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# Isolation and Purification of Fungus *Aspergillus funiculosus* G. Smith and its Enzyme Protease

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**Abstract:** In the present study fungal colonies were isolated from different protein sources by enrichment culture technique and the fungal isolates were screened for protease activity. Isolate G, the most potent producers of protease were identified as *Aspergillus funiculosus* G. Smith. The isolate G was found to show highest protease production (activity) in an optimized medium (Yeast extract 0.5%, lactose 0.1% and $KNO_3$ 1%) at pH 7.0, temperature 37°C and 5 days of incubation period. The crude enzyme of the isolate G was found to show its highest activity at pH 7.5 and temperature 35°C in enzyme substrate reaction. The molecular weight of enzyme of the isolate *Aspergillus funiculosus* (G) was found 51 kDa, which was determined by SDS-PAGE technique.

**Key words:** Isolates, protease, *Asperigillus funiculosus* G. smith

## INTRODUCTION

Enzymes are biocatalyzed extracted from plants, animals and microorganisms. Among them microbial enzymes are becoming important for its technical and economical advantages. Various kinds of microorganisms in nature degrade different type of proteins. This degradation indicates the proteolytic capabilities of microorganisms. About 80% of the enzymes produced annually, are simple hydrolytic enzymes and of which 60% are proteases[1]. Different bacteria, fungi and actinomycetes are the major sources of microbial proteases. The activity of an enzyme is due to its catalytic nature. The enzyme activity depends upon the substrate concentration, pH, temperature and other physical and chemical factors[2]. Enzymes have large molecular weights. When treated with acid or alkali, they can be denatured or yield mixtures of amino acids. Generally proteases are produced from the fungi that are active at neutral or acidic pH[3]. Now a days biotechnological companies searching for modified properties of proteins or enzymes to enhance their efficiency because of its multipurpose applications and some notable success have already been achieved by protein/enzyme engineering[1,4,5]. The chief objective of enzyme engineering is to produce an enzyme that is more useful for industrial and/or other applications. The present study was carried out for the isolation, purification and characterization of a protease producing microorganism and its enzyme protease.

## MATERIALS AND METHODS

**Fungal strain:** Various protein sources such as pulses, fish, dry fish and meat were collected and preserved carefully in the refrigerator and were tested for proteolytic microbes within few days using enrichment technique. After isolation, the isolated organisms were purified through repeated plating method in PDA media. For the identification of selected isolates different morphological and cultural characteristics (size, shape, arrangement, colour, growth on agar plate, agar slants, in liquid or in deep agar media etc) were observed. Finally the characteristics were compared with the standard description of 'A manual of soil fungi' by Gilman[6].

**Screening of the isolates:** Screening for protease was performed in two steps, primary and secondary. The primary screening was done by the egg albumin degradation, skimmed milk casein hydrolysis and gelatin hydrolysis. After primary selection, the isolates were selected for the protease activity in liquid medium by quantitative method.

**Quantitative method:** For the *in vitro* production of protease by the isolates, tryptone-dextrose-yeast extract broth medium containing tryptone 1.0%, dextrose 0.1%, yeast extract 0.5%, pH 6.5 was used[7]. The medium was dispensed at the rate of 50 ml per 100 ml conical flask and was sterilized. After cooling, the flasks were inoculated

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with selected isolates (4 days old fungal culture) and incubated at 27°C for 7 days. The fungal culture mat was filtered through Whatman no. 1 filter paper. The filtrates were then centrifuged at 2,000 rpm for 10 min. at 4°C temperature. The supernatant was used as crude enzyme. The enzymes were stored at 4°C temperature with few drops of toluene to avoid bacterial contamination. Enzyme activity was measured by the modified method of Hayashi *et al.*[8], as followed by Meyers and Ahearn[9]. The amount of amino acids released was calculated and compared with a standard curve plotted from a known concentration of tyrosine. The enzyme activity was expressed in Unit which was defined as the amount of enzyme that releases 1 μg of tyrosine $ml^{-1}$ of crude extract $h^{-1}$.

**Biomass yield:** Fungal biomass was measured by dry weight method. After collection of the supernatant, the biomass residue was dried at 70°C and the yield was expressed as mg $g^{-1}$ of protein.

**Optimization of culture conditions:** An attempt was also made to ascertain the optimum culture conditions such as pH, temperature, incubation period and carbon and nitrogen source requirements for their maximum growth and activities. The biomass characteristics, yields and protease production of the selected isolate were recorded.

**Incubation periods:** To ascertain the optimum incubation period of the isolate for maximum enzyme production, the supernatant were collected after 3, 5, 7, 10, 12 and 15 days of incubation.

**Medium pH:** To determine the optimum medium pH, for maximum enzyme production, selected medium of different pH (such as 5.0, 6.0, 7.0, 8.0 and 9.0) was inoculated with the isolate. The effects of medium pH on biomass characteristics, biomass yields and protease activities were recorded.

**Temperature:** To determine the optimum temperature for enzyme production, the culture medium was incubated at 30, 37 and 45°C temperature at optimum pH and incubation period. The effects of temperature on biomass characteristic, biomass yields and protease production was recorded.

**Carbon and nitrogen sources:** The production of extracellular proteases under different carbon and nitrogen availability was studied in the liquid culture medium[7] having yeast extract+carbon sources+nitrogen sources. Four carbon sources (glucose, lactose, cellulose and cellubiose) and four nitrogen sources [peptone, tryptone, $KNO_3$ and $(NH_4)_2HPO_4$] were used and the effects of these carbon and nitrogen sources on the production of protease, biomass characteristic and biomass yields were recorded.

**Crude enzyme activity:** To determine the optimum conditions during enzyme substrate reaction phase for maximum activity of crude protease, different temperature and pH were studied.

**Effects of pH:** The effects of pH on the protease (crude) activity were studied in 5 different phosphate buffer solutions ranged from 4.5 to 8.5[10].

**Effects of temperature:** The effects of temperature on the protease (crude) activity were also studied at different temperature such as 30, 35, 40, 45 and 50°C.

**Determination of molecular weight:** The molecular weight of the crude enzyme of the isolate G was determined by SDS-PAGE described by Laemmli[11] with the standard protein BSA (molecular weight 66 kDa), egg albumin (molecular weight 45 kDa) and casein (molecular weight 23.5 kDa).

## RESULTS AND DISCUSSION

**Microorganism:** Twelve fungal isolates were found to degrade or hydrolyse egg albumin, skimmed milk casein and gelatin in primary screening. Among them the fungal isolate (G) was the potent protease producer in liquid medium finally selected for protease and biomass production (Fig. 1). The selected fungal isolate was tested and compared with the standard description of 'A manual of soil fungi' [6]. On the basis of the characteristics of the fungal strain G was identified as *Aspergillus funiculosus* G. smith (Fig. 2).

**Effects of culture conditions**

**Incubation period:** Isolate G was found to show maximum enzyme activity after 5 days of incubation period but highest biomass yield was recorded after 7 days of incubation period and white aerial mycelial mat was observed without any submersed growth. The pH of the supernatant were 7.0 to 8.1 (Table 1).

**Medium pH and temperature:** To ascertain the optimum incubation temperature and pH, for maximum production of protease the selected isolate was incubated at different temperature such as 30, 37 and 45°C with different pH

Table 1: Effects of incubation periods on the biomass yield and the production of protease by the isolate G

| Incubation period (days) | Medium after incubation period: Colour | pH | Biomass characteristics | Biomass yield (mg $g^{-1}$ of protein) | Protease activity (U $ml^{-1}$) |
|---|---|---|---|---|---|
| 3 | Copper leaf | 7.0 | White mycelial | 136 | 29.93 |
| 5 | ,, | 7.3 | mat but no | 132 | 32.45** |
| 7 | Topaz | 7.8 | submerged | 211* | 24.33 |
| 10 | Pale mandarin | 7.7 | growth | 135 | 17.02 |
| 12 | ,, | 7.9 | | 129 | 23.12 |
| 15 | Sand gold | 8.1 | | 104 | 18.58 |

Note: Initial colour of the medium Golden brown, pH 6.5; incubation temperature 27°C. During enzyme substrate reaction temperature 35°C and pH 5.5 *Maximum biomass yield; **Maximum enzyme activity

Table 2: Effects of medium pH at different temperatures on the biomass yield and the production of protease by the isolate G

| Incubation temperature | pH of the medium before/after inoculation: Before | After | Biomass characteristics | Biomass yield (mg $g^{-1}$ of protein) | Protease activity (U $ml^{-1}$) |
|---|---|---|---|---|---|
| 30°C | 5.0 | 6.1 | Profuse aerial | 115 | 6.50 |
| | 6.0 | 6.6 | mycelium over | 119 | 7.09 |
| | 7.0 | 7.3 | the medium | 120* | 23.40** |
| | 8.0 | 7.6 | | 110 | 10.85 |
| | 9.0 | 7.9 | | 109 | 18.79 |
| 37°C | 5.0 | 7.7 | Profuse aerial | 162 | 9.89 |
| | 6.0 | 7.8 | mycelium over | 169 | 13.90 |
| | 7.0 | 7.8 | the medium | 175* | 24.96** |
| | 8.0 | 8.0 | | 149 | 21.48 |
| | 9.0 | 8.1 | | 142 | 19.93 |
| 45°C | 5.0 | 7.2 | Scanty growth | 70 | 2.06 |
| | 6.0 | 7.6 | | 72* | 2.70 |
| | 7.0 | 8.0 | | 40 | 2.84** |
| | 8.0 | 8.1 | | 52 | 1.49 |
| | 9.0 | 8.7 | | 20 | 121 |

Note: Incubation period = 5 days, During enzyme substrate reaction temperature 35°C and pH 5.5 *Maximum biomass yield; **Maximum enzyme activity

Table 3: Effects of carbon and nitrogen sources on the biomass yields and protease production by the isolate G

| | Carbon sources: Glucose | | Lactose | | Cellulose | | Cellobiose | |
|---|---|---|---|---|---|---|---|---|
| Nitrogen sources | B | PA | B | PA | B | PA | B | PA |
| Peptone | 148 | 1.70 | 170 | 6.52 | 164 | 1.42 | 181 | 20.07 |
| Tryptone | 124 | 2.30 | 153 | 1.276 | 171 | 2.62 | 154 | 2.70 |
| $KNO_3$ | 184 | 16.45 | 179 | 23.26** | 226* | 9.65 | 215 | 15.96 |
| $(NH4)_2HPO_4$ | 183 | 14.89 | 165 | 17.09 | 196 | 10.07 | 143 | 5.18 |

Note: Incubation period 5days, temperature 37°C and, medium pH 7.0. During enzyme substrate reaction temperature 35°C and pH 5.5. *Maximum biomass yield; **Maximum enzyme activity (B) Biomass yields (mg $g^{-1}$ of protein), (PA) Protease activity (U $ml^{-1}$)

Table 4: Effects of pH and temperatures on the protease (crude) activity of the isolate G (*Aspergillus funiculosus*)

| pH during crude enzyme substrate reaction phase | Protease activity (U $ml^{-1}$): Temperature during crude enzyme substrate reaction phase 30°C | 35°C | 40°C | 45°C | 50°C |
|---|---|---|---|---|---|
| 4.5 | 5.18 | 9.43 | 8.20 | 5.54 | 2.57 |
| 5.5 | 8.37 | 12.82 | 10.69 | 6.97 | 4.61 |
| 6.5 | 16.97 | 26.73 | 10.88 | 11.46 | 10.21 |
| 7.5 | 2.63 | 35.09* | 25.51 | 12.31 | 11.54 |
| 8.5 | 12.92 | 23.56 | 9.4 | 3.67 | 9.72 |

Note: Incubation period 5 days, temperature 37°C and, medium pH 7.0 *Maximum enzyme activity

such as 5.0, 6.0, 7.0, 8.0 and 9.0 and their growth characteristics were recorded (Table 2).

The isolate G was found to show maximum enzyme activity 24.96 U $ml^{-1}$ at 37°C temperature and in medium pH 7.0, respectively. The isolate G showed different biomass yield at different pH and temperature. The highest biomass yield 120 mg $g^{-1}$ of protein, 175 mg $g^{-1}$ of protein and 72 mg $g^{-1}$ of protein were recorded at incubation temperature 30, 37 and 45°C, respectively. The changes of pH in different incubation temperature were found to vary from 6.1 to 7.9 at 30°C, 7.7 to 8.1 at 37°C and 7.2 to 8.7 at 45°C. From the above described results it can be concluded that for the strain G the temperature 37°C and medium pH 7.0 is most suitable for maximum protease production and biomass yield (Table 2).

Protease production by the fungal isolate at different temperatures and at different pH were also reported by many workers. Fermor and Wood[3] reported the acid (pH 3.6), neutral (pH 7.0) and alkaline (pH 9.1) proteinase activities of cultural supernatant of *Agaricus bisporus*. Our results are in concurrence with their neutral protease production.

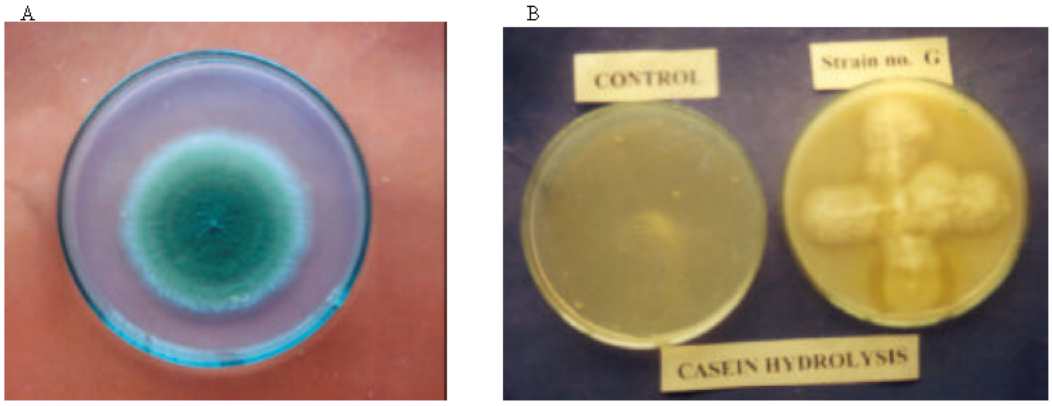


Fig. 1A: Colony of the isolate *Aspergillus funiculosus* on PDA medium
B: Primary screening by casein hydrolysis by the isolate

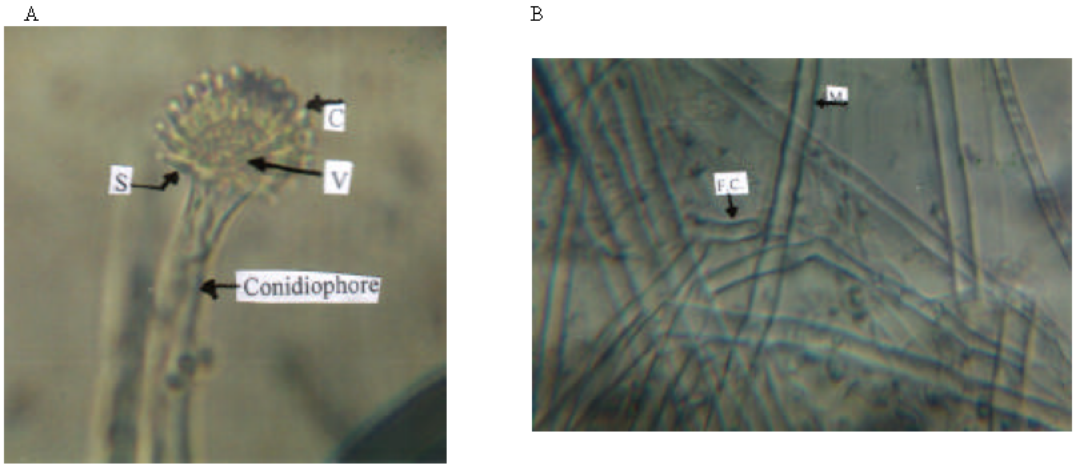


Fig. 2: Microscopic feature showing A= the conidia (C), Sterigmata (S), Vesicle (V) and Conidiophore of the *Aspergillus funiculosus* G. smith and B= Mycelium (M) and foot cell (F.C.)

Fig. 3: SDS-PAGE of fractions. SDS-PAGE was done as described by Laemmli[11] using 12.5% acrylamide. Lane-4 shows different molecular mass markers and Lane 3 show band of the enzyme of the isolate *Aspergillus funiculosus*.

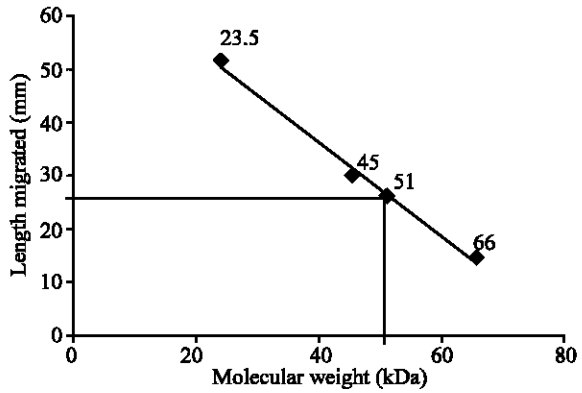


Fig. 4: Standard with known protein markers

**Carbon and nitrogen sources:** The pH, colour of the medium and biomass characteristics were found to changed with the changes of carbon and nitrogen sources of the medium after incubation period. Maximum biomass (226 mg $g^{-1}$ of protein) was recorded when the isolate G was allowed to grow with cellulose and $KNO_3$ as carbon and nitrogen sources, respectively. The maximum enzyme production (23.26 U $ml^{-1}$) was recorded with lactose and $KNO_3$ containing medium.

Our results suggest that use of $KNO_3$ and lactose induced the protease production by the isolate G (*Aspergillus funiculosus*). Similarly, induction of protease production with different carbohydrates were also reported by Micales[12], Sabita *et al.*[14], Suseela[13] and Marzan *et al.*[15].

**Determination of optimum conditions for the maximum protease crude activity:** To study the optimum temperature and pH for maximum activity of the proteases, the crude enzymes were collected and enzyme activity was assayed with casein as substrate at various temperature ranged from 30 to 50°C and pH ranged from 4.5 to 8.5. The maximum enzyme activity was recorded at 35°C in pH 7.5 (Table 4) during enzyme substrate reaction with crude enzymes of the isolate G (*Aspergillus funiculosus*). Similar protease activities at acid to neutral pH were reported by many workers[16-19].

**Molecular weight:** Using ammonium salt precipitation and fractional separation method the active protein (enzyme) thus obtained was subjected for molecular weight determination. The molecular weight of the crude enzyme of the isolate G (*Aspergillus funiculosus*) was found to 51 kDa (Fig. 3 and 4).

Results of this study suggested that *Aspergillus funiculosus* produce maximum protease when the incubation temperature is 37°C and the pH is 7.0 with the carbon source lactose and nitrogen source $KNO_3$. Proteases from the *Aspergillus funiculosus* were found to show active at 35°C temperature at 7.5 pH.

So, from the present study we can conclude that optimum pH, temperature, incubation period, carbon and nitrogen sources are the important limiting factors for the maximum protease production as well as enzyme activity. Further works with the split of each factor and interactions of factors may provide clear picture about maximum protease production and optimum protease activity of our isolates, which is variable for species to species.

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