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Optimization of Alkaline Protease Production from Fungus Isolated from Siruvani Hills in Western Ghats, Tamil Nadu

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ABSTRACT:

This study was conducted to monitor the production of protease enzyme derived from specific parts of medicinal plants found in Siruvani Hills, Tamil Nadu, Western Ghats. The main objective was to optimize the kinetic parameters of the enzyme in order to establish a large-scale production process. Among the six isolates, *Aspergillus flavus* and *Aspergillus niger* were identified as potential candidates for enzyme production using the plate assay screening method. The results revealed the highest enzyme production by these isolates at different temperatures and pH levels. Notably, *Aspergillus flavus* exhibited the highest activity in the optimized medium at pH 8.0 and a temperature of 30 ºC, while *Aspergillus niger* showed the highest activity at pH 7.0 and a temperature of 35 °C.

KEY WORDS:

Protease, *Aspergillus flavus* , *Aspergillus niger*, pH, temperature

INTRODUCTION:

Microbial proteases, such as those produced by the fungus *Aspergillus*, play a crucial role in various industries due to their wide range of applications and enzymatic activities. These proteases have the ability to break peptide bonds in proteins, making them essential for numerous physiological processes and cellular metabolic functions. In addition to their biological importance, microbial proteases from fungi like *Aspergillus* have found significant application in the industrial field. They are used in various sectors such as food, detergent, pharmaceutical, and leather industries for their ability to enhance the efficiency of processes like protein degradation and waste management. The use of microbial proteases from *Aspergillus* in these industries not only improves product quality but also reduces production costs and environmental impact Zheng, H., *et al.,* 2015. Furthermore, the production of microbial proteases from *Aspergillus* can be optimized through bioprocess technology and genetic engineering techniques, resulting in enzymes with improved characteristics and performance. Therefore, the use of *Aspergillus*-derived microbial proteases holds great promise for developing novel and efficient biocatalysts in various industries, paving the way for sustainable and eco-friendly manufacturing processes.

The proteases from *Aspergillus* are classified into two main types: acid and alkaline proteases. Acid proteases have an optimal activity in the acidic pH range (pH 3.0-5.5) and are used in food processing, leather manufacturing, and medical applications. Alkaline proteases, on the other hand, have an optimal activity in the alkaline pH range (pH 8.0) and are used in the detergent industry and other applications.

Aspartic proteases are a type of acid protease that are produced mainly by filamentous fungi, including *Aspergillus* species. They are endopeptidases with a molecular weight in the range of 30-45 kDa and are stable within the acidic to neutral pH range. Aspartic proteases are widely used in industrial processes such as food processing, the breading industry, leather manufacturing, and medical applications.

The production of proteases from *Aspergillus* species is typically carried out through fermentation processes. This can be done using submerged fermentation (SmF) or solid-state fermentation (SSF). The choice of fermentation method depends on the specific application and the desired properties of the protease.

Microbial proteases offer numerous advantages over proteases sourced from plants and animals. Various microorganisms, including bacteria, fungi, yeast, and actinomycetes, are capable of producing these enzymes. While most commercial proteases are derived from Bacillus species, fungi boast a wider array of proteases compared to bacteria. Fungi, particularly strains considered safe, produce extracellular enzymes that are easily recoverable from fermentation broth *Wei, M., et al.,*2023. *Aspergillus, Penicillium,* and *Rhizopus* molds are particularly valuable for protease production, as many species within these genera are deemed safe. In this study, protease-producing fungi were isolated from plant samples, screened, and identified. The enzyme was then produced in shake flasks, with critical production parameters such as pH and temperature being optimized.

MATERIALS AND METHODS:

Soil samples were gathered from five distinct plants situated in various areas of the Siruvani Hills, which are part of the Western Ghats. The collection of soil samples took place over three separate intervals spanning a period of six months. These samples were carefully collected from different plants, reaching a depth of up to 15cm, and placed in small sterilized polythene bags. Subsequently, they were transported to the laboratory for further analysis and investigation.

ISOLATION OF FUNGI:

The enumeration of soil fungi was conducted using the Soil Dilution method with Potato Dextrose Agar. The sterilization process involved subjecting the medium to a temperature of 121°C for 15 minutes at 15 lbs. Subsequently, 15 ml of the sterilized medium was dispensed into sterile petri dishes and set aside for future use. To each sterile Petri dish (triplicate for each dilution), 1 ml of microbial suspension from dilutions 10-3, 10-4, and 10-5 was added, along with 15 ml of sterile Potato Dextrose Agar. To prevent bacterial growth, a 1% streptomycin solution was introduced to the medium before pouring it into the Petri plates. The Petri dishes were then placed in a dark incubator at a temperature of 28°C. The plates were observed daily for a period of three days.

MICROSCOPIC IDENTIFICATION OF SOIL FUNGI:

The fungal morphology was analyzed through macroscopic observation of colony characteristics such as color and texture, as well as microscopic examination using lactophenol cotton blue staining to observe conidia, conidiophores, and spore arrangement under a compound microscope. Identification of the fungi was done with the assistance of literature resources.

SCREENING BY PLATE ASSAY METHOD:

Fungal isolates were tested for the production of proteolytic enzymes using the Plate assay method developed by Hankin and Anagnostakis. Gelatin served as the protein source in the growth medium. The isolates were inoculated onto petri dishes containing nutrient agar supplemented with 1% gelatin (Peptone, 5g; Beef extract, 3g; NaCl, 5g; Agar, 15g; Distilled water, 1 liter, pH 6). The petri dishes were then placed in an incubator at 28 ± 1°C for 3 days. After a week, the formation of a clearing zone around the fungal colonies indicated gelatin degradation.

Upon adding an aqueous saturated solution of mercuric chloride reagent to the plate, a distinct gelatinolysis zone was observed. The mercuric chloride solution interacted with the gelatin, resulting in the formation of a white precipitate that made the clearing zone visible. The size of the clearing zone was used as an indicator of the fungal strain's extracellular protease activity. Enzyme activity was quantified using the formula EA = D-d, where D represents the diameter of the colony plus the clearing zone, and d represents the diameter of the colony itself. Following the assessment, the two most effective isolates out of the six were selected for further investigation in production and kinetic optimization studies.

PROTEASE PRODUCTION:

The two chosen fungal isolates were introduced into an aseptic 100 ml of protease-specific fermentation medium with the following composition (% w/v): yeast extract 1.0, MgSO4 0.02, glucose 2.0, K2HPO4 0.1, pH 7.0. The flasks containing the inoculated isolates were then placed in a rotary shaker and incubated at 28˚C for a period of 5-6 days.

After the completion of the incubation period, the solutions in the flasks underwent filtration using Whatmann filter paper No. 1. Subsequently, the filtrates were centrifuged at 8,000 rpm at 4˚C for 10 minutes. The pellet resulting from centrifugation was discarded, while the clear supernatant was utilized as the protease enzyme source. This crude enzyme supernatant was then employed for additional research purposes.

MEASUREMENT OF ENZYME ACTIVITY:

Protease activity in the crude enzyme extract was assessed following the protocol established by Carrie Cupp-Enyard, utilizing casein as the substrate. Two test tubes were designated as test (T) and blank (B). A 0.65% casein solution was introduced into both tubes, after which the test tubes were incubated at 37 ˚C for 5 minutes. Subsequently, 1 mL of enzyme solution was added to the T-test tube, thoroughly mixed, and then incubated at 37 ˚C in a water bath for 30 minutes to allow the enzymatic reaction to take place. The reaction was halted by the addition of 5 mL of Trichloroacetic acid (TCA) solution to both test and blank tubes. In the blank test tube, only 1 mL of enzyme solution was added and left to stand for 15 minutes at room temperature. The solutions from both test tubes were filtered using Whatmann No 1 filter paper.

Two milliliters of the filtrate from the test and the blank were placed into separate test tubes and labeled as "test" (T) and "blank" (B). Sodium carbonate (5 mL) was then added to both test tubes, followed by the addition of 1 mL of Follin Ciocalteus phenol reagent that had been diluted two-fold. The resulting solutions in both test tubes were incubated in the dark at room temperature for 30 minutes to allow for the development of a blue color. The absorbance of the blue color compound was measured at 660 nm using tyrosine standard and compared against a reagent blank. The activity of the enzyme was determined by measuring the amount of tyrosine released per minute at pH 7.5 and 37 ºC. One protease unit was defined as the amount of enzyme that releases 1 μM of tyrosine per minute under these conditions. All experiments were performed in triplicate, and the mean value was reported.

OPTIMIZATION OF CULTURE CONDITIONS FOR ENZYME PRODUCTION:

The impact of pH on the production of the Protease enzyme: In order to identify the ideal pH level, fungus cultures were grown in a 150 mL flask filled with 50 mL of optimized medium. The pH of the medium was adjusted within a range of 4.0 to 8.0 using either 1 N HCl or 1 N NaOH. The flasks were then maintained in a stationary stage at a temperature of 28°C for a cultivation period of 5 days.

The Impact of temperature on the production of Protease enzyme: To establish the optimal temperature for protease production by the fungal species, fermentation was conducted at 5°C increments within the temperature range of 20, 25, 30, 35, and 40°C.

RESULT AND DISCUSSION:

Fungi that thrive in natural habitats experiencing changing environmental conditions hold great importance in terms of industrial applications. The composition of fungal species in soil is heavily influenced by several soil factors, such as pH, organic content, and moisture levels. These fungi have the ability to produce innovative metabolites or enzymes that exhibit exceptional catalytic properties.

ISOLATION AND IDENTIFICATION OF FUNGAL ISOLATES:

In the course of the investigation, a total of 60 fungal colonies belonging to 5 distinct fungal species (*Aspergillus sparsusi*, *A.ochraceous*, *A. flavus*, *A.terreus*, and *A.niger*) were identified. The majority of these fungal species were classified under Deuteromycotina. Notably, the genus *Aspergillus* was found to be the prevailing group among the majority of the isolates.

The pigmentation of the colony ranged from white to yellow-brown and black shades of green, growing uniformly with dense erect conidiophores. The conidial heads on LPCB microscopic mounts displayed hyaline characteristics; hyphomycetes with well-defined conidial heads featuring flask-shaped phialides arranged on vesicles were observed. Typically, the identification of Aspergillus species relies on the morphological traits of the colony and microscopic analyses. According to the cell morphology results, these isolates are classified within the Aspergillus sp. group, sharing common features such as globular vesicles, conidiophores that are translucent yellowish-green in shape, and semi-conidiospores that are round to round-shaped and light green to brownish green.

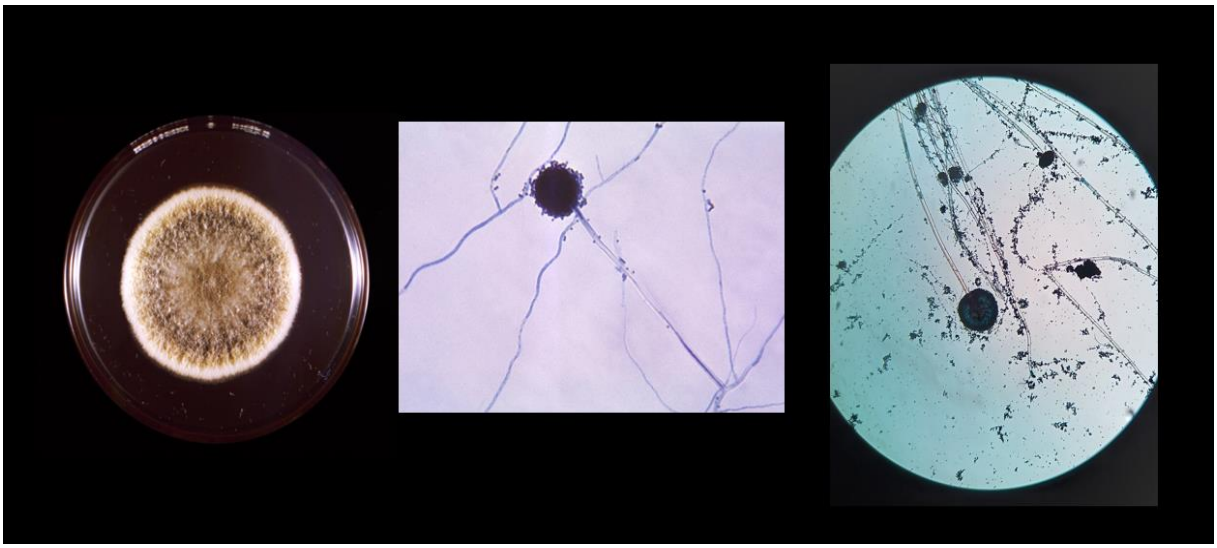

Fig. 1: Morphological and microscopic observation of fungus

SCREENING BY PLATE ASSAY METHOD:

Fungi were screened for protease activity through the hydrolysis of substrate incorporated in the medium using the plate assay method. Following an incubation period, enzyme activities were identified by the presence of zones surrounding the fungal colonies. *Aspergillus flavus* and *Aspergillus niger*, two fungal species out of the five isolates, exhibited the largest zones around the colonies and were selected for further investigation. *Aspergillus, Penicillium, Rhizopus,* and *Rhizomucor* are recognized as prominent producers of proteases among certain genera, displaying activity across a broad pH range.

ENZYME PRODUCTION: ENHANCING KINETIC PARAMETERS THROUGH OPTIMIZATION:

Impact of pH on the production of protease enzymes:

The growth medium pH is a crucial physical parameter that influences morphological changes in microbes and enzyme secretion. pH fluctuations during microbial growth impact product stability in the medium. The optimal pH level varies among different microorganisms and enzymes. Both isolates were cultured in media with pH levels ranging from 4.0 to 8.0. *Aspergillus flavus* exhibited maximum enzyme production (30 IU/ml) in pH 8.0 medium, while *Aspergillus niger* showed the highest enzyme production (26 IU/ml) in pH 7.0 medium.

It has been widely documented that protease production from microbial sources can result in either acidic or alkaline proteases, as indicated by numerous researchers, depending on the organisms and the source of isolation. Nascimento and Martins *et al* and Sookkheo *et al*. have also noted that the optimal pH for protease activity falls between 7.0 and 8.5. Borris *et al* ., have reported that alkaline protease production tends to be higher within the pH range of 9-13. Similarly, studies conducted by Sonia Sethi and Saksham Gupta *et al* ., have shown that the maximum protease enzyme activity was observed in a medium with a pH of 9.0 in the case of *Penicillium chrysogenum*, followed by *Aspergillus niger*.

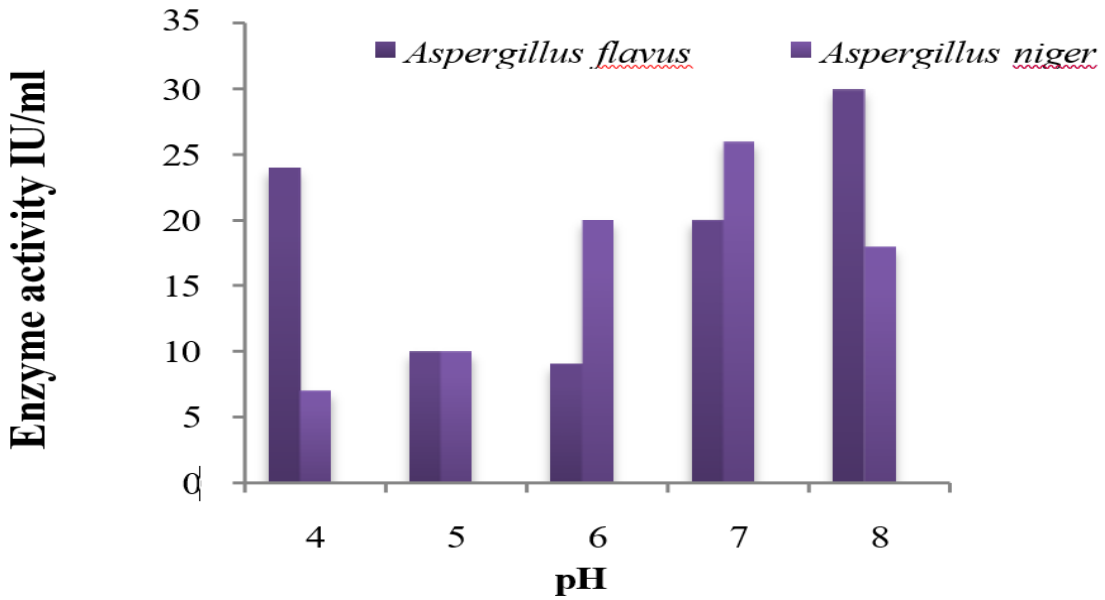

Fig. 2: Effect of pH on Protease production

Impact of temperature on the production of protease enzymes:

Incubation temperature significantly influences the metabolic activities of microorganisms. Even minor temperature variations can impact enzyme production. A recent study examined the optimal temperature for maximum protease production through incubation of the inoculated media at temperatures ranging from 20 to 45°C. The highest enzyme production was observed at 30°C, while complete inactivation occurred at 45°C. Results from tests conducted at various temperature

levels indicated that the ideal temperature for protease production by *A. flavus* was 30°C, resulting in 28 IU/mL , whereas *A. niger* achieved 31 IU/mL at 35°C.

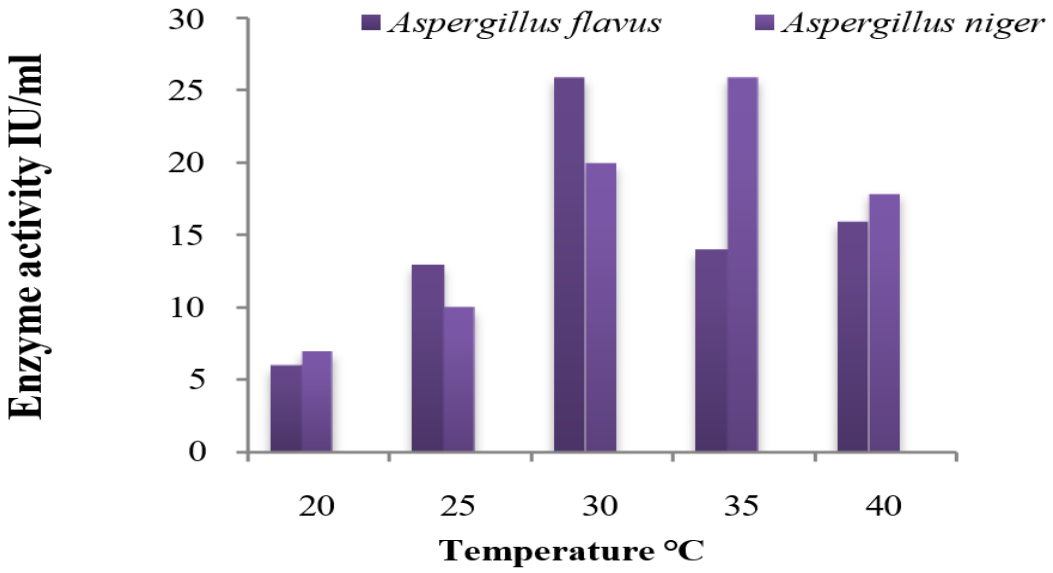

Fig. 3: Effect of Temperature on Protease production

During the exponential growth phase, enzymes are produced as secondary metabolites. However, subjecting the incubation process to high temperatures can result in inadequate growth and subsequently decrease the yield of enzymes . In a similar vein, Ganesh Kumar *et al*. discovered that the optimal temperature for protease production by the mesophilic fungi Synergistes species was 35°C. Beyond this temperature, both growth and protease production ceased, as observed by Morimura *et al*. in their study on *Aspergillus usami*.

The fermentation process for enzyme production was analyzed based on the results obtained, identifying major parameters and determining optimal levels. Microorganisms tightly regulate enzyme production, and enhancing productivity can be achieved by refining these controls. The yields of proteases seem to be influenced by a complex interplay of factors such as inoculum size, pH, temperature, aeration, growth time, among others. This research indicates that the diverse protease-producing fungi found in soil can be utilized for biotechnological purposes. The significant enzyme activity observed at pH levels of 8.0 and 7.0 suggests that *A. flavus* and *A. niger* have the potential to be valuable protease producers for various industries. Further purification studies on this protease enzyme are still pending. In summary, soil-dwelling microorganisms, particularly fungi like *Aspergillus flavus, Aspergillus niger,* Aspergillus and *Penicillium sp*., produce hydrolytic enzymes that hold industrial and biotechnological significance. The protease enzymes from these fungi possess the capability to break down proteolytic materials, which have diverse applications across multiple fields.

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