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Production of alkaline protease from *Aspergillus oryzae* isolated from seashore of Bay of Bengal

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Abstract
Aspergillus oryzae isolatedon Potato dextrose agar from soil samples of kottakoduru seashore of Bay of Bengal, Andhra Pradesh, India seashore of Bay of Bengal by spread plate method and was screened for alkaline protease production on Skim milk containing agar plates and identified by clear zones of protein hydrolysis around colonies. Different physical and chemical parameters such as pH, temperature, substrate concentration and incubation time were optimized for the better production of alkaline protease. The maximum protease activity was found at pH of 8 containing 10% wheat bran at 30^0C, after 72 hours of fermentation.$ZnSO_4$was effective activator for protease activity and sodium dsulphate had shownmore than 50% inhibition of enzyme activity. Among the different oil cakes used for the production of enzyme the Sesame oil cake proved to be suitable substrate after wheat bran for the production of protease by *Aspergillus oryzae.*

Keywords: Alkaline protease, *Aspergillus oryzae*, Temperature, Wheat bran

INTRODUCTION

Proteases are enzymes that break down protein molecule through peptide bond hydrolysis.The molecular weight of protease range from 18-90 kilo Daltons(Lester and Sidney, 1972). Microbial proteases are preferred than plant and animal sources in view of various advantages. A variety of microorganisms such as bacteria, fungi, yeast and actinomycetes are known to produce these enzymes (Madan *et al.*,2002). The genera *Aspergillus, Penicillium* and *Rhizopus* are especially useful for producing proteases, as several species of these genera are generally regarded as safe (Sandhya *et al.*, 2005).

Protease, a hydrolytic enzyme shares 60% of total worldwide sale of industrial enzymes (Godfrey *et al.*,1996). A large proportion of commercially available proteases are currently derived from bacillus strains. Fungal proteases offer a distinct advantage over bacterial enzymes in terms of ease of down stream processing. The microbial proteases of *Aspergillusoryzae* in particular, have been studied in detail since they are known for their capacity to secrete high levels of enzymes in their growth environment. Several of these secreted enzymes, produced in a large-scale submerged fermentation, have been widely used in the food and beverage industry for decades (Salihi *et al.*, 2017).

Fungi elaborate a wide variety of proteolytic enzymes than bacteria. The filamentous fungi have a potential to grow under various environmental conditions of time, pH and temperature, utilizing a wide variety of substrates as nutrients (Ikram *et al.*, 2006). Fungal proteases have attracted the attention of environmental biotechnologists because fungi can grow on low cost substrates and secrete large amountof enzymes into culture medium which could ease downstream processing (Anitha and Palanivelu, 2013).

In the last three decades there has been a spectacular rise in the production of industrial enzymes. The growth of industrial enzyme market has expanded to nearly 85 enzymes, which are currently in commercial production. With the discovery of a variety of new and more active enzymes, the enzyme market reached US $ 2.5- 2.8 billion in 2006 (Mohana *et al.*, 2012). The protease enzyme constitutes two thirds of total enzymes used in various industries and this dominance in the industrial market is expected to increase as year gone-by (Gupta *et al.*, 2002).

Proteases are widely used in several industrial sectors such as detergent, food, pharmaceutical, chemical, leather, silk, cleaning contact lenses/

denatures, dewooling of animal skin, recovery of soluble proteins and amino acids from chrome leather wastes and waste treatment etc (Malathi and Chakraborty, 1991; Rajkumar *et al.*, 2011). Protease is one of the most important commercial enzymes and is used in food processing, detergents, dairyindustry and leather making (Negi andBenerjee, 2006). The use of enzymes in textile industry is one of the most rapidly growing field in industrial enzymology.

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 intracellularly (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). Industrial enzymes are widely accepted in food and beverage applications, owing to their functional properties. They play an important role in determining the desired attributes such as taste, mouthfeel, texture, appearance, and flavor in food & beverage applications and are also used for the production of biofuels. Next-generation enzymes such as psychrozymes have expanded the application areas of industrial enzymes in the food & beverage, animal feed, textile & leather, and biofuel sectors, thus driving the overall market for industrial enzymes.

Alkaline proteases are generally produced by submerged fermentation. In addition solid state fermentation has been exploited to a lesser extent for production of these enzymes (Kumar *et al.*, 1999). Solid-State fermentation has several advantages over submerged fermentation, especially in the economic production of microbial enzymes using agro industrial waste materials as substrates (Pandey, 2003). Though several studies have revealed that fungal strains are much more suitable for solidstate fermentation than bacterial strains, success in the production of microbial products is also achieved with bacterial strains under solid-state fermentation conditions (Agrawal, *et al.*, 2004; Uyar *et al.*, 2004).

Alkaline proteases of neutrophilic as well alkalophilic bacterial, fungal and insect origins are utilized for commercial exploitation (Anwar and Saleemuddin, 1998). Several microbial species are reported to produce alkaline protease. The most extensively studied alkaline protease producing bacteria are the genera of *Bacillus* (Kalisz 1998; Gupta *et al.*, 2002), *Pseudomonas* (Bayoudh *et al.*,2000) and *actinomycetesStreptomyces species* (Petinate *et al.*, 1999), among Fungi the genera of *Conidiobolus* (Bhosale *et al.*, 1995), *Rhizopus* (Banerjee and Bhattacharyya, 1992) and *Aspergillus,* and among yeasts is the genus *Candida*.

Various solid substrates, such as wheat bran, soybean meal etc. have been used in SSF. These substrates were employed individually without any supplementation with other carbon and nitrogen sources. In the SSF process, the solid substrate not only supplies the nutrients to the culture, but also serves as an anchorage for the microbial cells. There are several reports describing production of various enzymes using oil cakes as substrate in solid state fermentation (SSF). Oil cakes are ideally suited nutrient support in SSF rendering both carbon and nitrogen sources, and reported to be good substrate for enzyme production using fungal speices. Sandhya *et al.*(2005) have reported protease production using oil cakes as nutrient source. In the present study an attempt has been made to produce and optimize the alkaline protease by *Aspergillus oryzae* under various physiological conditions such as pH, temperature and also using different oil seed cakes such as ground nut oil cake, sesame oil cake and cotton seed oil cake.

MATERIALS AND METHODS

Soil samples were collected from 3 different sites of Kothakoduru seashore area, Nellore dist. Andhra Pradesh. 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 comprised 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 ^{0}C. The positive fungal strain which produced maximum zone of clearance was morphologically identified as *Aspergillus oryzae*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 1bs/inch pressure), cooled and inoculated with the fungal stain (10^6 spores/ml)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-1of crude enzyme per hour.

Optimization studies: Production of protease from *Aspergillus oryzae*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_4NO_3), 0.2% Potassium di hydrogen phosphate (KH_2PO_4), 0.2% Magnesium sulphate ($MgSO_4$) 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^{0}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 (24,48 and 72 hrs).

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 ($ZnSO_4$), Calcium chloride ($CaCl_2$) and Ferrous sulphate ($FeSo_4$) 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

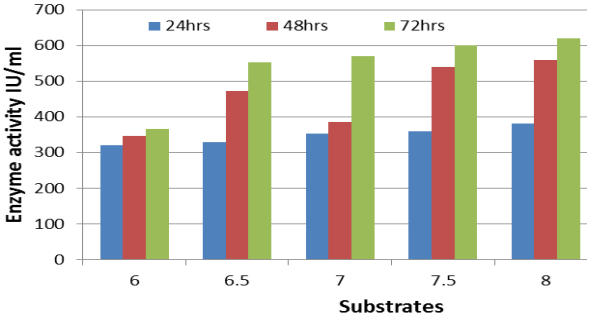

Fig. 1. *Effect of different substrates on protease production by Aspergillus oryzae.*

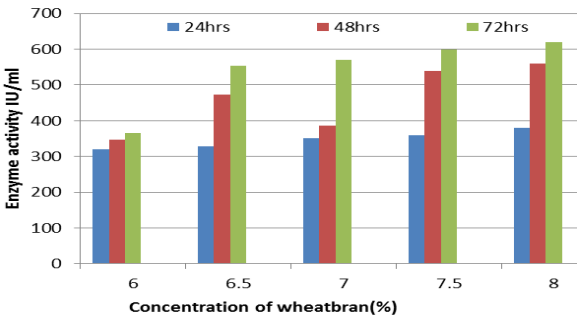

Fig. 2. *Effect of different concentrations of wheat*

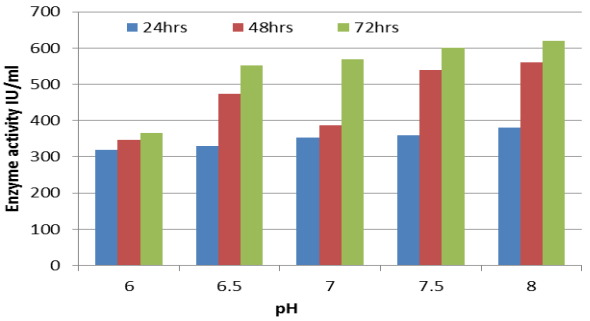

Fig. 3. *Effect of pH on protease production by Aspergillus oryzae.*

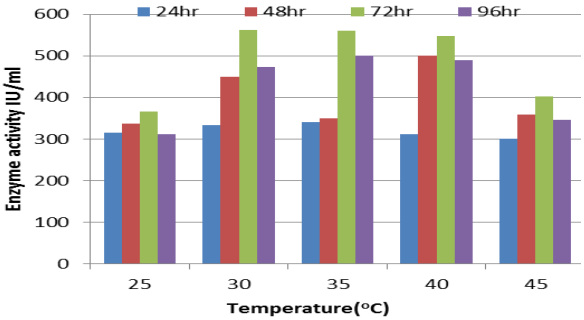

Fig. 4. *Effect of temperature on protease production by Aspergillus oryzae.*

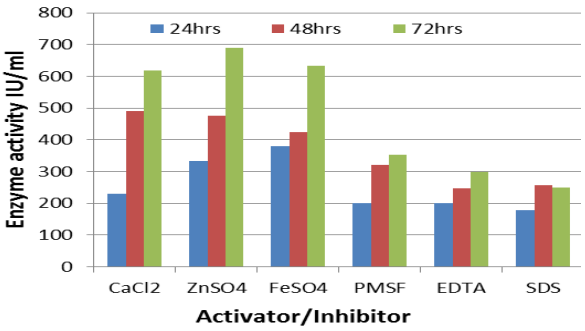

Fig. 5. *Effect of Activator/Inhibitor on protease production by Aspergillus oryzae.*

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 600 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*. Sandhya *et al.* (2005) reported that wheat bran was found to be effective substrate for *Aspergillus oryzae*. Murthy and Naidu (2010) reported that coffee cherry husk was found to be suitable substrate for *Aspergilusoryzae* for protease production. However Wang *et al.* (2005) reported that a combination of soyabean and wheat flour as substrate for protease production with *A.oryzae*.

Chutmanop *et al.* (2008) reported that a protease activity was obtained on substrate that had a wheat bran to rice bran ratio of 0.33by dry weight. Chuenjit *et al.* (2012) reported that a high content of soyabean in koji showed high protease activity. 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. 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).

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 oryzae*. There was gradual increase in protease production from pH 6.5 to 8 and maximum production was observed at pH 8 with 600 IU. Similar results were reported byM.J.Gracia *et al.* (2008) and Salihi *et al.* (2017). Sandhya *et al.* (2005) and Chutmanopet *et al.* (2008) reported the maximum protease production at pH 7.5 in *Aspergillus oryzae*. However maximum protease production by *A. oryzae at* pH 9was reported by Karthic *et al.* (2014). Pagare *et al.* (2009) reported an optimum pH of 8 for extracellular protease production from *Aspergillus niger* and *Bacillus subtilis*.

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. Li-Jung Yin *et al.*, (2013) reported an optimum pH of 3 for *A.oryzae.*

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 isolationAccording 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 (25-45^{0}C) was studied for protease production by *Aspergillus oryzae*. A gradual increase in enzyme production was observed with increase in temperature up to 30^{0}C and reached the peak with production of 562 IU. Then it decreased and minimum production was recorded at 25^{o}C (366 IU) .Similar results were reported by Sandhya *et al.* (2005) and Chutmanop *et al.* (2008) in *Aspergillus oryzae*. García *et al.* (2009) that the maximum proteolytic found at 50^{0}C temperature. Ruann (2014) reported that the biochemical characterization of a protease from *A.oryzae*, the maximum activity was at 55-60^{0}C temperature. Murthy and Naidu (2010) reported that coffee cherry husk was found to be suitable substrate for *Aspergilus oryzae* for protease production at 60^{0}C. Karthic *et al.* (2014) reported that *A. oryzae* synthesizes protease at optimum temperature 35^{0}C.

Osman *et al.* (2014) reported that the purified enzyme from *Aspergillus terreus*was active at 55oC and stable till 45^{o}C isolated from soil. Gopalkumar*et al. (*2016) reported that the maximum protease activity at 60^{o}C temperature for *Aspergillus terreus BAB-346* isolated from paper mill area, Banana farm and poultry farm area. Niyonzima *et al. (*2015) reported that higher enzyme activity at 50^{o}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^{o}C respectively, isolated from soil around leather industry. Sethi*et al. (*2015) reported that optimum temperature for pro-

duction of protease was 30^{o}C by *Aspergillus terreus* isolated from isolated from chuckling vetch. This indicates that variation in temperature requirement for enzyme production is strain dependent.

Effect of inhibitors and activators: While studying the effect of activators it was observed that ZnSO4 enhanced the protease activity up to 690 IU followed by Fe SO4 (634 IU) and Ca Cl2 (619 IU). Karthik *et al.* (2014) reported that the purified enzyme activity was induced up to 121% by Cu and the enzyme did not display the activity in presence of SDS in *Aspergillus oryzae*.. In the present study, SDS was found to be an effective inhibitor for the production. After SDS, PMSF and EDTA also inhibited the enzyme production. Salihi *et al.*, (2017) reported that SDS decreased enzyme activity in *A.oryzae.* Li-Jung-Yin *et al.* (2013) reported that purified acidic protease from *A.oryzae* was completely inhibited bypepstainin and partially inhibited by leupeptin and TPCK, no significant loss observed on samples with EDTA, IAA, PMSF. Chellapandi (2010) reported that the enzyme activity of *Aspergillus flavus* completely inhibited by indicating that the protease produced by *Aspergillus terreus*was a serine protease and. PMSF was found to be an effective inhibitor for the production. Sumantha*et al. (*2005) reported that protease was activated through Ca^{2+}, Fe^{2+} and Mg^{2+}.

Conclusion

Microbial proteases play a major role in detergent, food, leather, chemical, silk and pharmaceutical industries. So far many bacterial species have been evaluated for their production of proteases and their applications. But studies on marine organisms especially marine fungi as a source of protease producers are very few. The present study revealed that the A. oryzae isolated from sea shore was very good source of protease production especially the alkaline protease. Hence it can be concluded that the organism can be further evaluated for its use in the production of alkaline protease at the industrial level using cheap oil cakes like sesame oil cake.

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