STUDIES ON THE PRODUCTION OF PROTEASE BY *ASPERGILLUS ORYZAE* NCIM 637 UNDER SOLID-STATE FERMENTATION USING MIXED SUBSTRATES OF PRAWN'S SHELL AND FISH MEAL POWDER

Abstract

Proteases are the enzymes that catalyze the breakdown of protein molecules into peptides and amino acids. In the present study, Because of the wide range of uses of these proteases in the present study, the production of protease from Aspergillus oryzae NCIM 637 was carried out under solid-state fermentation. The highest yield of the enzyme was screened using two substrate powders (Prawn's shell and fish meal powder), and it was observed that combined substrate powder has a higher potential to serve as a substrate for neutral protease synthesis by the fungal strain Aspergillus oryzae NCIM 637. Fermentation time (5 days), fermentation temperature (35°C), optimum pH (7), initial moisture content (46.40%), inoculum age (4 days), and inoculum volume were all optimized (1.0ml). The influence of additives such as carbon source maltose (2%) and nitrogen source casein (2%) was investigated, with a maximum production of 562.57U/gds. When the enzyme was partially purified using ammonium sulphate precipitation, the activity of the protease enzyme was found to be 570U/gds. Proteases have a wide range of applications in the food, pharmaceutical, and leather industries, as well as brewing.

Keywords: Protease, *Aspergillus oryzae*, Prawns shell and fish meal powder, Solid-state fermentation, Enzymes

1. Introduction (Time New Roman, Font 10, Bold)

Proteases that catalyze hydrolytic cleavage of protein molecules into peptides and amino acids. Microorganisms are a good source of proteases than plants and animals because they can be produced in large quantities in a relatively short period of time using proven fermentation procedures. Microbial proteases can be stored for weeks

in ideal conditions without losing significant activity. Microbial proteases, in general, are extracellular in origin and immediately secreted into the fermentation broth by the producer. Proteases accounting for, accounting for over 60% of all enzyme sales globally.

Proteases are used mostly in food processing, and in the health care industry. Protease enzymes have gotten a lot of interest in the industrial world. These proteases are available in soluble and immobilized forms. In a shrimp and crab shell powder media, San-Lang Wang et al. isolated and characterized a serine protease generated extracellularly by Aspergillus fumigatus.

Submerged fermentation (SMF) and solid-state fermentation (SSF) are two ways for producing enzymes. However, when compared to SMF, SSF has been proven to be a superior approach, with numerous advantages such as high volumetric outputs, simpler equipment use. Sandhya *et al.*, reported that the yield in SSF was 3.5 times higher than SMF.

Filamentous fungi are the most common type of fungus in the SSF process, and they may grow on minimal substrates. Natural substrates are more readily available and less expensive than synthetic substrates.

The present study was carried out using a fungal strain aspergillus oryzae NCIM 637 and natural substrates prawns' shells and fish meal powder under solid-state fermentation (SSF) for the optimal production of neutral protease.

2. MATERIALS AND METHODS

2.1 Microorganism and culture conditions:

The fungus strain Aspergillus oryzae NCIM 637 utilized in this study was obtained from the National Collection of Industrial Microorganisms (NCIM) in Pune, India. The culture was grown on potato dextrose agar slants and incubated for 7 days at 280°C. Slants were kept at a temperature of 40°C. Every month, the organism was sub-cultured and employed in the following research experiments.

2.2 Inoculum preparation:

The spores from a 7-day old fungal slant culture were dispersed in a 0.1 percent Tween-80 solution with a sterile inoculation loop to make the fungal homogenous spore suspension.

2.3 Substrate

Prawns' shells and fish waste are collected from the Visakhapatnam harbor, dried in sunlight, and ground to powderto use as a substrate.

2.4 fermentation medium and culture conditions:

Solid-state fermentation and culture conditions were maintained as Paranthaman *et al*., 2009. Fermentation was carried out in 250ml Erlenmeyer flask containing 5gm of Prawns shell and fish meal powder as the substrate and moistened with 10ml of salt solution and its composition is as follows.

Table 1 Composition of the salt solution

Mineral salts	**Composition (%w/v)(g/100ml)**
Ammonium nitrate	0.5
Potassium dihydrogen orthophosphate	0.2
Sodium chloride	0.1
Magnesium sulfate	0.1

At 121.5^{0}C (15 lb) the flasks were sterilized for 15 min, cooled, aseptically inoculated with 1ml of fungal spore suspension (10^6 spores/ml), and incubated at 35^{0}C for 5 days.

2.5 Extraction of crude enzyme:

TWEEN-80 (0.1 percent) solution was added to 100ml distilled water, and 25ml of this water was added to the 5grams of the fermented substrate, which was homogenized for 1 hour on a rotary shaker at 180 rpm. The particles were removed from the homogenate by centrifuging at 8000 rpm for 15 minutes at 40°C, and the clear supernatant was used for analytical tests.

2.6 Assay for protease:

In a test tube, 5ml of casein (1%) and 300 l of 0.2 mol/l phosphate buffer (pH 7) were combined, and 2ml of crude enzyme extract was added. The reaction mixture was incubated at 60^{0}C for 10 minutes before being ceased by

adding 1 mL of 10% trichloroacetic acid 8. 5ml of 0.4 mol/l Na_2CO_3 and 1ml of 3-fold diluted Folin-phenol Ciocalteau's reagent was added to the supernatant after centrifugation at 8000 rpm at 40C for 15 minutes. The resultant solution was incubated for 30 minutes at room temperature, and the absorbance of the blue color formed was measured at 660 nm using a visible spectrophotometer, and its concentration was estimated using a tyrosine standard curve.

Under assay conditions, one unit of enzyme activity was defined as the quantity of enzyme that liberated 1g of tyrosine from a substrate (casein) per minute. The activity of neutral protease per gram of dry substrate was used to calculate the enzyme yield. (U/gds).

3.RESULTS AND DISCUSSION

For the production of neutral protease, the SSF was carried out using Aspergillus fungus and natural media prawn shells and fish meal powder mixture, and optimization of process parameters was carried out.

3.1 Optimization of fermentation time:

The effect of fermentation time on the production of neutral protease was studied at different time intervals (1, 2, 3, 4, 5, 6, 7, 8) of 1 to 8 days. The enzyme production was observed maximum on the fifth day of fermentation. The maximum enzyme activity observed in 504.8 u/gds is shown in figure 1. The results it is observed the gradual increase in enzyme activity observed up to 5 days and reached a maximum on the 5th day (504.8)u/gds. Later an increase in enzyme activity was observed. This may be due to the depletion of the fermentation medium. Similar results were reported by Srividya Shivakumar, 2012 maximum protease activity was obtained after 120hrs of incubation for medium with wheat bran & gelatin 1% w/vas substrates.

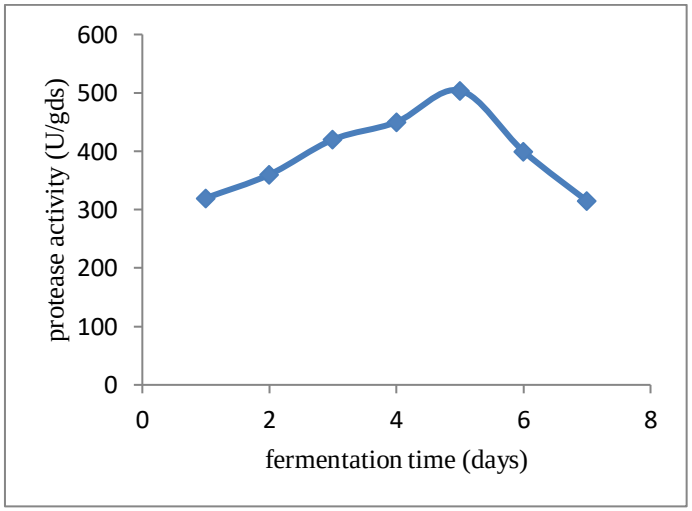

Figure 1 Effect of Fermentation time on protease production

3.2 Optimization of fermentation Temperature:

Fermentation temperature has a great influence on fungal growth and protease production. So the effect of temperature was studied using various temperature ranges from 20^0C, 25^0C, 30^0C, 40^0C, 45^0C, 50^0C,55^0C. The maximum activity (509.2u/gds)of the enzyme was observed at temperature 35c as shown in figure 2. Increased temperature causes an increase in activity up to a certain point after which it has a determinate effect on the microorganism's growth and may denature the extracellular enzyme. Nehra et al., (2002) reported an optimum temperature of 35^0C for the production of alkaline protease by Aspergillus sp., under solid-state conditions.

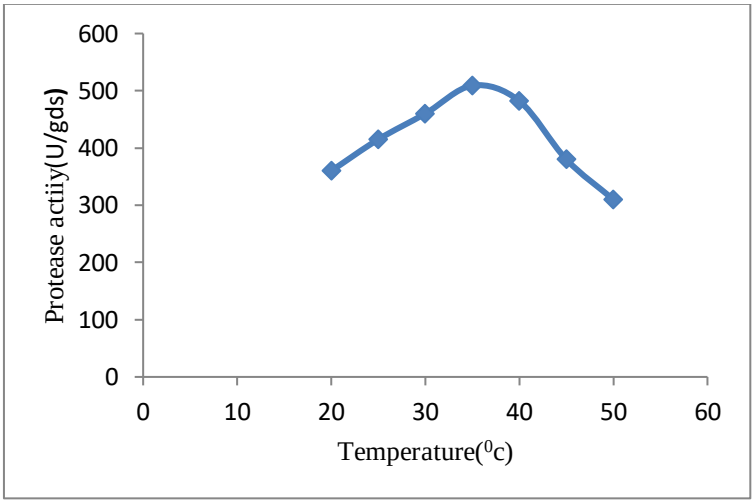

Figure 2 Effect of fermentation temperature on protease production

3.3 Effect of initial moisture content:

To determine the optimal moisture content the initial moisture content of the fermentation substrate is varied to various levels of 40-55%. For neutral protease synthesis, moisture content of 46.40% was shown to be optimal and the activity of the enzyme was observed as 525.3u/gds (Fig 3)**.** The results showed that moisture content had a substantial impact on enzyme activity. Lower moisture content causes solubility issues for nutrients in the substrate. Whereas greater moisture content diminished the porosity of the substrate, limiting oxygen transmission.

Jurun chutmonov et.al.,(2008) reported similar findings in the synthesis of protease utilizing rice bran and wheat bran as substrates with a moisture level of 50%.

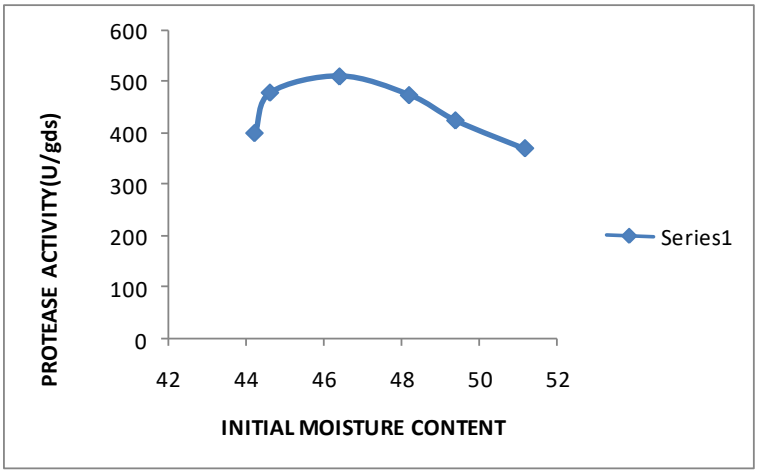

Figure 3 Effect of initial moisture content on protease production

3.4 Effect of pH:

The pH of the medium strongly influences the growth of the organism and protease production. The fermentation was carried out at different pH levels of the substrate medium 2-9 ph. The maximal neutral protease activity (516.2 U/gds) was obtained at neutral pH 7(Fig 4). Paranthaman *et al.*, 2009 reported similar results using rice mill waste as substrate. To produce the neutral protease.

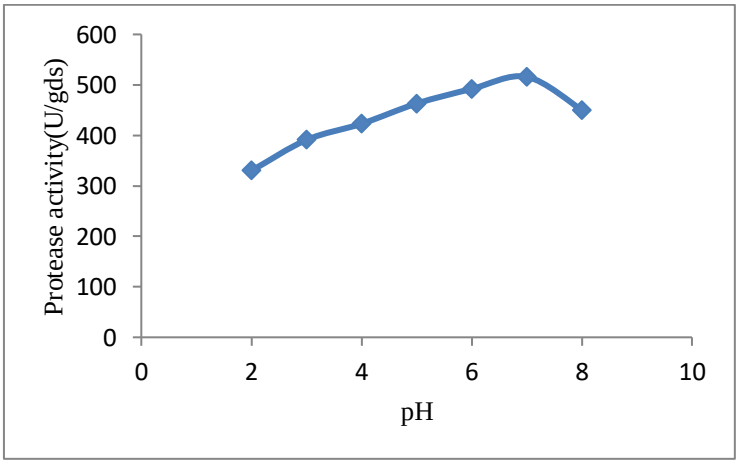

Figure 4 Effect of pH on protease production

3.5 Effect of inoculum volume:

The effect of inoculum volume was investigated, and it was observed that increasing inoculum volume up to 1ml increased activity. Maximum protease **(**519.12 U/gds**)** was obtained from a 1ml volume of 4-day-old culture (Aspergillus oryzae NCIM 637) culture. The findings are shown in (Figure 5) increased inoculum volume resulted in decreased inactivity. (Fig 5). The greatest neutral protease production was reported by Abdul Rauf et al., 2010 with a 1ml volume of inoculums (1x106 spores/ml).

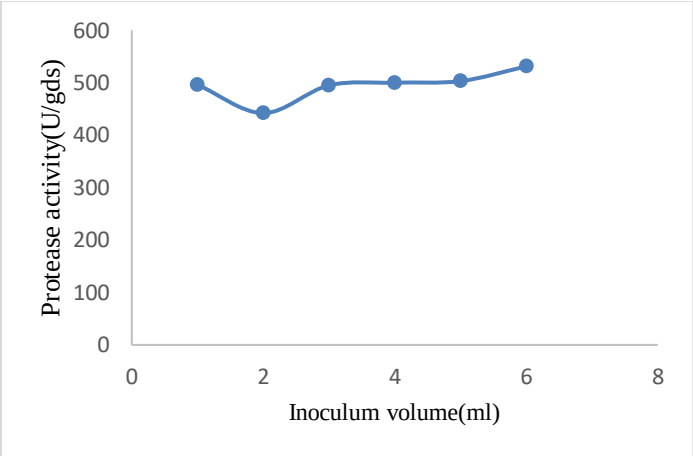

Figure 5 Effect of inoculum volume on the production of protease

3.6 Effect of inoculum age:

The Effect of inoculum age on Protease activity was highest in a 4-day old A. oryzae culture, with a maximum activity of 523.32U/gds. Figure 6 represents the complete results. With Rhizopus oligosporus ACM 145F, Ikasari and Mitchell (1994) found that the 4-day old inoculum yielded the best protease yield.

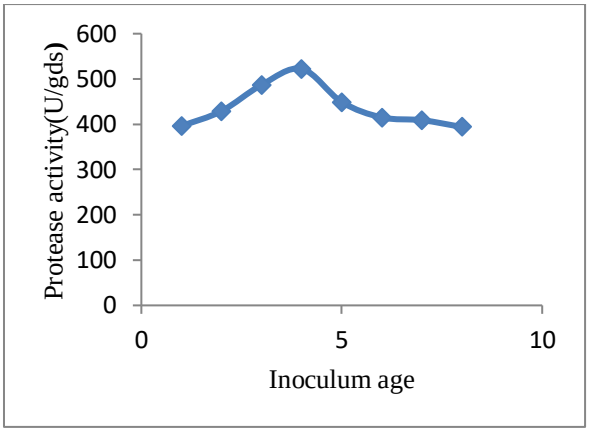

Figure 6 Effect of inoculum age on production of protease

3.7 Effect of substrate concentration:

The effect of substrate concentration was studied using varied concentrations of production medium. Each flask was inoculated and kept in a slanting posture after being prepared at pH 7.0 in 250 ml flasks. The results are shown in Figure 7. At a concentration of 5g, the enzyme activity increased, reaching a maximum of 525 U/gds.

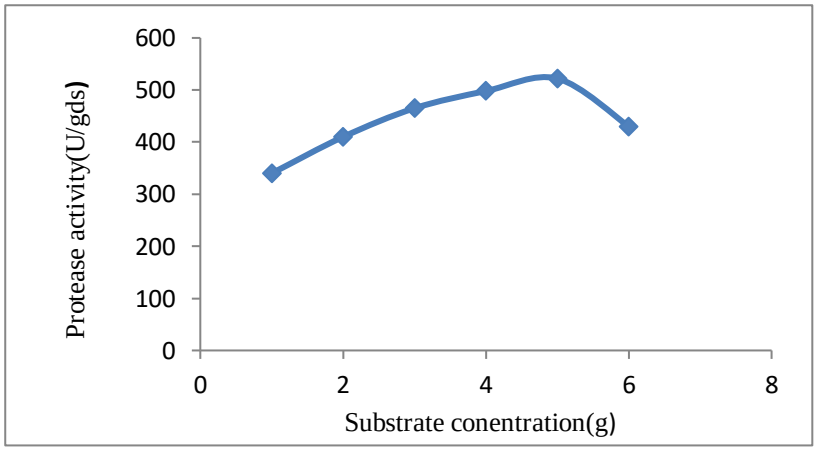

Figure 7 Effect of substrate concentration on protease production

3.8 Effect of Enrichment with carbon sources:

To determine several carbon sources such as Fructose, cellulose, sucrose, Dextrose, Lactose, maltose, Soluble starch, Mannitol were added to the solid medium at a concentration of 2.0 percent. Maltose was found to be an effective carbon supplement among the other carbon supplements evaluated. Figure 8 shows the highest protease activity of 531.57U/gds.

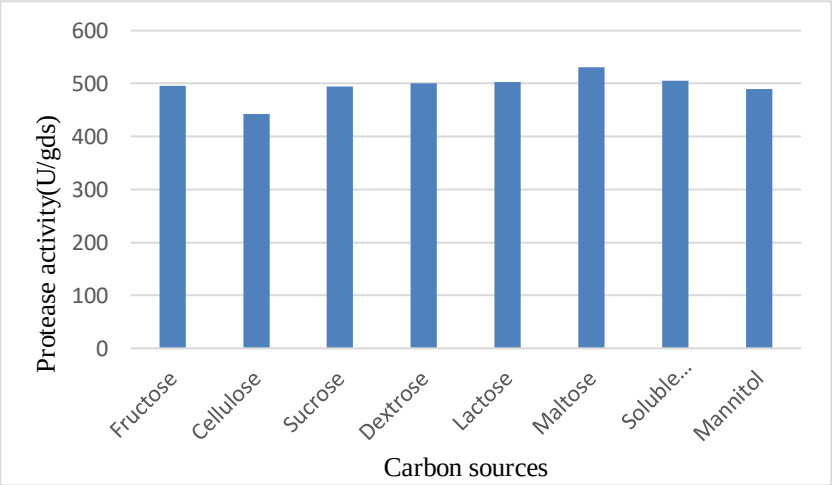

Figure 8 Effect of Carbon Source on protease production

3.9 Effect of enrichment with nitrogen source:

Various organic (yeast extract, beef extract, malt extract peptone, urea, casein) and inorganic (potassium nitrate, ammonium nitrate, ammonium sulfate, sodium nitrate, ammonium chloride) nitrogen sources were supplemented in the fermentation media, and it was observed that when 2.0 % casein was added, the maximum yield of protease (562.57U/gds) was obtained. Other nitrogen sources utilized in the experiment produced considerable amounts of enzymes during the fermentation process. Similar results were reported by Algarswamy, sumantha et.al.,(2006) using casein as an organic nitrogen supplement.

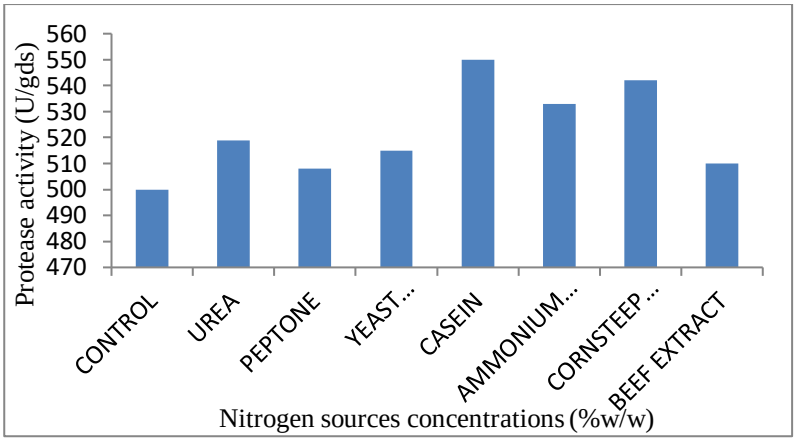

Figure 9 Effect of various nitrogen sources on protease production

4. Conclusion

According to the present studies it is observed that the fungi Aspergillus oryzae NCIM 637 is the more effective potential microbe for the production of protease using mixed substrates. By use of prawn shell and fish waste powder as substrate increased enzyme yields (562.24U/gds) and resulted in the production of highly active protease. Because the substrate has plenty of nutrients. It aided fungi growth and resulted in the production of highly active protease. They are a high-protein source that can be used as a low-cost substrate for the production of microbial enzymes. Marine wastes include fish heads, tails, fins, viscera, and chitinous prawn shell materials. Processing these wastes for the production of commercial value-added products may lead to a decrease in costs of production. Furthermore, we can eliminate pollution of the environment and health issues caused by improper waste disposal.

Conflict of Interest

This manuscript has not been submitted to, nor is under review at, another journal or other publishing venue. The authors have no affiliation with any organization with a direct or indirect financial interest in the subject matter discussed in the manuscript.

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