

Original Research Article

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Optimization of a Novel Alkaline Protease from Cashew Industrial Soil Associated *Aspergillus niger*

G. Parvathy*, C. Prabhakumari, Nair Sreecha Chandran and Sonia John

Department of Biotechnology, CEPC Laboratory and Research Institute,
Mundakkal, Kollam, Kerala, India

*Corresponding author

ABSTRACT

Protease the leading industrial enzyme accounts for about 60% of the total enzyme market. It has wide application in different sectors. The search of proteases with better activity and potential of different application is very important. The current paper screened cashew nut processing units' soil near Kollam for the isolation of effective protease producers. The isolated fungal strains isolated from soil by serial dilution method were screened by casein hydrolysis for alkaline protease production. Out of the ten isolated strains of fungi A, B, G and H are protease producers. After the optimization the isolate strain H showed highest activity in the optimized medium at pH 11.0, temperature 35°C, with glucose as carbon source incubated for 7 days. The highest enzymatic activity is showed in the 4th day of batch fermentation at 96hr of incubation. The most potent protease produced strain was identified based on morphological characterization characteristics in National Fungal Culture Collection of India (NFCCI & FIS) Pune as *Aspergillus niger*. Proteases represent one of the largest groups of industrial enzymes and find application in detergents in the removal of different kinds of stains and also in dehairing process practised in leather industry.

Keywords

Protease,
Aspergillus niger,
NFCCI.

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Introduction

Proteases catalyze hydrolytic reactions of protein molecules resulting the formation peptides and amino acids. These enzymes have remarkable applications in both physiological and commercial fields. Among all the different commercial enzymes, microbial protease in particular, represents about 60% of all the industrial enzyme's sales in the world due to their applications in several industrial sectors like in the detergent, food, pharmaceuticals, chemicals, leather, paper and pulp and silk industries (Singh *et al.*, 2016). Proteases have physiological role in all living organisms and therefore, they are

present in a wide range of sources such as animals, plants and microorganisms (Rao, 1998) Microbial origin proteases are preferred over other proteases for industrial application due to the broad biochemical diversity and their susceptibility to genetic manipulation as well as economic advantages (Souza *et al.*, 2015; Ferid Abidi *et al.*, 2008).

The protease mediated leather processing is an efficient alternative in an environmental friendly manner to improve the quality of leather, help to shrink waste and, save time and energy (Zambare *et al.*, 2011; Adrio and

Demain, 2014). This paper reports the isolation characterization and optimization of a novel extracellular alkaline protease from *Aspergillus niger* that has an wide application in different industry.

Materials and Methods

Isolation of protease producer from soil sample

Soil samples were collected from Cashew Industry near Kollam. Enrichment method was done for that all the soil samples were pooled and inoculated in to protease producing media containing the following: (g/L) glucose 5.0; peptone 7.5; MgSO₄7H₂O 5.0, KH₂PO₄ 5.0, and FeSO₄7H₂O 0.1 and incubated for 48 hours at 37⁰ C for isolating efficient protease producers. After the incubation the sample was plated in to Casein agar plates at pH 7. The protease production was confirmed by the formation of clear zones around the colonies. The individual isolates which show clear zone was selected and grown in 100ml of protease producing media the protease that released in to the fermentation media was quantitatively measured Sigma-Aldrich method.

Protease assay

The protease activity was determined by the Sigma-Aldrich method. The reaction mixture contains 450 µl of 1% casein in 50 mM phosphate buffer (pH 7) and 50 µl of the enzyme. After 20 min incubation at 37°C the reaction was stopped by adding 750 µl of TCA solution followed by 30 min incubation at room temperature and centrifugation (15000 x g, 15 min).

In 2 ml above filtrate after centrifugation, 5 ml of 0.4M sodium carbonate and 1 ml of Folin's reagent was added, mixed and incubated at 37⁰C for 30 minutes. The

developed blue colour was read at 660 nm. One unit of the enzyme activity was defined as the amount of enzyme which releases 1 µmol of tyrosine per min under the assay conditions.

Optimisation studies on protease production

Effect of pH on protease production

The effect of pH on alkaline protease production by the fungi was determined by growing the isolates in assay medium with different alkaline pH in the range of 4-11 using required concentrations of 1N NaOH and 1N HCl. Protease activity was determined in the supernatant.

Effect of incubation temperature on protease production

The effect of temperature on protease production by the fungi was determined by growing it in assay media at different temperatures (25-40⁰C with an interval of 5⁰C) with the pH and time of incubation remains constant at 11 and 24hr respectively. The protease assay was carried out to determine the concentration of the enzyme.

Effect of different carbon source on protease production

The effect of various simple carbon sources such as glucose, sucrose and lactose on the production of alkaline protease by fungi was studied.

These carbon sources were added individually in the production medium at different concentration of 0.5-10% (w/v). The pH, temperature and incubation time of the medium was set at 11.0 at 37⁰C for 24 hr, respectively.

Effect of different incubation period on protease production

To study the effect of time for optimization of protease production, the isolate was inoculated in to the assay medium with pH 11. Further, the organism was incubated at 37°C in shaker to obtain a uniform growth. The alkaline protease activity was monitored at regular time intervals of 24, 48, 72 and 96 hour's duration.

Identification of isolate

The isolate which show maximum enzyme production was sent to National Fungal Culture Collection of India (NFCCI & FIS) Pune for morphological identification.

Results and Discussion

Isolation of protease producer from soil sample

Screening of protease producing microorganisms usually involves growth on the medium that contains protein as the selective substrate. The growth of microorganisms on skim 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 zone of protease 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 A, B, G, H produced clear zone

in the media the protease producing strains with 25-40 mm diameter of proteolytic zone were characterized and hence was confirmed to be protease producers.

Isolation of protease producer from soil sample

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Optimisation studies on protease production

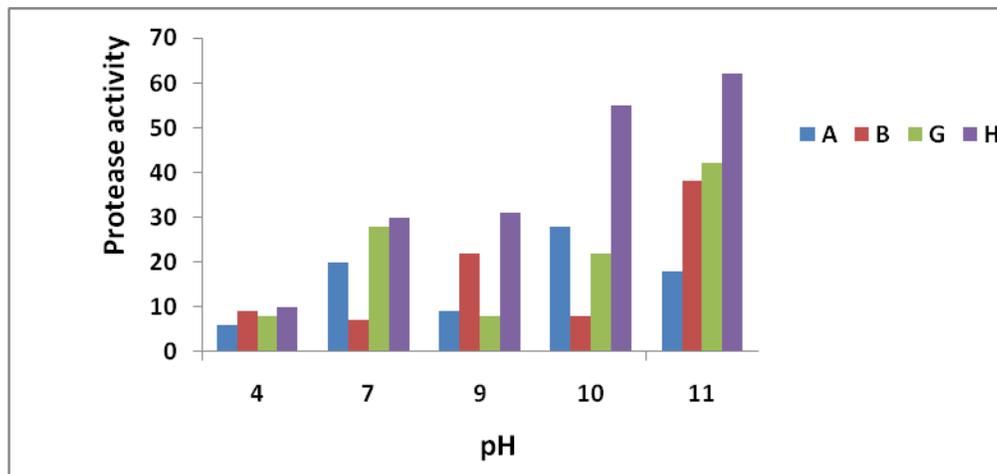
Effect of pH on protease production

Among physical parameters, pH of the growth medium plays an important role by inducing morphological changes in microbes and in enzyme secretion. The pH change observed during the growth of microbes also affects product stability in the medium. The optimal pH varies with different microorganisms and enzymes. All the four isolates were allowed to grow in media of different pH ranging from 7.0 to 11.0. Maximum enzyme activity

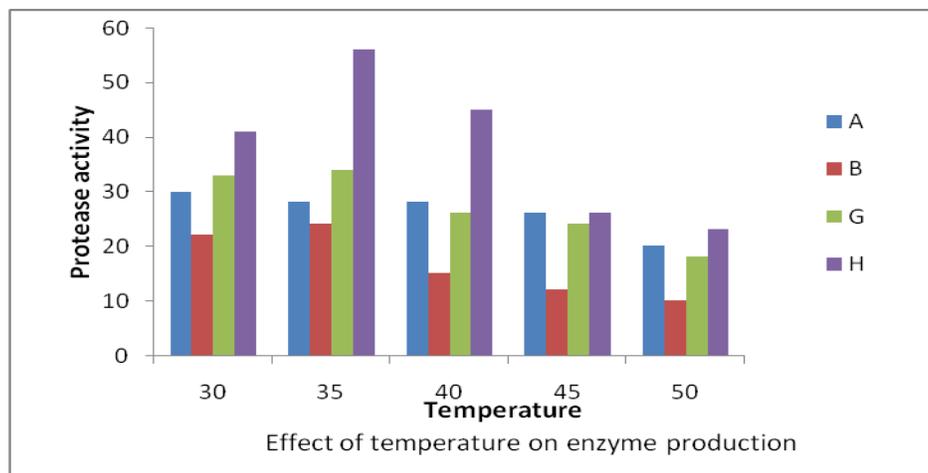
was observed in medium of pH 11.0 in case of strain H. It has been reported widely that protease production from microbial source can be acidic or alkaline proteases as reported by many researchers depending on the organisms and source of the isolation.

Among the four fungal strains A, G, H shows high protease activity and hence undergoes the large scale production of these strains at optimized pH 11 (Sookkheo *et al.*, 2000) also reported the optimum pH for protease activity to be between 7.0 and 8.5 (Borris, 1987) reported alkaline protease production is found to be higher at pH 9-13 (Graph 1).

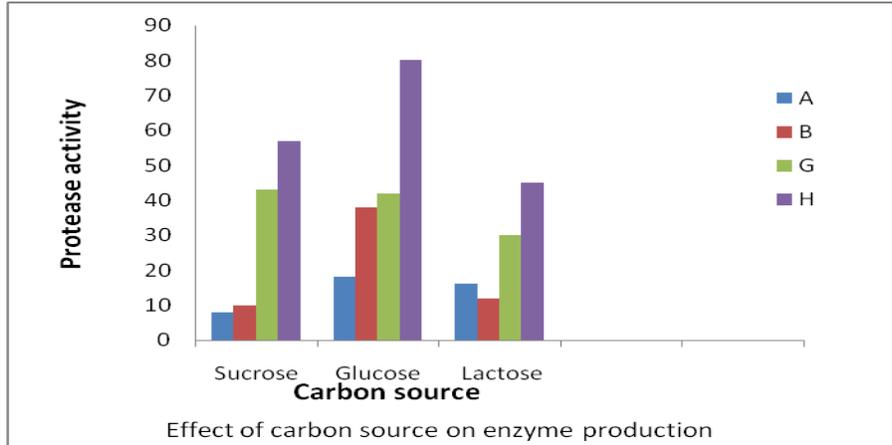
Graph.1 Effect of Incubation pH on protease production



Graph.2 Effect of temperature on protease production



Graph.3 Effect of different carbon sources on protease production



Graph.4 Effect of incubation period on protease production

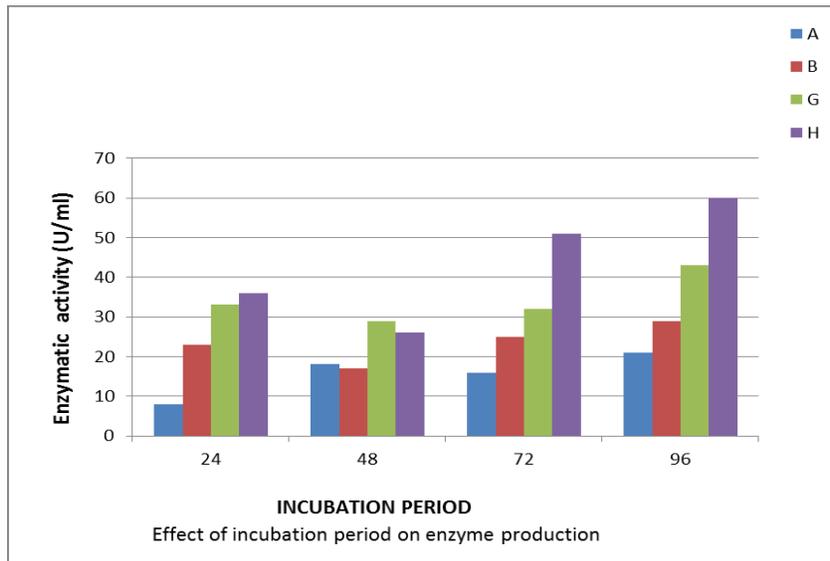


Fig.1 Isolate on PDA slant

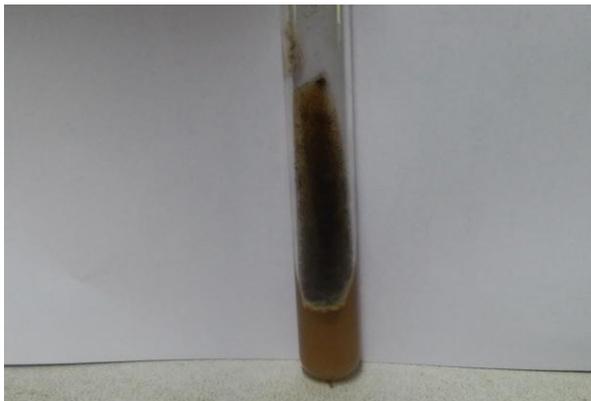
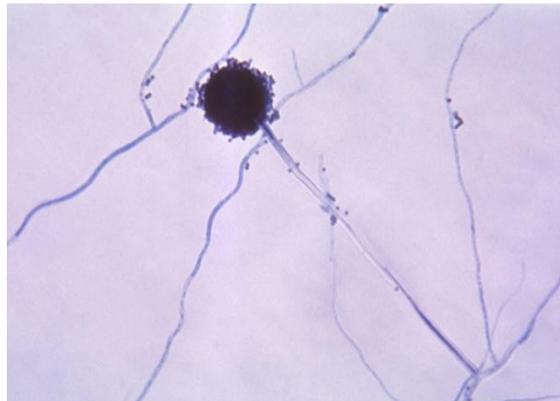


Fig.2 Microscopic image of the isolate



Effect of incubation temperature on protease production

Incubation temperature plays an important role in the metabolic activities of a microorganism. Even slight changes in temperature can affect enzymes production. Presently, the optimal temperature for maximum protease production was at 35°C by the fungal strain H with production decreasing at higher temperature. Since enzyme is a secondary metabolite produced during exponential growth phase, the incubation at high temperature could lead to poor growth and thus a reduction in enzyme yield (Sabu *et al.*, 2012). Among the four fungal strains, A, G, H shows high protease activity at 35°C and hence undergoes large scale production. Assay mixture was incubated at different temperature ranging from 30-60°C and enzyme activity was found to be highest at 35°C. However, the enzyme was completely inactivated at 60°C. The optimum temperature for protease production for by was in mesophilic fungi *Synergistes* species at 35°C (Graph: 2).

Effect of different carbon source on protease production

Various sources of carbon such as sucrose, lactose and glucose were used in optimization of production of protease enzyme. Results obtained showed that strain H in presence of glucose brought about the maximum protease production compared to other carbon sources (lactose and sucrose). After optimization of the effect of the carbon source for protease production, selected strains A, G, H shows high activity and hence undergoes large scale production of these strains at glucose as carbon source. There are general reports showing that different carbon sources have different influences on extracellular enzyme production by different strains (Nehra *et al.*, 2002). The carbon sources like glucose,

maltose, and starch were indispensable components for protease production by *S. albidoflavus* in submerged fermentation (Narayana *et al.*, 2008). (Graph: 3)

Effect of incubation period on enzyme production

After optimizing the production parameters, fermentation was carried out under optimized conditions on shake flask. The amount of tyrosine liberated/g of substrate was found to be 63 U/ml (graph 1). So the maximum production of protease enzyme at the fourth day i.e, at 96 hr of incubation, with other parameters remaining the same. After optimization of incubation period on enzyme production, the selected fungal strains A, H, G have high protease activity and so undergo large scale production of these strains at 96 hr of incubation. The lower production could be due to increase in volume of the medium and lower aeration level (Graph: 4).

Identification of isolate

Ten different isolates are isolated, out of which one fungus with high protease production was selected and identified on the basis of morphological characteristics in National Fungal Culture Collection of India (NFCCI & FIS) Pune as *Aspergillus niger* (Fig. 1 and 2).

In the present work, the protease producing strains are screened by casein hydrolysis and after optimization were done for the maximum protease production. The optimization parameters are pH, incubation temperature, incubation period, carbon source etc. The most potent protease producing strain was identified based on morphological characteristics in National Fungal Culture Collection of India (NFCCI & FIS) Pune. The maximum enzyme production was at 35°C and at pH 11. Glucose is the

selected carbon source for protease production. The enzyme activity of protease is shown more in the 4th day of batch fermentation. Thus the crude and partially purified enzyme was isolated and stored at 4°C for future use.

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