CHARACTERIZATION OF PROTEASE PRODUCING FUNGI \textit{ASPERGILLUS FUNICULOSUS} AND \textit{A. TAMARII}, AND THEIR PROTEASES.

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ABSTRACT

Two \textit{Aspergillus} spp. namely \textit{A. funiculosus} (Z$_a$) and \textit{A. tamarii} (Z$_c$) were isolated, purified, identified and studied for their protease activity under some selected environmental condition and nutritional factors. The strains showed the maximal activity of protease after 5 days (Z$_a$) and 6 days (Z$_c$) of incubation at 27ºC in a medium containing peptone as nitrogen source with pH 5.0. Isolate $Z_a$ showed highest protease activity in the presence of fructose as carbon source, at 35 0C, with pH 6.0 and 2.5% casien as substrate concentration. Where as for $Z_c$ it was starch as carbon source, temperature 40 0C, with pH 7.0 and 2% casien, the isolate showed highest enzyme activity.

\textbf{Key words:} \textit{Aspergillus funiculosus}, \textit{Aspergillus tamarii}, Protease.

INTRODUCTION

Proteases are complex group of enzymes collectively known as peptidyl-peptide hydrolase. These may include proteinases and peptidases that hydrolyze the peptide bonds in protein molecules. Microbial proteases have to a certain extent replaced the traditional proteases from animal and plant origin and in addition, have found further applications due to their special properties and types namely acid, neutral and alkaline(Dilip \textit{et.al}. 2004). A number of fungi produce proteases in an appreciable good yield Thus the large scale production of fungal proteases has legitimately made use of a large cross section of fungal species; such as \textit{Mucor delemar}, \textit{Aspergillus flavus}, \textit{Aspergillus oryzae}, \textit{Aspergillus awenti} \textit{Aspergillus funiculosus}, \textit{Aspergillus tamarii}, \textit{Amylomyces rouxi} Proteases account for a major share of global enzyme market. They have been used for the production of value-added products in diverse fields in various industries such as Detergent industry, Food and Dairy industry, Lather industry, Medical industry etc. Proteases are also known for their catalytic use in synthetic chemistry. It can solubilize proteinaceous wastes and so lower the BOD of waste systems. For the

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above concern the present study was conducted for the isolation, identification and characterization of protease producing fungi and their proteases.

MATERIALS AND METHODS

Microorganisms
The isolates *Aspergillus funiculosus* and *A. tamarii* were isolated, purified and identified from spoiled pulse seeds.

Isolation and screening of the isolates
For the isolation of proteolytic microorganisms enrichment technique was followed. Primary screening was done by three methods - Egg albumin degradation, Skimmed milk casein hydrolysis and Gelatin hydrolysis. After primary selection, the isolates were examined for the protease activity in liquid medium.

Measurement of Enzyme Activity
Protease activity was determined by the modified method of Hayashi *et al.* (1967), as followed by Meyers and Ahearn (1977). Three ml of culture filtrates, 3 ml of citrate phosphate buffer and 3 ml of 1% casein were mixed and incubated at 35°C in a water bath. After reaction, 5 ml of 20% TCA was added with the solution to stop the reaction. After one hour, the solution was filtered by Whatman no. 40 (Ashless). One ml enzyme substrate mixture was taken into a test tube and 2 ml of 20% Na₂C₀₃ and 1 ml of Folin Ciocalteu Reagent was added and mixed well. Six ml distilled water was added to it and after 30 minutes absorbance of the solution was measured at 650 nm in a spectrophotometer and calculated the amount of amino acids released from a standard curve plotted from known concentration of tyrosine. Enzyme activity was expressed in Unit, which was defined as the amount of enzyme that releases 1 µg of tyrosine /ml of crude extract /hour.

Biomass Yield
Biomass was determined by dry weight method.

Optimization of Culture Conditions
A. Effect of incubation time, temperature and medium pH
To observe the effect of culture conditions the organism were studied at different incubation time (3, 4, 5, 6 and 7 days), at different pH (5.0, 6.0, 7.0, 8.0 and 9.0) and temperature (30±2°C, 37±2°C, and 45±2°C). Biomass characteristics, biomass yield and protease production was also recorded.
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B. Effect of carbon sources
The production of extracellular proteases using different carbon source and the original nitrogen source (peptone) was studied in the liquid culture medium (Matta et al. 1975). Five carbon sources (sucrose, dextrose, fructose, starch, and maltose) were added to the medium and their effect on the production of protease, extracellular protein and biomass yield were recorded.

Factors affecting enzyme activity
Temperature and pH
The effect of pH on protease activity was determined by incubating the reaction mixture at pH values ranging form 5.0 to 9 using citrate phosphate buffer. Optimum temperature for enzyme activity was determined by conducting the assay at various temperatures from 35 to 45±2°C during enzyme substrate reaction.

Enzyme substrate reaction time
To ascertain the effect of incubation time on enzyme activity the enzyme substrate reaction mixture were incubated for different incubation time i.e. 10, 20, 30, 40, 50, 60, 70, 80 and 90 minutes and the results were recorded.

Substrate concentration
The effect of substrate concentration was measured using different concentrations of casein (i.e. 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0%)

Substrate specificity
Four different types of natural proteins such as Casein, Egg albumin, BSA and Gelatin were used to observe the substrate specificity and enzyme activity was measured separately for each substrate.

RESULTS AND DISCUSSION
Using enrichment techniques 16 microbial isolates were isolated from spoiled pulses. These isolates were purified, preserved and tested for their proteolytic activity.

Screening and identification of selected isolates
Among the isolates the fungal isolate $Z_a$ and $Z_c$ exhibited higher proteolytic activity in broth medium and were selected for further studies. The isolates were identified on the basis of their colony color, spore size, shape, arrangement, different types of conidiophores and sporangiophores, presence or absence of other special morphological features etc. The isolates were found to
belong to the genus *Aspergillus*, and closely related to the species *Aspergillus funiculosus* G. Smith and *Aspergillus tamarii* kita G. Smith while compared with the standard description given by Gilman, 1957.

**Optimization of Different Cultural Conditions**

**Effects of incubation time**

The fungal isolates showed varied degrees of protease activity at different incubation periods (Fig: 1a). The isolate Zₐ (*Aspergillus funiculosus*) showed maximum activity after 5 days of incubation period. It was also reported by Shumi *et.al* (2004) and the isolate Zₖ (*Aspergillus tamarii*) showed maximum enzyme production after 6 days of incubation.

**Effects of temperature**

Both the isolates Zₐ and Zₖ showed maximum protease activity and highest biomass yield at 27°C(Fig:1c).

**Effects of pH**

Maximum protease production was recorded at pH 5.0 and 6.0 by the fungal isolates Zₐ and Zₖ respectively (Fig: 1b).

**Effect of carbon source**

In the present study effect of carbon source were tested by using peptone as nitrogen source. Different carbon sources such as sucrose, fructose, maltose, starch, and dextrose were used. The isolate Zₐ showed maximum protease activity when fructose was used and isolate Zₖ showed maximum activity when starch was used as a carbon source(Fig:1d)


**Factors involved in enzyme activity**

Enzyme activity depends upon the pH, temperature, incubation time, substrate concentration and many others factors. So it is necessary to find out different limiting factor for maximum activity of proteases.

The crude enzyme of fungal isolates Zₐ (*Aspergillus funiculosus*) showed highest protease activity at 40°C for 70 minutes incubation and 80 minutes at 40°C for Zₖ (*Aspergillus tamarii*) (Fig:2). The isolates were allowed to react with different substrate concentrations (0.5 to 3%) and maximum activity was found with 2.5% for Zₐ and 2.0% for Zₖ, while casein was used as
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substrate(Fig:3). Temperature and pH are also most important limiting factors, which markedly influenced enzyme activity. Maximum protease activity of crude enzyme extract of fungal isolate Z_a was recorded at reaction mixture temperature 35°C and pH was 6.0(Fig:4), where as for Z_c temperature was 40°C and pH 7.0(Fig:5). Similar work at acid to neutral pH and different temperature was also done by Shumi et al. 2004, and Das et al. 2005.

Using Aspergillus spp. many researchers (Chao and Gruen.1987, Channe and Shewal.1998, Liu et al.1998, Shumi et al.2004 and Hossain et al. 2006) worked to understand the nature of protease enzyme and their catalytic potentiality. From the present study, it can be said that both the species A funiculosus and A tamarii showed a little difference in their protease activity. Further investigation on these two species can possibly reveal their potentiality as a source of protease for any biotechnological approach.

FIG.1: EFFECTS OF DIFFERENT FACTORS ON THE PRODUCTION OF PROTEASE BY A. FUNICULOSUS (Z_a) AND A. TAMARI (Z_c). (A) INCUBATION TIME (B) MEDIUM PH (C) INCUBATION TEMPERATURE (D) DIFFERENT CARBON SOURCES USING PEPTONE AS NITROGEN SOURCE.
FIG. 2: EFFECTS OF INCUBATION TIME ON PROTEASE (CRUDE) ACTIVITY OF THE ISOLATES A. FUNICULOSUS (ZA) AND A. TAMARI (ZC).

FIG. 3: EFFECTS OF SUBSTRATE CONCENTRATION (%) ON PROTEASE (CRUDE) ACTIVITY OF THE ISOLATES A. FUNICULOSUS AND A. TAMARI.

FIG. 4: EFFECTS OF PH AND TEMPERATURE ON PROTEASE (CRUDE) ACTIVITY OF THE FUNGAL ISOLATE A. FUNICULOSUS (Z_A).

FIG. 5: EFFECTS OF PH AND TEMPERATURE ON PROTEASE (CRUDE) ACTIVITY OF THE FUNGAL ISOLATE A. TAMARI (Z_C).

REFERENCES


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