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Production of Protease by *Aspergillus flavus* Through Solid State Fermentation Using Different Oil Seed Cakes

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Abstract: *Aspergillus* species are known to produce various types of proteases. The isolated *A. flavus* was shown to be a better good producer of protease at pH 7.5 and 45°C temperature indicating slight thermo stability. Various metal ions were shown to be effective activators of protease activity and PMSF had given approximately 50% inhibition. Among the selected oil seed cakes sesame oil was shown to be a suitable substrate after wheat bran for the production of protease by *A. flavus*.

Key words: Protease · Ground Nut Oil Cake · PMSF · Mineral Medium.

INTRODUCTION

Proteases is are essential constituents of all forms of life on earth including prokaryotes, fungi, plant and animals and are highly exploited enzymes in various industries, representing worldwide sale at about 60% of total enzyme market [1]. Extracellular enzymes are usually capable of digesting insoluble nutrient materials such as cellulose, proteins and starch [2]. Proteases of fungal origin have an advantage over bacterial protease as mycelium can be easily removed by filtration. Protease produced by *Aspergillus* sp. is of greater importance due to its higher protease producing ability.

Solid-state fermentation (SSF) has many advantages including superior volumetric productivity, use of inexpensive substrate, simpler downstream processing, lower energy requirement and low wastewater output [3] and the production of enzyme using different oil seed cakes is having a commercial importance and highly cost effective. This procedure of protease production with the use of low cost substrate and cost effective method allows possible production and application of crude enzyme for various industrial processes [4]. For the production of enzymes for industrial use, isolation and characterization of new promising strain is a continuous process [5]. These are generally produced by solid state fermentation. Solid state fermentation has been established as a superior technique for the production of enzymes [6]. Solid state fermentation involve microbial modification of a solid, undissolved substrate in which microbial cultures are grown on moist solid with little or no free water, although capillary water may be present [7]. The product can be recovered in highly concentrated form as compared to those obtained by submerged fermentations. It doesn't only provide a natural substrate for fungal growth and fermentation, but also results in improved value of these agro-industrial residues [8].

The environmental conditions of the fermentation medium play a vital role in the growth and metabolic production of microbial population.The most important among them are medium pH and incubation temperature. The pH of fermentation medium was reported to have a substantial effect on production of proteases [9]. The purpose of this study was to isolate and produce protease from fungi and study some properties of this protease.

MATERIALS AND METHODS

Isolation and Characterization of the Studied Fungus: A fungus was isolated on PDA plates from soil samples collected from Sri Ramakrishna Degree College surroundings, Nandyal, India and subjected to serial dilution. The fungus identification was done using microbiological atlas [10]. This fungus was further maintained on PDA slants for further use.

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Substrates and Diluents: Five oil seed cakes selected including sesame oil cake; ground nut oil cake; cotton seed oil cake and mustard oil cake along with wheat bran were used as substrates in this study. The composition of moistening agent (mineral medium) in percentage included 0.5 ammonium nitrate (NH_4NO3), 0.2potassium dihydrogen phosphate (KH_2PO4), 0.2 magnesium sulphate (MgSO4) and 0.1sodium chloride (NaCl) in water.

Production of Protease Using Wheat Bran as Substrate: The wheat bran was used as substrate for the production and optimization studies of protease. Ten grams of wheat bran mixed with 15 ml of moistening agent after sterilization and cooed to room temperature. The slants of 5-7 days old culture was wetted by adding 10 ml of distilled water; a homogeneous suspension was obtained by shaking for approximately 1 min. One ml spore suspension was used for inoculation and was incubated for about 72 hrs in an incubator under room temperature. Protease activity was measured at different time intervals.

Preparation of Enzyme Extract: A 250 ml conical flasks containing 10 g of substrates with 15 ml of moistening agent were sterilized at 121°C (15 1bs/inch pressure), cooled, inoculated and incubated at 30°C for 72 hrs. After incubation, 80 ml of distilled water was added to the culture flask, flask was shaken for 14 hrs at 200 rpm. The content of flask was filtered and filtrate was analyzed for enzymatic activity[180.

Protease Assay: The Protease activity in the crude enzyme extract was assayed by using 1% casein in citrate buffer (pH 7). The reaction mixture contained 1ml casein and 1ml crude enzyme extract and was allowed to stand for 1hr at the room temperature. After 1hr, 5 ml trichloro acetic acid (TCA) solution was added to stop the enzymatic reaction. After addition of the TCA, the tubes were shaken and then contents were centrifuged at 10000 rpm for 15mins for the sedimentation ofthe pellet. The supernatant was collected from the centrifugal tubes and to this supernatant 5ml of NaOH solution was added and allowed to stand for another 15mins. Finally 0.5ml of Folin-Ciocalteu reagent (FC reagent) was added and the intensity of blue color was measured at 700nm within half an hour. One unit of enzyme activity is defined as the amount of the enzyme that releases 1µg of tyrosine mL^{-1} of crude enzyme per hour [4].

Optimization of Media Parameters: Production of protease from *Aspergillus flavus* was optimized by controlling different physico chemical parameters like carbon source, nitrogen source and other components in the medium like magnesium sulphate (MgSO4), Potassium chloride (KCl), Dipotassium hydrogen phosphate (K_2HPO4), pH range and temperature for the maximum yield of enzyme. The optimization experiments were conducted uniformly by varying one compound at a time and keeping the other conditions constant. Optimization studies were carried out at 24hrs, 48hrs and 72hrs.

Optimization of Substrate Concentration: Wheat brawn at different concentrations (2.5 to 12.5%) moistened with mineral medium was used for the determination of optimum concentration of substrate for the better production of protease.

Optimization of Nitrogen Source: Sodium nitrate was the nitrogen source in the medium. To determine the optimum concentration of Sodium nitrate ($NaNO_3$) varied concentrations of $NaNO_3$ were taken from 3 to 7%. Afterincubation protease activity was studied.

Optimization of KH_2PO_4 and $MgSO_4$: These two were also optimized by taking at different concentrations (0.1 to 0.3%) for the better production of protease.

Optimization of NaCl: To determine the optimum concentration of sodium chloride (NaCl), different concentrations of NaCl range 0.025% to 0.125% were taken and fermentations were carried out.

Optimization of Temperature: One of the most important factors is the incubation temperature, which is important for the production of proteases by microorganisms. For temperature optimization, different temperatures ranging from 25°C to 45°C were taken and the activity of protease was studied.

Optimization of pH: The pH can effect growth of microorganisms either directly or indirectly by affecting the availability of nutrients or directly by action on cell surfaces. The metabolic activities of microorganisms are sensitive to pH changes and the pH of culture media has marked effect on the type and amount of enzyme produced. Changes in pH may also cause denaturation of enzyme resulting in the loss of catalytic activity [10]. In the present study, the pH range for the optimization of pH selected varied from 6 to 8.

Effect of Activators and Inhibitors: Various chemicals were tested at 0.1M concentration as activators and inhibitors while assaying the protease activity.

The activators used were mostly metal ions like zinc sulphate ($ZnSO_4$), calcium chloride ($CaCl_2$) and ferrous suphate ($FeSO_4$) and the inhibitors included PMSF (phenyl methyl sulphonyl fluoride), SDS and EDTA.

RESULTS AND DISCUSSION

The protease was successfully produced from the isolated fungus, *A. flavus* using wheat bran as substrate. 10% of the substrates have given a maximum activity of 640 IU after 72 hrs of incubation after which the production has declined (Fig. 1).

Optimization of Media Parameters: Mineral media components viz. NH_4NO_3, KH_2PO_4, $MgSO_4$ and NaCl were optimized for the better production. 0.5, 0.2, 0.2 and 0.1% concentrations of the above salts had given maximum enzyme activity of 525, 515, 455 and 464 IU respectively.

Effect of Inhibitors and Activators: While studying the effect of activators it was observed that $ZnSO_4$ enhanced the protease activity up to 698IU followed by FeSO4 and CaCl2. The selected inhibitors were found to be less effective against the protease activity (Fig. 2) but the crude enzyme of *A. flavus* showed approximately 50% inhibition in the presence of PMSF indicating that the enzyme is an alkaline serine protease [10]. Work on serine protease has been reported by many researchers [11, 12].

Effect of Temperature and pH: To further enhance the production of protease pH and temperature were also optimized. Maximum production was obtained at 40°C and pH 7.5. Most alkaline proteases have been reported to have optimum activity in the pH range 8-9 [13]. Protease activity at 45°C was also reported by Shumi *et al.* [14] while working with the protease of *Fusarium tumidum.* Fungal proteases are usually thermolabile and show reduced activities at high temperatures [15]. Higher temperature is found to have some adverse effects on metabolic activities of microorganism [16] and cause inhibition of the growth of the fungus. The enzyme is denatured by losing its catalytic properties at high temperature due to stretching and breaking of weak hydrogen bonds within enzyme structure [17].

Production Using Different Oil Cakes: Among the selected oil seed cakes in place of wheat brawn it was found that none of them were shown to be effective substrates for the production of protease with *A. flavus* than wheat bran (Fig.1). To some extent the sesame oil cake was found to be an effective substrate after wheat bran. In a study by Rajmalwar and Dabholkar [18] soya bean oil gave higher production followed by sesame oil cake.

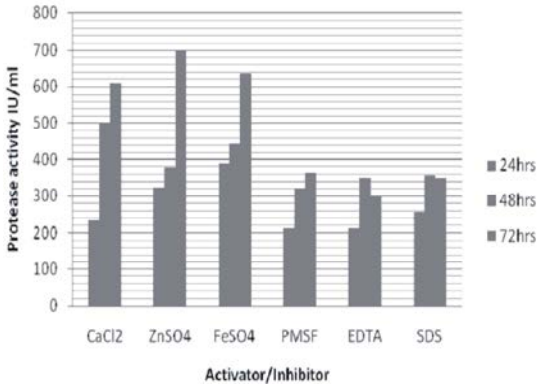

Fig 1: Effect of activators and inhibitors on enzyme activity

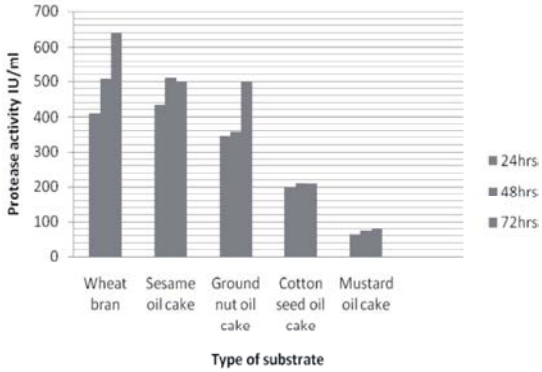

Fig 2: Effect of selected substrates on protease production.

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