0. Vishwantha and Appu [14],who reported pH 5.0 as the best initial pH for the production of protease from *Aspergillus oryzae* MTCC 5341.Our findings are in close agreement with the earlier findings of Francis et al., 2011, they showed that pH 6 was the suitable for maximum protease production by using *Aspergillus carbonarius* and it shows 0.14 IU.

Incubation temperature is an important environmental factor for the production of proteases by microorganisms because it affects growth rates of microorganisms, regulates the synthesis of the enzyme and also the enzyme production by changing the properties of the cell wall[15]. Keeping this in view, experiments were conducted to understand the effect of temperature on protease production by *Aspergillusoryzae*KS5. The present study revealed that the 35 ^{0}C is suitable and maximum production of protease with *Aspergillusoryzae*KS2. The results reveal that the maximum production of protease was observed at temperature 35^{0}C by using *Aspergillus oryzae* KS 5and it showedapproximately1.36 IU/ ml, similar observations were reported by Francois et al [13] reported that the maximum production of protease was observed at temperature 37 ^{0}C by using *Aspergillus terreus* and it showed approximately7 IU/ ml,. As such our findings are close agreement with Francois et al [13].

Importance of inoculum size on microbial fermentation process is widely accepted. Out of five inoculum size tested (0.25, 0.50, 0.75, 1.0 and 1.25 ml) and 1.0 ml inoculum was found to be the most suitable for high production of protease by *Aspergillus oryzae* KS 5in submerged fermentation at 72 hrs of fermentation. From Fig. 3, it is clear that the protease production steadily increased with the increasing in the size of the inoculum until it reaches to the magnitude when enzyme productivity became maximum, thereafter no appreciable change in production of protease with high inoculum size could be observed. The maximum enzyme activity was showed at 1.71 IU. at 1.0 ml inoculum size and least enzyme activity 0.74 IU was showed at 1.25 ml of inoculum size.

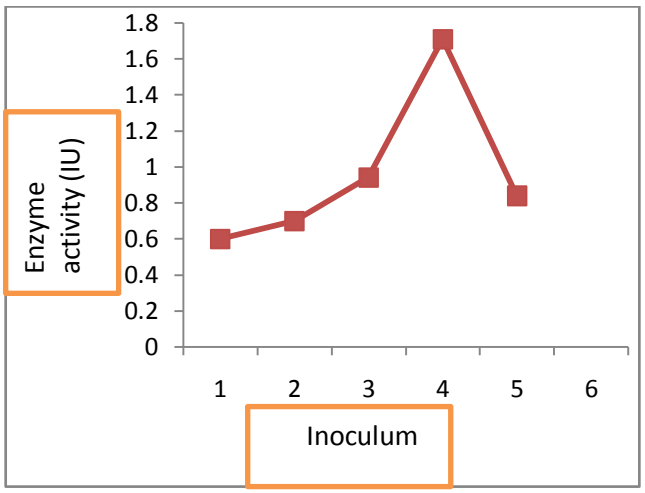

Fig 3: Effect of Inoculum Size on protease production

Francois et al [13] reported that 2% of fungal spores as an inoculum. The maximal protease production (9.021 PU/ml) was observed when an inoculum concentration of 2% fungal culture was added. At lower and higher inoculum levels, poor protease production was observed. It is very important to provide an optimum inoculum level in fermentation processes were reported by various inoculum levels in the range of 1 to 10% were found to maximally produce alkaline proteases by different *Aspergillus* sp.[16,17]. The inoculum concentration has thus profound effects on protease production depending upon the characteristics of the strains. The maximum enzyme production (1.7 IU/ ml) was observed in 1.0% of initial inoculum supplemented conditions. Our findings are coincides with the report of Francois et al(2013)

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