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### RESEARCH ARTICLE

#### OPTIMIZATION STUDIES ON ALKALINE PROTEASE FROM *ASPERGILLUS TERREUS* ISOLATED FROM MARINE SOIL.

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#### Abstract

Extracellular proteases have high commercial value and multiple application in detergents, food, dairy, pharmaceutical, leather, diagnostics, waste management and several recovery industries. The *Aspergillus terreus* isolated from kottakoduru seashore, Nellore, was found to produce alkaline protease. Maximum enzyme production was recorded at pH 8 and temperature of 30 °C in a medium containing 10% wheat bran. ZnSO<sub>4</sub> is effective activator for protease activity and PMSF had given approximately 50% inhibition, indicating that it is a serine protease. Sesame oil cake proved to be suitable substrate after wheat bran for the production of protease by *Aspergillus terreus*.

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#### Introduction:-

Microbial enzymes gained much attention due to their industrial application in clean, environmental-friendly and cost effective biotechnological processes. A protease (E.C No.3.4) breaks down proteins. A protease is an enzyme that conducts proteolysis, that is, begins protein catabolism by hydrolysis of the peptide bonds that link amino acids together in the polypeptide chain forming the protein. Extracellular proteases are involved in the hydrolysis of proteins in the outer cell environment and enable the cell to absorb and utilize hydrolytic products (Joo et al., 2005).

Extracellular proteases have high commercial value and multiple application in detergent, food, dairy, pharmaceutical, leather, diagnostic, waste management and silver recovery. The microbial protease represents 60% of the worldwide market with sales of more than \$350 million annually (Kirk et al., 2002). Proteases are among the most important class of industrial enzymes, which constitute more than 65% of the total industrial applications, such as laundry detergent, i.e., help in removing protein based stains from clothing (Banerjee et al., 1999), leather preparation, meat tenderization, peptide synthesis, food industry, de-hairing process, pharmaceutical industry in contact lens eye cleaners and as an active component in enzymic debriders in view of its ability to digest various protein substrates, (Anwar and Saleemudin, 2000) as well as in bioremediation process (Gupta et al Roy, 2002; Oskouie et al., 2008).

Solid state fermentation (SSF) was chosen for the present study as it has been reported to be of more graded productivity than that of submerged fermentation (Ghildyal et al., 1985). Economically, SSF offers many advantages including superior volumetric productivity, use of simpler machinery, use of inexpensive substrates, simpler downstream processing, and lower energy requirements when compared with submerged fermentation (Paranthaman et al., 2009). Fungi elaborate a wide variety of proteolytic enzymes than bacteria. The filamentous fungi have a potential to grow under varying environmental conditions such as pH and temperature and utilizing a wide variety of substrates as nutrients (Haq et al., 2004). Several fungal species (*Aspergillus flavus*, *A. melleus*, *A. niger*,

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*Chrysosporium keratinophilum*, *Fusarium graminearum*, *Penicillium griseofulvum*, *Scedosporium apioserum*) and bacteria (*Bacillus licheniformis*, *Bacillus firmus*, *B. alcalophilus*, *B. amyloliquefaciens*, *B. proteolyticus*, *B. subtilis*, *B. thuringiensis*) are reported to produce proteases (Ellaiah et al., 2002).

Alkaline proteases find diagnostic and therapeutic applications. Among the total microbial proteases the most commercially important are the alkaline proteases, especially those from fungal source. The use of immobilized alkaline protease from microbial source having therapeutic properties was studied for the development of soft gel-based therapeutic formulas, gauze, non-woven tissues and bandage materials. Proteases from *Aspergillus oryzae* were orally used as a therapeutic and diagnostic aid to treat certain lytic enzyme deficiency syndromes. Despite the extensive research done on protease, the need of a novel protease with wide applications in industrial application is increasing day by day. In the present study the optimization of protease production by *Aspergillus terreus* was carried out.

### Materials and Methods:-

Soil samples were collected from 3 different sites of kothakoduru seashore area, Nellore dist, AP. The samples were collected randomly from one feet depth in seashore area. Serial dilutions were prepared using the representative composite soil samples collected from the sites, spread on the PDA plates and incubated for 72 hours at room temperature. The isolated colonies obtained were sub cultured on the slants and these pure cultures were used in this study.

### Qualitative Screening of Alkaline Protease:-

Skim milk agar medium was used for qualitative screening for alkaline protease production (Sharma et al., 2006). The medium comprises of skim milk powder 100 gm, peptone 5 g and agar 20 g per litre with pH 8.0. The isolated fungi from the pure slants were inoculated on to the skim milk agar plates and incubated at room temperature for 72 hours. After incubation the fungi which produce clear zone around the colony were selected and sub cultured and finally transferred to Potato dextrose agar slants and maintained at 4 °C. The positive fungal strain which produced maximum zone of clearance was morphologically identified as *Aspergillus terreus* and used in this study.

### Preparation of Enzyme Extract:-

Conical flasks (250 ml) containing 10 g of wheat bran (substrate) with 15 ml of moistening agent were sterilized at 121°C (15 lbs/inch pressure), cooled and inoculated with the fungal stain and incubated at 30 °C for 72 hrs. After incubation, 80 ml of distilled water was added to the flask, and was shake on a rotary shaker for 14 hrs at 200 rpm. The content of flask was filtered and filtrate was analyzed for enzymatic activity.

### 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 1 ml casein and 1ml crude enzyme extract and was allowed to stand for 1hr at the room temperature. After 1hr, 5 ml tri chloroacetic acid (TCA) was added to stop enzymatic reaction. After addition of the TCA, the tubes were shaken and then the contents were centrifuged at 10000 rpm for 15 min. To the supernatant 5ml of Na OH solution was added allowed to stand for another 15 min. Finally 0.5ml of Folin- ciocalteu reagent (FC reagent) was added and intensity of blue color developed was measured at 700 nm within half an hour using Spectrophotometer. The amount of enzyme produced can be calculated by standard graph of tyrosine. One unit of enzyme activity is defined as the amount of the enzyme that releases 1µg of tyrosine mL G<sup>-1</sup> of crude enzyme per hour.

### Optimization studies:-

Production of protease from *Aspergillus terreus* was optimized by controlling different physico chemical parameters like pH, temperature, metal ions together with different substrates for the better yield of enzyme. These optimization studies were carried out at different times of incubation viz., 24, 48, 72 and 96hrs.

### Standardization of substrate:-

Four oil seed cakes, including sesame oil cake, ground nut oil cake, cotton seed oil cake along with wheat bran were used as substrates in this study. The moistening agent (mineral medium) was prepared with the composition of 0.5% ammonium nitrate (NH<sub>4</sub>NO<sub>3</sub>), 0.2% Potassium di hydrogen phosphate (KH<sub>2</sub>PO<sub>4</sub>), 0.2% Magnesium sulphate (MgSO<sub>4</sub>) and 0.1% Sodium chloride (Na Cl) in water.

Among the four substrates, for the best proved substrate, the effective concentration was identified by screening the enzyme production at different concentrations (2.5 to 12.5%) of the substrate.

#### Optimization of temperature and pH:-

For temperature optimization, enzyme production at different temperatures ranging from 20 to 40°C was estimated. Similarly for pH optimization, enzyme production at different pH ranging from 6.5 to 8.5 was estimated at three incubation times.

#### Effect of activators and inhibitors:-

Various chemicals were tested at 0.1 M concentration as activators and inhibitors while assaying the protease activity. The activators used were metal ions like zinc sulphate (ZnSO<sub>4</sub>), Calcium chloride(CaCl<sub>2</sub>) and Ferrous sulphate (FeSO<sub>4</sub>) and the inhibitors included PMSF (phenyl methyl sulphonyl fluoride), SDS and EDTA.

#### Results and discussion:-

The use of natural and cheap substrate in the process of enzyme production has been investigated using various agro industrial products. Wheat bran has been reported to be a potent substrate among various agro by products used in different growth systems (SSF,SMF and Two phase system) by several workers (Kaur et al., 2001; Sumantha et al., 2005; Naidu and Devi, 2005). In the present study maximum protease production of 580 IU was recorded after 72 hrs incubation when 10% of wheat bran was used as fermentation medium. With further increase in substrate concentration the enzyme production decreases. Similar type of results were reported by Kranthi *et al.*,(2012) with *A. flavus* and *A. oryzae*. However Osman *et al.*, (2014) reported that 4% wheat bran is optimum for *Aspergillus terreus* and also reported that at 5% concentration there is a decline in enzyme production. Muthulakshmi *et al.*,(2011) reported that 3% wheat bran was optimum for *Aspergillus flavus*.

Next to the wheat bran, sesame oil cake was found to be effective substrate for maximum protease production. Oil cakes have been widely used as substrates for the production of industrial enzymes using fermentation process since they provide both carbon and nitrogen sources in the nutrient medium (Ramchandra *et al.*, 2007).

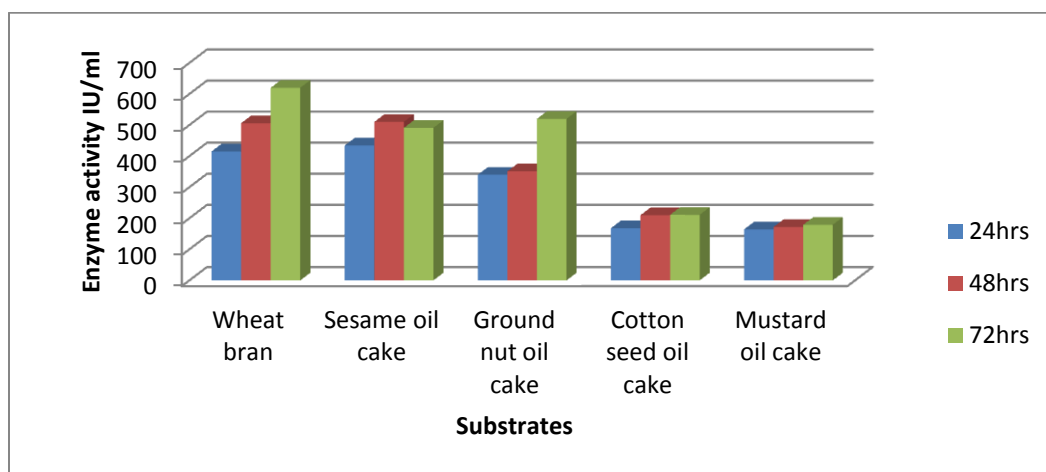


Fig 1:- Effect of different substrates on protease production by *Aspergillus terreus*

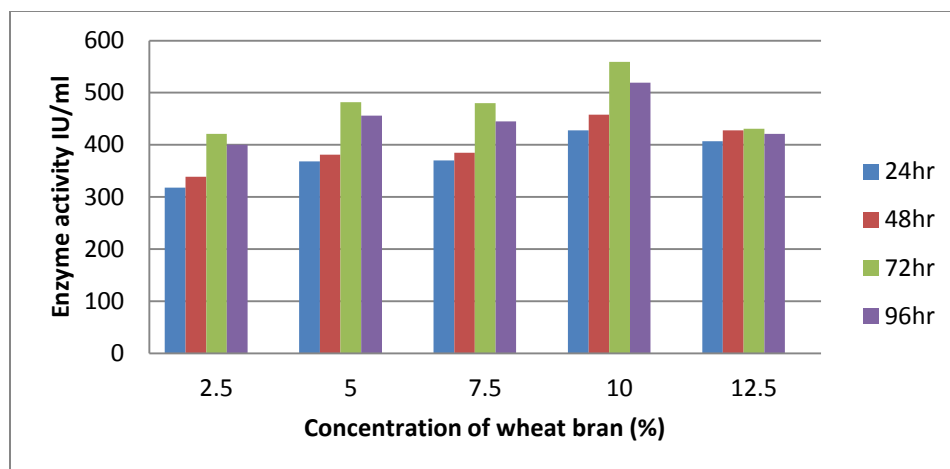


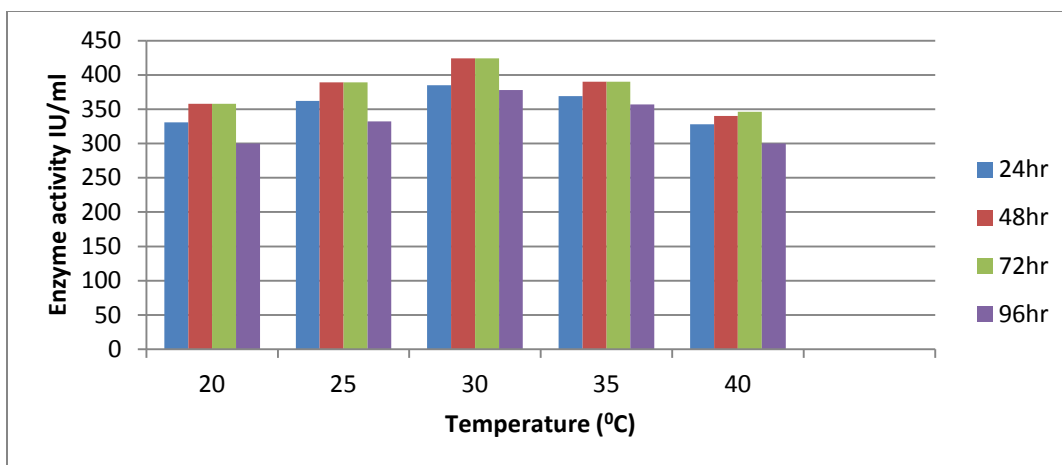
Fig. 2:- Effect of different concentrations of wheat bran on protease production by *Aspergillus terreus*

#### Effect of pH and Temperature:-

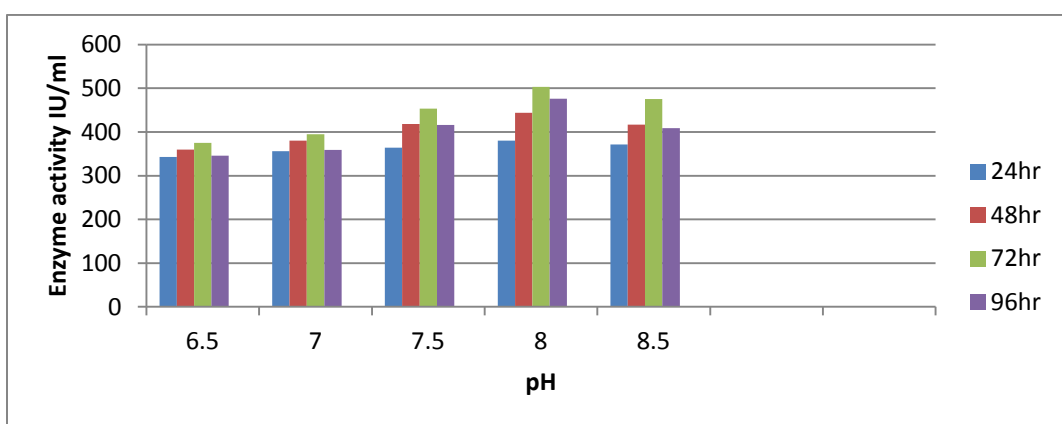
The important physical factors that determine rate of bioprocessing are pH and temperature. The productivity of enzyme greatly depends on pH of the medium. Therefore the effect of pH from 6.5 to 8.5 was studied for protease production by *Aspergillus terreus*. There was gradual increase in protease production from pH 6.5 to 8 and maximum production was observed at pH 8 with 510 IU. Similar results were reported by Gopal Kumar *et al.*, (2016), Chellapandi (2010) and Hussain *et al.*, (2010) in *A. terreus*. However acidic protease production by *A. terreus* was reported by Sethi *et al.*, (2015) and Chakrabarthy *et al.*, (2000) at 5.5 pH indicating the strain variation within the species.

Alkaline proteases reported to have optimum activity in the pH range 8-9. It was reported that protease production from microbial source can be acidic or alkaline depending on the type of organism and source of the isolation. Pagare *et al.*, (2009) reported an optimum pH of 8 for extracellular protease production from *Aspergillus niger* and *Bacillus subtilis*. According to Borris, (1987) alkaline protease production by microbes was found to be higher at pH range of 9-13. Osman *et al.*, (2014) reported that at pH 7 the maximum enzyme activity by *Aspergillus terreus* isolated from soil. Muthulakshmi *et al.*, (2011) also reported that at pH 7 the maximum enzyme activity by *Aspergillus flavus*.

In the present study the effect of temperature (20-40°C) was studied for protease production by *Aspergillus terreus*. A gradual increase in enzyme production was observed with increase in temperature up to 30 °C and reached the peak with production of 425 IU. Then it decreased and minimum production was recorded at 40°C (351 IU) which is almost similar to that observed at 20 °C. As a secondary metabolite this enzyme was produced during exponential phase of the growth, the incubation at high temperature could lead to poor growth and thus a reduction in enzyme yield (Sabu *et al.*, 2002). Chandrasekaran *et al.*, (2015) showed similar type of results. Osman *et al.*, (2014) reported that the purified enzyme from *Aspergillus terreus* was active at 55°C and stable till 45°C isolated from soil. Gopalkumar *et al.*, (2016) reported that the maximum protease activity at 60°C temperature for *Aspergillus terreus* BAB-346 isolated from paper mill area, Banana farm and poultry farm area. Niyonzima *et al.*, (2014) reported that higher enzyme activity at 50°C by *Aspergillus terreus*. Chellapandi (2010) reported that there was optimum enzyme activity for *Aspergillus flavus* and *Aspergillus terreus* at temperature of 45 and 60°C respectively, isolated from soil around leather industry. Sethi *et al.*, (2015) reported that optimum temperature for production of protease was 30 °C by *Aspergillus terreus* isolated from isolated from chuckling vetch. This clearly indicates that variation in temperature requirement for enzyme production is strain dependent.



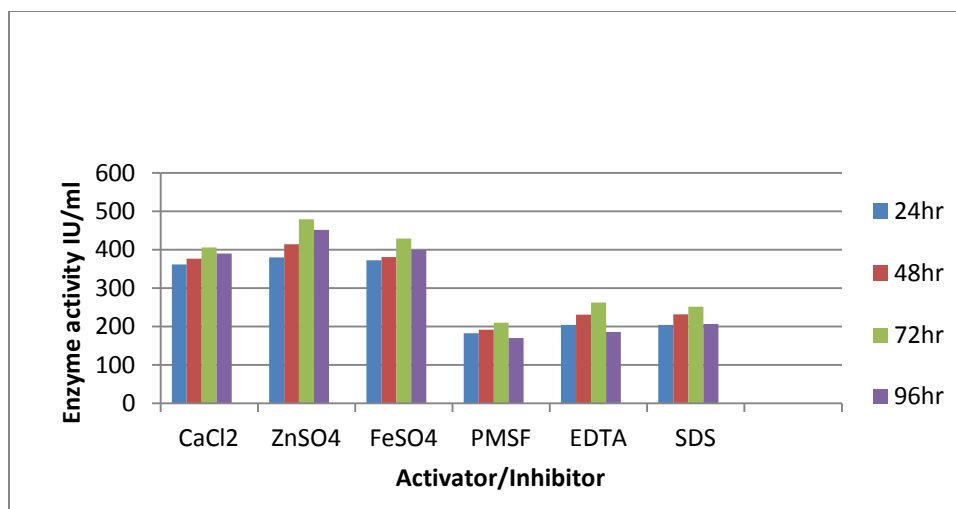
**Fig.4:-** Effect of temperature on protease production by *Aspergillus terreus*



**Fig.5:-** Effect of pH on protease production by *Aspergillus terreus*

#### Effect of inhibitors and Activators:-

While studying the effect of activators it was observed that  $ZnSO_4$  enhanced the protease activity up to 510 IU followed by  $FeSO_4$  (461 IU) and  $CaCl_2$  (412 IU). Gopalkumar *et al.*, (2016) reported that  $Zn^{2+}$  inhibited the enzyme activity up to 80%, but  $Cu^{2+}$  enhanced the enzyme activity up to 122% in *Aspergillus terreus* –BAB-346. Sethi *et al.*, (2015) reported that  $Fe^{2+}$  affects the enhanced production of protease by *Aspergillus terreus*. Sumantha *et al.*, (2005) reported that protease was activated through  $Ca^{2+}$ ,  $Fe^{2+}$  and  $Mg^{2+}$ . In the present study, PMSF was found to be an effective inhibitor for the production, indicating that the protease produced by *A. terreus* was a serine protease. Similar results were reported by Srilakshmi *et al.*, (2014) and Oman *et al.*, (2014). After PMSF, EDTA and SDS also inhibited the enzyme production. Chellapandi (2010) reported that the enzyme activity of *Aspergillus flavus* completely inhibited by EDTA. This clearly indicated the presence of serine metallo type protease.



**Fig-6:-** Effect of Activator/ Inhibitor on protease production by *Aspergillus terreus*

### Conclusions:-

*Aspergillus terreus* isolated from marine soil could produce serene metello type alkaline protease of 580 IU when 10% of wheat bran was used as substrate, at pH 8.5 and 30 °C temperature after 72 hrs of incubation.

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