

Optimization Studies of Alkaline Protease from *Aspergillus Flavus* Isolated From Seashore Soil of Bay of Bengal

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Abstract: Proteases constitute the most essential enzymes owing to their wide variety of functions and have immense applications in various industries viz., medical, pharmaceutical, biotechnology, leather, detergent, and food industries. An *alkaline protease*-producing strain *Aspergillus flavus* was isolated from soil samples of Kothakoduru seashore of Bay of Bengal, Andhra Pradesh, India and enzyme production was optimized under solid-state fermentation conditions. Different physical and chemical parameters such as pH, temperature, substrate concentration, and incubation time were optimized for better alkaline protease production. The maximum protease activity was found at a pH of 8.5 containing 10% wheat bran at 30°C, after 72 hours of fermentation. ZnSO₄ was an effective activator for protease activity, and EDTA had shown inhibition of enzyme activity. Among the different oil cakes used to produce the enzyme, the Sesame oil cake proved to be a suitable substrate after wheat bran for the production of protease by *Aspergillus flavus*.

Keywords: Alkaline protease, *Aspergillus flavus*, Temperature, Wheat Bran

Introduction

Proteolytic enzymes account for nearly 60% of the industrial market and find applications in many biotechnological processes, pharmaceutical industry, leather industry, detergent industry, etc. Such enzymes may be of commercial significance and hence, exploited to assist protein degradation in various industrial processes (Phadatar S.U., D. V. (1993)., Rao M.B., T. A. (1998)., Kumar C.G., a. T. (1999)., Gupta R., B. Q. (2002)). Proteases are associated with all the cellular functions in living organisms and distributed ubiquitously (Kirk *et al.*, 2002). Proteases are extracellular enzymes that can be produced by both submerged fermentation and solid-state fermentation. Solid-state fermentation is especially suited for the growth of fungi because of their lower moisture requirements compared with the bacteria. It is simple, low cost, and provides high yields of appropriate enzymes. Commercial proteases are produced exclusively from molds of the genera *Aspergillus*, *Penicillium*, and *Rhizopus*, as several species of these genera are generally regarded as safe. Proteases are capable of cleaving proteins into peptides and amino acids, and they are characterized by their optimal pH (acid, neutral or alkaline), their temperature, their ability to hydrolyze specific proteins (collagenase, keratinase, etc), their homology to well-characterized enzymes as chymosin, chymotrypsin, pepsin and trypsin (trypsin-like, pepsin-like, etc.), and their stability. The major uses of free proteases are in dry cleaning, detergents, meat processing, cheese making, silver recovery from photographic film, production of digestive and specific medical

treatments of inflammation and virulent wounds. Fungi elaborate a wide variety of proteolytic enzymes than bacteria. The filamentous fungi can grow under varying environmental conditions such as pH and temperature and utilize a wide variety of substrates as nutrients (Haq et al., 2004). Several fungal species (*Aspergillus flavus*, *A. melleus*, *A. niger*, *Chrysosporium keratinophilum*, *Fusarium graminearum*, *Penicillium griseofulvum*, *Scedosporium apiosermum*) and bacteria (*Bacillus licheniformis*, *Bacillus firmus*, *B. alcalophilus*, *B. amyloliquefaciens*, *B. proteolyticus*, *B. subtilis*, and *B. thuringiensis*) are reported to produce proteases. (Ellaiah et al., 2002).

Alkaline proteases of microbial origin possess considerable industrial potential due to their biochemical diversity and wide applications in tannery and food industries, medicinal formulations, detergents, and processes like waste treatment, silver recovery, and resolution of amino acid mixtures (Rao et al., 1998; Agarwal et al., 2004). The alkaline proteases find their most prominent use in the household laundry with a worldwide annual production of detergents of approximately 13 billion tons (Nehra et al., 2002). Alkaline proteases were the first enzyme produced in bulk. Alkaline proteases: Alkaline proteases (E.C.3.4.21-24, 99) from different sources have been viewed for their production, their role in decomposition, downstream processing and commercial applications have been reviewed by Sumantha et al., (2005). The proteolytic enzymes hydrolyze the peptide links of proteins to form smaller sub-units of amino acids and are produced both extracellularly and intracellular (Gajju et al., 1996; Kumar et al., 2002; Potumarthi et al., 2007). These industrial applications account for over 80% of the global market of enzymes (Van Oort, 2010). In the present study, an attempt has been made to produce and optimize the alkaline protease by *Aspergillus flavus* under various physiological conditions such as pH, temperature, and different oil seed cakes such as ground nut sesame oil cake and cottonseed oil cake.

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 the 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 skim milk powder 100 gm, peptone 5 g, and agar 20 g per liter with pH 8.0. The isolated fungi from the pure slants were inoculated onto the skim milk agar plates and incubated at room temperature for 72 hours. After incubation, the fungi that produce a 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 that produced the maximum clearance zone was morphologically identified as *Aspergillus flavus* 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 strain and incubated at 30 °C for 72 hrs. After incubation, 80 ml of distilled water was added to the flask and

shook on a rotary shaker for 14 hrs at 200 rpm. The content of the flask was filtered, and the 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 room temperature. After 1hr, 5 ml trichloroacetic acid (TCA) was added to stop the enzymatic reaction. After the 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 1 μ g of tyrosine mL G-1of crude enzyme per hour.

Optimization studies

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

Standardization of substrate

Four oil seed cakes, including sesame oil cake, groundnut oil cake, cottonseed oil cake, along wheat bran, were used as substrates in this study. The moistening agent (mineral medium) was prepared with the composition of 0.5% ammonium nitrate (NH₄NO₃), 0.2% Potassium di hydrogen phosphate (KH₂PO₄), 0.2% Magnesium sulphate (MgSO₄) and 0.1% Sodium chloride (Na Cl) in water. Among the four substrates, the effective concentration was identified for the best-proved substrate 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 45⁰C 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 (ZnSO₄), Calcium chloride(CaCl₂) and Ferrous sulfate (FeSO₄) and the inhibitors included PMSF (phenyl methyl sulphonyl fluoride), SDS and EDTA.

Results and discussion

The use of natural and cheap substrates in enzyme production has been investigated using various agro-industrial products. Wheat bran has been a potent substrate among various agro by-products used in different growth systems (SSF, SMF, and Two-phase systems) by several workers (Kaur et al., 2001; Sumantha et al., 2005; Naidu and Devi, 2005). In the present study, maximum protease production of 76 IU has recorded after 72 hrs incubation when 10% of wheat bran was used as fermentation medium. With a further increase in substrate concentration the enzyme production decreases. Similar results were reported by Kranthi et al. (2012) and Mulimani and patil (1999). Chinnasamy muthulakshmi (2011) reported that protease production in fermentation medium was maximum when 3% wheat bran was used. Malathi and chakraborty (1990) also reported that wheat bran was the effective substrate for protease production by *Aspergillus flavus*. However, Osman et al, (2014) reported that 4% wheat bran is optimum for *Aspergillus terreus* and also reported that at 5% concentration, there was a decline in enzyme production. Next to the wheat bran, sesame oil cake was an effective substrate for maximum protease production. Oil cakes have been widely used as substrates for industrial enzymes using the fermentation process since they provide both carbon and nitrogen sources in the nutrient medium (Ramchandra et al., 2007).

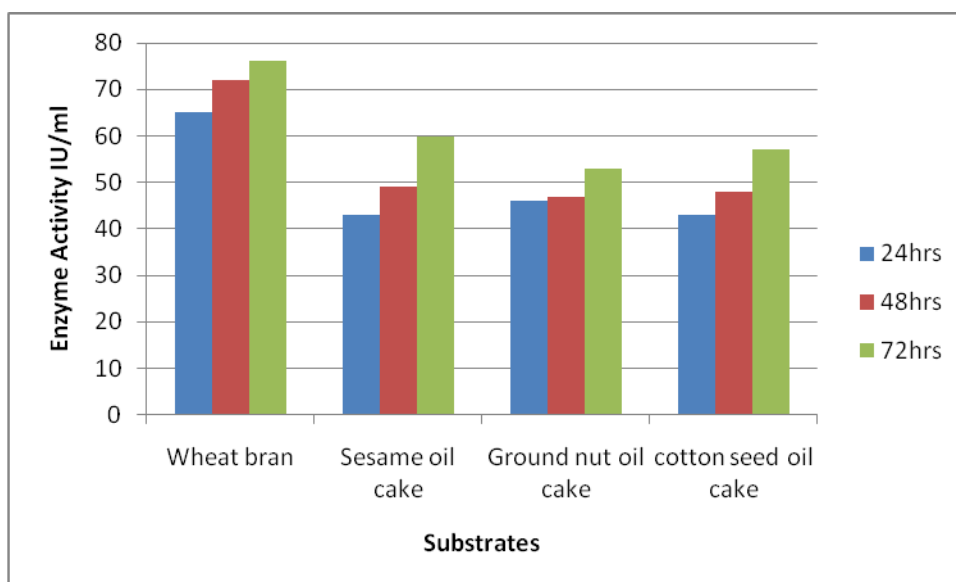


Fig 1 Effect of different substrates on protease production by *Aspergillus flavus*

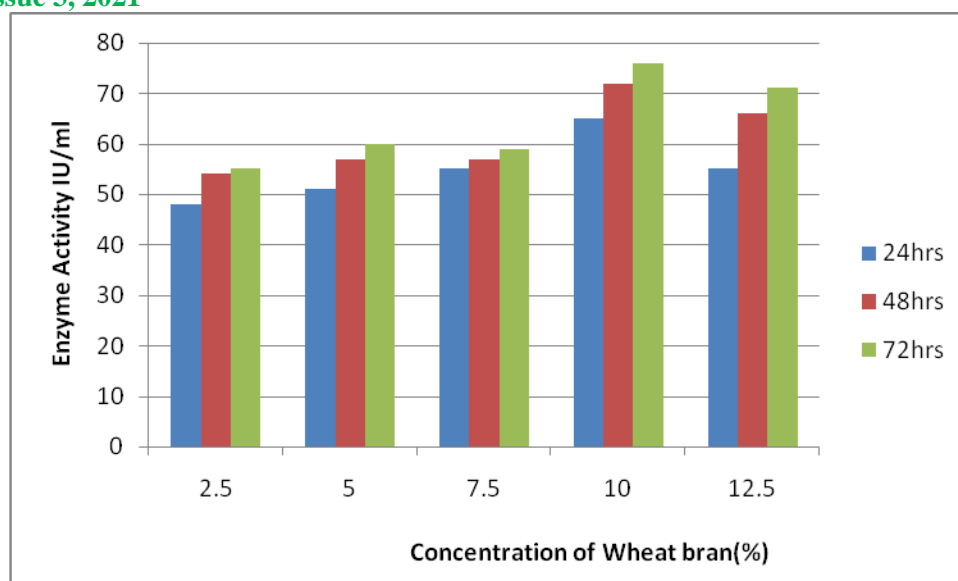


Fig. 2 Effect of different concentrations of wheat bran on protease production by *Aspergillus flavus*

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 flavus*. Maximum protease production was observed at pH 8.5 with 76 IU. Chinnasamy muthulakshmi (2011) reported that optimum pH was 4 for protease production in *Aspergillus flavus*. Chelapandi (2010) recorded maximum protease activity at pH 9-11 in *Aspergillus flavus*. Kranthi et al.,(2012) and Mulimani et al., (2002) and Malathi and Chakraborty (1990) reported that *Aspergillus flavus* was shown maximum proteolytic activity at pH 7.5. Subha et al., (2012) and Sankeerthana et al.,(2013) reported that maximum enzyme production at pH 7. Roshni choubey et al.,(2016) ,Oyeleke et al.,(2010) and Hossain et al., (2006) reported that in *Aspergillus flavus* ,alkaline protease production was found to be highest at pH 8. However Oseni (2011) reported that at pH 7 *Aspergillus flavus* was good producer for protease. Vishwantha et al. (2010) reported that pH-5 as the best initial pH for production of protease from *A.oryzae*. Ruann (2014) reported that biochemical characterization of a protease from *A.oryzae*, the enzyme was most active over the pH-5-5.5. LiJung Yin et al., (2013) reported an optimum pH of 3 for *A.oryzae*.

The present study studied the effect of temperature (20-40°C) for protease production by *Aspergillus flavus*. A gradual increase in enzyme production was observed with increase in temperature up to 30°C and reached the peak with production of 76 IU. Then it decreased and minimum production was recorded at 40°C (47 IU) which is almost similar to that observed at 20°C. Oyeleke et al.,(2010) and Chinnasamy muthulakshmi (2011) showed similar type of results. Chelapandi (2010) reported that optimum temperature for production of protease was found as 45-60°C. Kranthi et al., (2012) reported that *Aspergillus flavus* was good producer of protease at the temperature of 45°C. Roshni choubey et al.,(2016) recorded that in *Aspergillus flavus*, alkaline protease production was found to be highest at temperature of 28°C. Sankeerthana et al.,(2013) reported that maximum protease production at 37°C. Bommi .V.Subha et al., (2012) and Malathi and Chakraborty (1990) reported that the optimum temperature for protease activity produced by *Aspergillus flavus* was 35°C and

32°C respectively. However Oseni (2011) recorded that maximum protease production at 60°C. 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 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*.

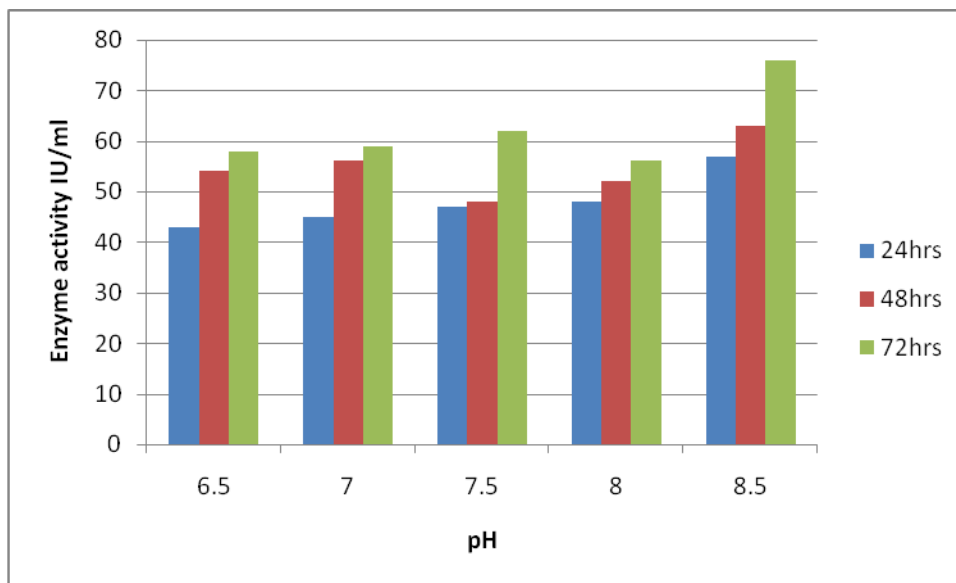


Fig.3 Effect of pH on protease production by *Aspergillus flavus*

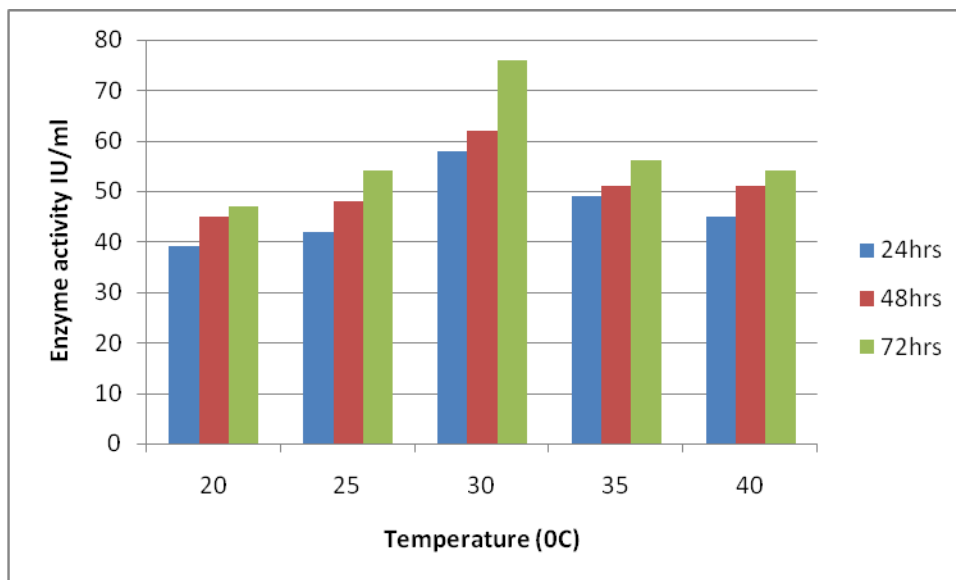


Fig.4 Effect of temperature on protease production by *Aspergillus flavus*

Effect of inhibitors and activators

While studying the effect of activators it was observed that ZnSO₄ enhanced the protease activity up to 82 IU followed by FeSO₄ and CaCl₂. A similar type of results was reported by Kranthi et al., (2012). Chinnasamy muthulakshmi (2011) studied the effect of various metal ions on activity of protease from *Aspergillus flavus* was shown that the metal ions Zn⁺⁺ and Cu⁺⁺ supported the

maximum enzyme activity where as Na^+ , Ca^{++} drastically inhibited the protease activity particularly Mg^{++} was found to be potent inhibitor of protease. In the present study, EDTA was found to be an effective inhibitor for the production. Chelapandi (2010) and Sankeerthana et al., (2013) reported similar type of results. Roshni choubey et al., (2016) recorded that in *Aspergillus flavus*, alkaline protease production was found to be highest with Mn^{++} ion. Salihi et al., (2017) reported that SDS decreased enzyme activity in *A.oryzae*. Sethi et al., (2015) reported that Fe^{2+} affects the enhanced protease production by *Aspergillus terreus*. Sumantha et al., (2005) reported that protease was activated through Ca^{2+} , Fe^{2+} and Mg^{2+} . Oludumila et al., (2015) reported that EDTA, Cu^{++} , Fe^{++} , Mg^{++} and Ca^{++} inhibited and Na^+ enhanced the alkaline protease activity produced by *Aspergillus niger*.

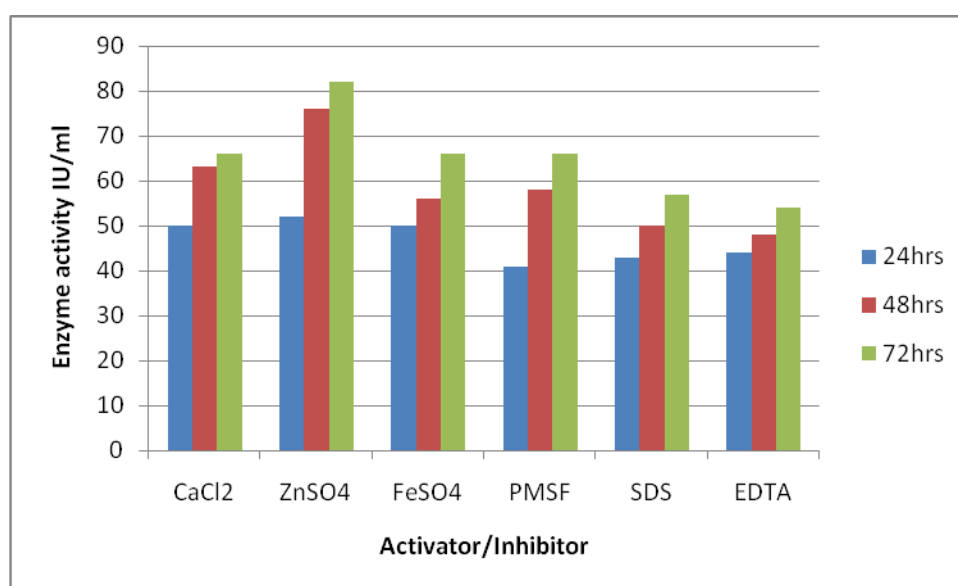


Fig-5 Effect of Activator/ Inhibitor on protease production by *Aspergillus flavus*.

Conclusion

Protease enzymes are of great industrial importance. This study indicates that *Aspergillus flavus* is a potent protease producing fungi. The alkaline protease production with *Aspergillus flavus* was found to be highest when 10% of wheat bran was used as the substrate, at temperature (30°C), pH 8.5, incubation time 72 hrs. The present study results demonstrate that seashore soil can be used as potential source for isolating alkaline protease-producing fungi.

Conflict of Interest: Authors are declared no conflict of interest.

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