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Comparative Production of Proteaseby Locally Isolated Aspergillus Species Using Different Oil Seed Cakes

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Abstract: Two strains of Aspergillus species; *Aspergillus flavus* and *Aspergillusoryzae* were evaluated and wheat bran based solid state fermentation was selected for protease production. Along with wheat bran, four oil seed cakes namely sesame oil cake, groundnut oil cake, cotton seed oil cake and mustard oil cake were selected and results showed that enzyme production was maximum when wheat bran was used as a substrate for both *A. flavus* and *A. oryzae*. With *A. oryzae*, groundnut oil cake was also found to be effective substrate after wheat bran. Various process parameters influencing protease production including temperature, pH and nutrient supplements were optimized under solid state conditions. After 72hrs, *A. flavus* was shown to be a good producer of protease at pH 7.5 and 40°C temperature and whereaswith *A. oryzae* the maximum activity was at pH 7.0 and 35°C temperature after which the production was declined in both the cases. Among the various activators used *A. flavus* protease activity was enhanced with $ZnSO_4$ but for *A. oryzae* the activity was increased with $FeSO_4$. In case of inhibitors EDTA inhibited the protease activity of *A. flavus*and PMSF inhibited *A. oryzae* protease activity.

Key words: Wheat bran · Oil cakes · PMSF and activators

INTRODUCTION

Proteasesareessential constituents of all forms of life on earth including prokaryotes, fungi, plant and animals and are highly exploited enzymes in various industries, representing a worldwide sale at about 60% of total enzyme market [1]. Extracellular enzymes are usually capable of digesting insoluble nutrient material 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 great importance due to its high 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 which 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 a highly concentrated form as compared to those obtained by submerged fermentations. It does not only provide a natural substrate for fungal growth and fermentation, butalso results in improved value of these agro-industrial residues [8]. The environmental conditions of the fermentation medium

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play a vital role in the growth and metabolic production of a microbial population.The most important among them are medium pH and incubation temperature. The pH of fermentation medium was reported to have substantial effect on production of proteases [9]. The purpose of this study was to isolate protease from high protease producing fungi; *A. flavus*and *A. oryzae*.

MATERIALS AND METHODS

Microorganisms and Maintenance of Culture: Aspergillus species were isolated from soil samples collected from Sri Ramakrishna Degree College surroundings, Nandyal, India. Identification of fungi was done [10]. The fungi were grown and transferred to potato dextrose agar slants. The cultures were maintained by weekly transfer onto fresh slants of PDA and stored in refrigerator at 4°C for further use.

Substrates: In the present study, wheat bran was used as a substrate. Along with wheat bran four oil seed cakes such as sesame oil cake; ground nut oil cake; cotton seed oil cake and mustard oil cake were selected and used as substrates.

Screening of Fungi for Protease Production: Wheat bran was used as the substrate for screening of neutral proteases. Ten grams of wheat bran was mixed with 15 ml of moistening agent after sterilization and cooling 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 hrsat room temperature. Protease activity was measured at different time intervals [4].

Fermentation Procedure: The fungalcellswere grown in Erlenmeyer flasks containing 250 ml medium. 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 72hrs. 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 [4].

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. 5ml of tri chloro acetic acid solution was added to stop the enzymatic reaction and the tubes were shaken and then contents were centrifuged at 10000 rpm for 15mins for the sedimentation of the 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 FolinCiocalteu reagentreagent was added and the intensity of the blue color was measured at 700nm within half an hour. One unit of enzyme activity is defined as the amount of enzyme that releases 1µg of tyrosine mL^{-1} of crude enzyme per hour under the assay conditions [11].

Optimization of Media Parameters: Production of protease from *Aspergillusflavus* and *Aspergillusoryzae*was optimized by controlling different physico chemical parameters like carbon source, nitrogen source and other components in the medium like $MgSO_4$, KCl, K_2HPO_4, 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 for about 72hrs.

Optimization of Substrate Concentration: Wheat bran at different concentrations (2.5 to 12.5%) moistened with mineral medium was used as substrate for both *Aspergillusflavus* and *Aspergillusoryzae* 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 $NaNO_3$ for both *Aspergillusflavus* and *Aspergillusoryzae* varied concentrations of $NaNO_3$ were taken from 0.3 to 0.7%. The protease production was studied after different incubation periods.

Optimization of KH_2PO_4 and $MgSO_4$: Different concentrations (0.1 to 0.3%) of KH_2PO_4 and $MgSO_4$ were used for the better production of protease with *A. flavus*and *A. oryzae* and protease activity was measured at different time intervals.

Optimization of NaCl: To determine the optimum concentration of NaCl, different concentrations of NaCl between 0.025 to 0.125% were taken and fermentation was carried out.

Table 1: Optimization of media parameters for protease production

Component	Concentration of the Component at which the activity was maximum	Enzyme activity of *Aspergillusflavus* (IU)	Enzyme activity of *Aspergillusoryzae* (IU)
NH_4NO_3	0.5	525	634
KH_2PO_4	0.2	515	660
$MgSO_4$	0.2	455	609
NaCl	0.1	464	605

Optimization of Temperature: One of the most important factors is the incubation temperature, which is important for the production of proteases by microorganisms [11, 12]. For temperature optimizationfor both *A. flavus* and *A. oryzae*, fermentation wascarried out for both fungi under different temperatures ranging from 25 to 45°C and the activity of protease was studied.

Optimization of pH: In the present study, protease production was studied under different pH ranging from 6 to 8 for both *A. flavus* and *A. oryzae*.

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 weremostly metal ions like $ZnSO_4$, $CaCl_2$ and $FeSO_4$ and the inhibitors included PMSF (phenyl methyl sulphonyl fluoride), SDS and EDTA.

RESULTS AND DISCUSSION

Theprotease was successfully produced from the isolated fungi, *A. flavus* and *A. oryzae* using wheat bran as substrate. 10% of the substrates gavea maximumactivity of 640 IU with *A. flavus* and 697 IUwith *A. oryzae* after 72 hrs of incubation after which the production declined to 400 IU in *A. flavus* and 657 IU in *A. oryzae*.

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 respectively gave maximum enzyme activity for both the species (Table1).

Effect of Temperature and pH: Maximum production was seen only after 72 hrs of incubation in both the organisms. With *A. flavus* Protease activity was 647 IU at 40ºC but in *A. oryzae* it was 685 IU at 35°C and after which the production was declined in both the cases (Fig. 1). After 72 hrs of incubation enzyme activity was 610 IU with pH 7.5 in *A. flavus* and in *A. oryzae* maximum activity was 721 IU with pH 7.0 (Fig. 2). 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 *Fusariumtumidum* Sherbakoff. Fungal proteases are usually thermo labile and show reduced activities at high temperatures [14]. High temperature is found to have some adverse effects on metabolic activities of microorganism [15] 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 [16].

Effect of Inhibitors and Activators on Protease Production: After 72 hrs of incubation *A. flavus* protease activity was enhanced with $ZnSO_4$ to 698 IU but in *A. oryzae* the activity was increased with $FeSO_4$ to 792 IU. Among the inhibitors used EDTA and PMSF were found to inhibit the enzyme activity with *A. flavus* and *A. oryzae*

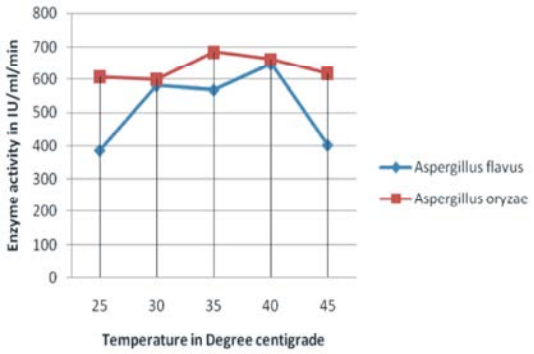

Fig. 1: Effect of temperature on protease activity

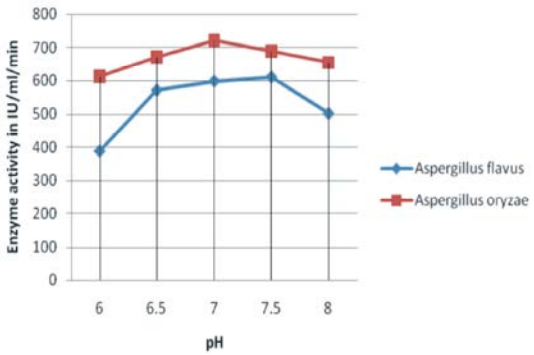

Fig. 2: Effect of pH on protease activity

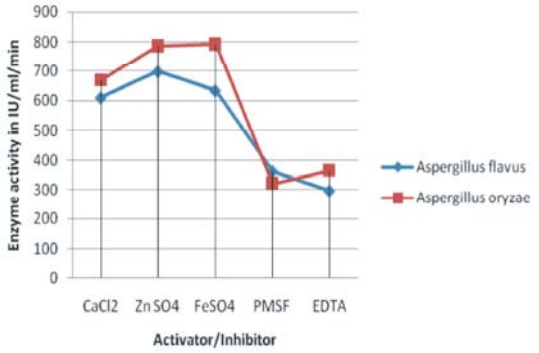

Fig. 3: Effect of activators and inhibitors on protease activity

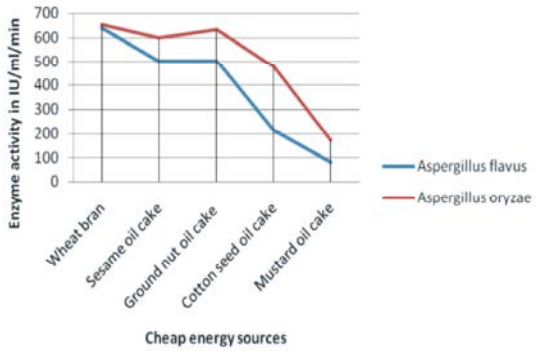

Fig. 4: Production of protease using different substrates

to 298 and 321 IU respectively. Work on serine proteases has been reported by many researchers [17, 18] (Fig. 3).

Protease Production Using Different Oil Cakes: Among the selected oil seed cakes in place of wheat bran it was found that none of them were shown to be effective substrates for the production of protease with *A. flavus* than wheat bran. With wheat bran the production was maximum with 640 IU after 72 hrs of incubation. In *A. oryzae* also maximum production was seen with wheat bran and to some extent the ground nut oil cake was found to be an effective substrate after wheat bran (Fig. 4). In a study by Rajmalwar and Dabholkar [19] soya bean oil gave highest production followed by sesame oil cake. It can be concluded that both species were good producers of protease. The Optimization studies have elevated the maximum capability of both organisms for production of protease. Under physico chemical conditions both the organisms were shown to be good producers of protease with selected cheap energy source.

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