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ISOLATION, SCREENING AND IDENTIFICATION OF PROTEASE PRODUCING FUNGI FROM RHIZOSPHERE SOIL AND OPTIMISATION OF pH, INCUBATION TIME AND INDUCER CONCENTRATION FOR ENHANCED PROTEASE PRODUCTION.

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ABSTRACT

Protease is any enzyme that conducts proteolysis and accounts for about 60% of the total enzyme market. These enzymes find applications in waste treatment, bioremediation process, in detergents and in leather industry. In the present research work protease producing microorganisms were isolated from soil samples collected from rhizosphere and non-rhizosphere region by spread plate technique. The isolated organisms were screened for their protease producing abilities. The strain that gave positive result in screening was identified as *Aspergillus niger* by analyzing the colony morphology and using LCB staining test. The important production parameters like pH, inducer concentration and incubation time were optimized. Maximum enzyme activity of 307.5 µmol/ml-min was observed at pH 9, 0.05% inducer concentration and after 96 hours of incubation.

KEY WORDS

Protease, *A.niger,* Enzyme activity, Screening

INTRODUCTION

There has been a spectacular rise in the production of industrial enzymes in the last three decades. The growth of industrial enzyme market has expanded to nearly 85 enzymes, which are currently in commercial production. With the discovery of a variety of new and more active enzymes, the enzyme market reached US $ 2.5- 2.8 billion in 2006.

Proteases, account for about 60% of total enzyme market and are among the most valuable commercial enzymes. They are the single largest class of enzymes occupying a pivotal position due to their wide application in the various industrial processes. Plants, animals and microbial sources are employed for protease production. Microbes serve as the preferred source of proteases because of their rapid growth, limited space required for their cultivation, and the ease with which they can be genetically manipulated to generate new enzymes with altered properties (Rao *et al*., 1998). Fungal extra cellular alkaline proteases are important enzymes and are mainly used in detergents to facilitate the release of proteinaceous stains such as blood, milk, egg and meat. The organisms most often used are *Aspergillus niger* and *Aspergillus oryzae.* They account for approximately 40% of the total worldwide enzyme sale. The performance of protease is influenced by several factors such as pH, ionic strength, temperature, mechanical handling *etc*. (Nascimento and Martins, 2006).

Protease preparations are mainly used for the improvement of taste, yield of extract, nutritional content and the physical properties of products. There are proteinases and peptidases. Proteinases hydrolyze large protein molecules to smaller peptide chains. Peptidases release amino acids from the terminals of proteins and peptides. Protein is generally degraded to smaller molecules by protease enzyme from filamentous fungi rather than bacterial sources (Kumar *et al*., 1993 and Moreina *et al*., 2003).

Proteases account for nearly 60% of the industrial enzyme market and have wide applications in many industries *viz*., textiles, detergents, food processing especially for cheese ripening, meat tenderizing, animal nutrition, pharmaceuticals, paper industry and food industry (Negi and Banerjee, 2006). These enzymes are also reported to have a significant role in development and manifestation of dreadful diseases such as AIDS and cancer. (Yadwad *et al.,* 1996). Although extensive work has been reported using fungal strains, still work on isolation and characterization is being carried out to isolate microorganisms having better activity and clinical importance. (Chandran *et al.,* 2004).

Fungal alkaline proteases are advantageous due to the ease of downstream processing to prepare a microbe-free enzyme. An alkaline protease from *Conidiobolus coronatus* was found to be compatible with commercial detergents used in India. (Phadatare *et al.,* 1993). Alkaline proteases with hydrated lime and sodium chloride are used for dehairing, resulting in a significant reduction in the amount of wastewater generated. Endo- and exoproteinases from *Aspergillus oryzae* have been used to modify wheat gluten by limited proteolysis. The cost of enzyme production is a major obstacle in the successful application of proteases in industry. Protease yields have been improved by screening for hyperproducing strains and/or by optimization of the fermentation medium. Industrial applications of proteases have posed several problems and challenges for their further improvements. (Rao *et al.,* 1998).

In the present investigation, protease producing fungi were isolated from soil sample, screened and identified. The enzyme was then produced in shake flask and the

critical production parameters like pH, incubation time and inducer concentrations were optimised.

MATERIALS AND METHODS

Sample Collection and Sampling Methods: Soil samples were collected at a total depth of 20 cm using direct penetration technology (DPT), equipped with sterile spatula from rhizosphere and non rhizosphere region. The site from where the samples were collected was located at Mala in Trissur District of Kerala, India. (Vishvesh *et al.,* 2002).

Isolation of microorganisms: Microorganisms present in the soil sample were isolated using spread plate technique. The diluted samples were spread on PDA and the plates were incubated at 30˚C for 3 days. (Cappuccino and Sherman, 2002).

Screening for protease producing fungi: Skimmed milk agar medium was used for screening of protease producing fungi. 1 mg/1000 ml ampicillin was added to the media to restrict the bacterial growth. The pH of the medium was maintained at 7.5 and contained per litre yeast extract, 5g; beef extract, 3g; sodium chloride, 5g; skimmed milk powder, 5g; potassium dihydrogen phosphate, 1g; potassium monohydrogen phosphate, 1g; dextrose, 3g and agar, 15g. (Cappuccino and Sherman, 2002).
The fungal isolates were streaked on the medium and incubated at 30˚C for 3 days. The strain that exhibited maximum clear zone was selected for protease production after identifying it.

Identification of isolated fungi: Based on the colony morphology and Lacto phenol Cotton Blue (LCB) test, the protease producing fungi were identified. (Holt *et al.,* 1994).

Inoculum Preparation: The inoculum was prepared by dispersing the spores from a week-old fungal slant culture in the potato dextrose broth using a sterile inoculation loop.

Fermentation process: Fermentation was carried out in Erlenmeyer flasks (250 ml) containing production media. The production media contained the following components per litre: potassium dihydrogen phosphate, 2g; magnesium sulphate, 0.3g; ammonium sulphate, 1.4g; calcium chloride, 0.3g; urea, 0.3g; yeast extract, 5g; glucose, 3g; manganese sulphate, 0.156g; iron sulphate, 0.5g and tween 80, 0.5ml. (Tsuzuki *et al*., 1993). The autoclaved broth was inoculated with 2 ml of spores suspension aseptically and incubated at 30°C. Fermentation was conducted under various experimental conditions.

Optimisation of Parameters: Optimization of various parameters was carried out by varying all parameters simultaneously at selected levels. The study was carried out in triplicates. *Aspergillus niger* is a known alkaline protease producer. So the pH values selected for optimization were pH 8, 9, 10 and 11. Also, casein acts as an inducer for protease production. Hence, different concentrations of the inducer like 0.03, 0.04, 0.05 and 0.06% were supplemented to the production media at different pHs *i.e*., pH 8, 9, 10 and 11. Control samples were also maintained with 0% inducer, same pH and same amount of inoculum. Samples were collected from both test and control at the end of 48, 72, 96 and 120 hours. Protease assay was performed and the total protein content estimated. Specific activities of the samples were calculated. The optimum values of the parameters initial pH, incubation time and inducer concentration were selected based on the production parameters that gave maximum specific enzyme activity.

Protease activity assay: The protease activity was assayed by casein digestion method. (Keay and Wildi, 1994). The reaction mixture contained suitably 0.5ml of crude enzyme extract, 2ml 1% casein solution, 0.5ml phosphate buffer, 0.1M and pH 7.2.

The reaction mixture was incubated at 37°C for 30 min. The reaction was terminated by the addition of 2 ml of 15% trichloroacetic acid. The terminated reaction mixture was centrifuged at 8000 rpm for 10 min. The absorbance of the supernatant was measured at 280 nm. Tyrosine was used as standard. One unit of protease activity is defined as the amount of enzyme which liberates one micromole of tyrosine per minute per gram dry substrate under experimental conditions. Protein was estimated by the method of Lowry et al. (1951).

RESULTS AND DISCUSSION

Isolation of microorganisms from soil sample: From the soil samples, four different fungal species were isolated. The isolated colonies are shown in figures 1.a, 1.b, 1.c and 1.d. The colony morphology of the isolated fungal species is as given in table 1.

Table 1
Colony morphology of isolated fungal strains

S. No.	Sample collection site	Strain name	Colony morphology
1.	Soil from rhizosphere region	F1	White colonies that turned black as culture matured.
2.	Soil from rhizosphere region	F2	Green colored colonies.
3.	Soil from rhizosphere region	F3	Grey colored colonies
4.	Soil from non-rhizosphere region	F4	White colonies that turned pink as culture matured

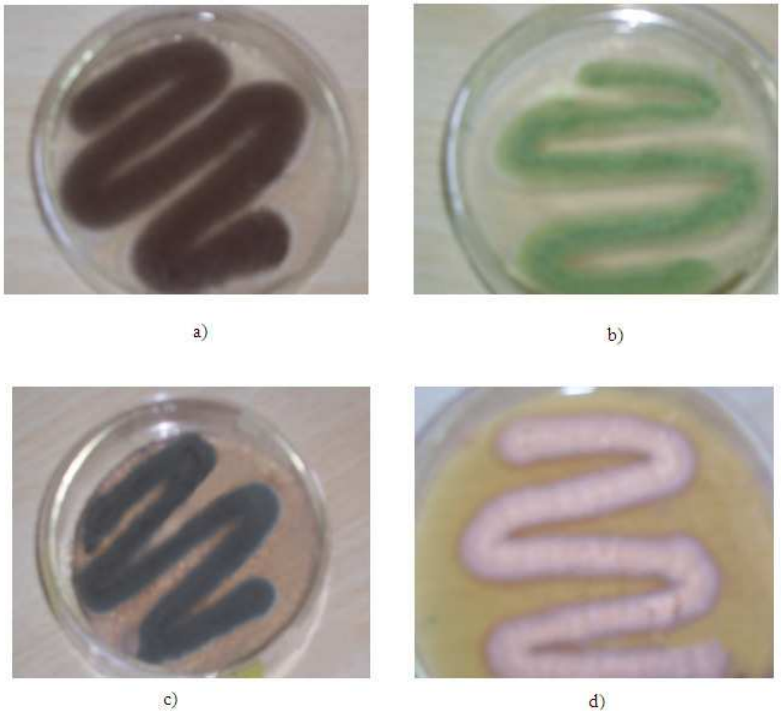

Figure 1
Pure culture of isolated fungal strains in PDA plate. a, b, c and d are strains F1, F2, F3 and F4 respectively.

Screening for protease producing microorganisms: Screening of protease producing microorganisms usually involves growth on the medium that contains protein as the selective substrate. The growth of microorganisms on skimmed milk agar was tested (Sharma et al., 2006). Following inoculation and incubation of the agar plate cultures, organisms secreting proteases, exhibited a zone of proteolysis, which was demonstrated by clear area surrounding the microorganism's growth. This loss of opacity is a result of a hydrolytic reaction yielding soluble, non-colloidal amino acids and it represents a positive reaction. In the absence of protease activity, the medium surrounding the growth of the organism remains opaque, which is a negative reaction (Cappuccino and Sherman, 2002).

In this present investigation, skimmed milk powder was used as the selective substrate, in which the isolated organisms were streaked. The fungal strain F1 produced clear zone in the media and hence was confirmed to be protease producing as is shown in figure 2.

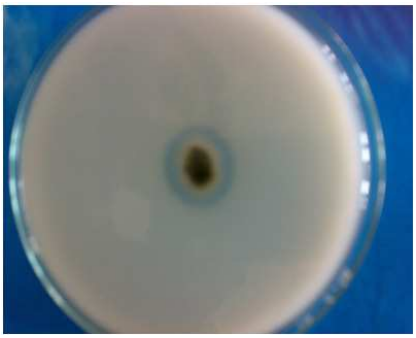

Figure 2
Strain F1 showing positive result for protease production

Identification of protease producing fungal strain: In this work, the protease producing fungal strain F1 was identified to be *Aspergillus niger.* The F1 fungal strain showed white colonies that turned black as culture matured. (Cappuccino & Sherman, 2002). By performing LCB staining, the morphology of the organism was observed under the microscope (Figure 3). The organism was observed to be single celled with circular spores (conidia) in chains that developed at the end of sterigma, arising from the terminal bulb of the conidiophores. It contained vesicles and long conidiophores arose from aseptate mycelium.

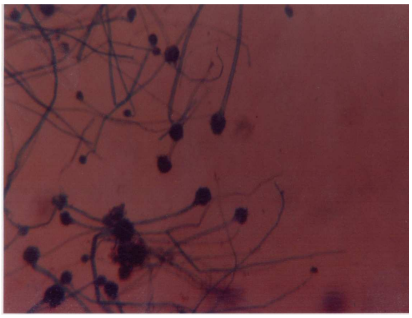

Figure 3
Microscopic view of Aspergillus niger

Optimisation of parameters: For protease production, pH, incubation time and inducer concentration were optimized. By comparing the enzyme activities and specific enzyme activities, it was found that maximum enzyme production is at pH 9, 0.05% inducer concentration and after 96 hours of incubation. Figure 4.1, 4.2, 4.3 and 4.4 shows the specific activities of protease produced at varying levels of pH, incubation time and inducer concentration.

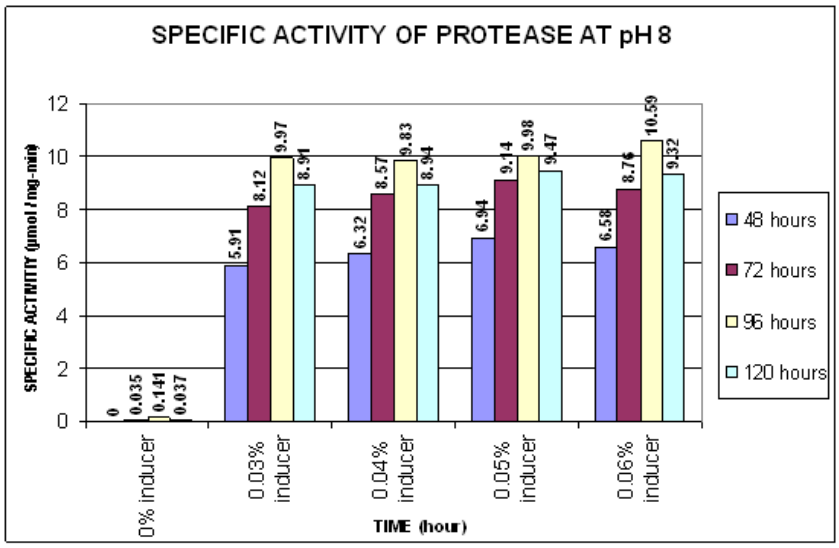

Figure 4.1
Specific activity of protease at pH 8

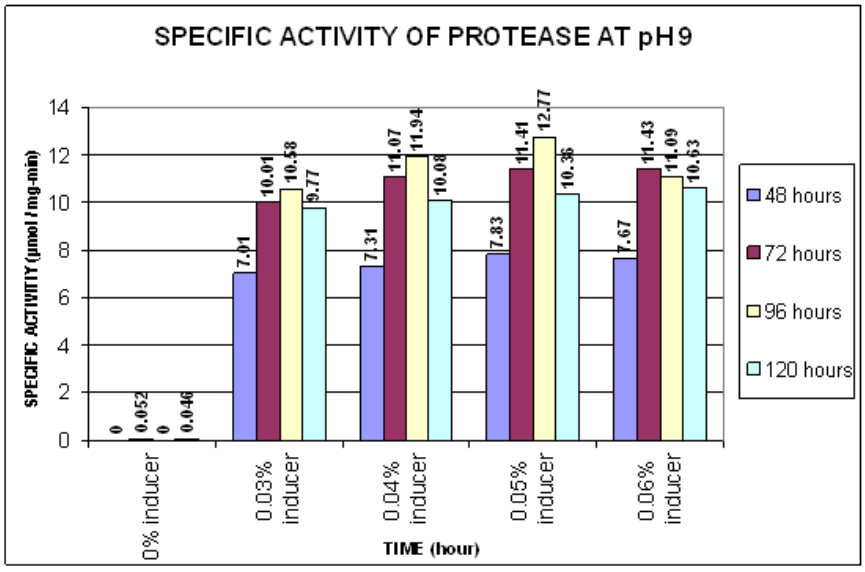

Figure 4.2
Specific activity of protease at pH 9

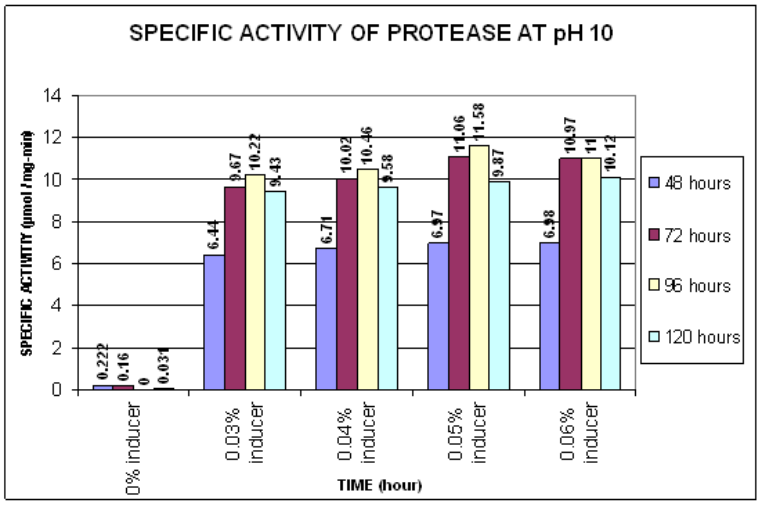

Figure 4.3
Specific activity of protease at pH 10

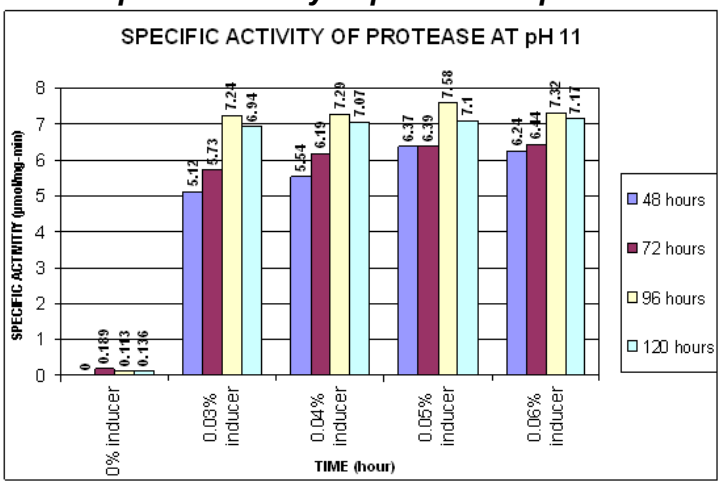

Figure 4.4
Specific activity of protease at pH 11

Figure 4: Specific activities of protease produced at different pH and inducer concentration at varying incubation times. 4.1, 4.2, 4.3 and 4.4: Specific activity of protease produced at initial pH 8, 9, 10 and 11.

The specific activities of the enzymes produced at pH 8 and different inducer concentrations show a similar pattern. For all the inducer concentrations, maximum specific activity was observed after 96 hours of incubation. In this case, maximum specific activity of 10.59 µmol/mg-min was observed when the organism was grown in media containing 0.06% inducer. The enzymes produced at pH 9 have maximum specific activities. The maximum specific activity was found to be 12.77 µmol/mg-min for the enzyme produced at 0.05% inducer concentration and after 96 hours of incubation. In this case, a maximum specific activity of 11.58 µmol/mg-min was observed when the organism was grown in media containing 0.05% inducer. The enzymes produced at pH 11 have very low specific activities. The maximum specific activity was found to be 7.58 µmol/mg-min for the enzyme

produced at 0.05% inducer concentration and after 96 hours of incubation.

As in the case of enzyme activity, maximum specific activity was found for the enzyme produced at pH 9, 0.05% inducer concentration and after 96 hours of incubation. Minimum activity was observed when the organisms were grown in media containing 0.03% inducer concentration. Production was also found to be minimum during 48th hour of incubation. In all the cases, there was an increase in specific activity till 96th hour which was followed by a decrease in activity by 120th hour. The control samples showed insignificant specific activity. This indicates that presence of inducer is a critical factor for optimum protease production.

CONCLUSION

From the results of the study, it could be concluded that the optimum pH for the production of alkaline protease by *Aspergillus niger* is 9. The optimum inducer concentration was found to be 0.05%. A higher concentration of inducer inhibits enzyme synthesis. The enzyme activity in the control samples was found to be negligible. Hence, it could be concluded that *Aspergillus niger* is unable to produce protease in the absence of inducer. The optimum incubation time was identified to be 96 hours. Before 96 hours, the production increases with increasing incubation time. But after 4 days, the enzyme production decreases considerably. This may be due to nutrient stress, aging of culture, accumulation of toxic end products, degradation of medium proteins by protease *etc*. Also, since the enzyme is alkaline in nature, it can be efficiently used as an additive in detergent industry, in pharmaceutical industry, in the bioremediation of alkaline wastes etc.

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