JOURNAL OF AGRICULTURE & SOCIAL SCIENCES
1813–2235/2006/02–1–23–25
http://www.fspublishers.org

Production of Protease by *Penicillium chrysogenum* Through Optimization of Environmental Conditions

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ABSTRACT

Present investigation describes the biosynthesis of neutral protease by *Penicillium chrysogenum* IHH_5 through submerged fermentation in shake flasks. Different environmental parameters such as time course fermentation, medium pH and incubation temperature were optimized for the production of protease. It was found that the maximum production of neutral protease by the mold culture was obtained after 72 h of batch fermentation. Similarly, the medium pH of 7.0 and incubation temperature of 30°C were found optimum for enzyme production by the microorganism. The maximum production of protease during the course of present studies was 12.79 U mL^{-1}.

Key Words: Production of protease; *Penicillium chrysogenum*; Environmental conditions

INTRODUCTION

Proteases are among the oldest enzymes known to man. These are degradative enzymes, which catalyze the total hydrolysis of proteins (Raju *et al.,* 1994). The molecular weight of proteases ranges from 18 – 90 kDA (Sidney & Lester, 1972). These enzymes are found in a wide diversity of sources such as plants, animals and microorganisms but they are mainly produced by bacteria and fungi. Microbial proteases are predominantly extracellular and can be concentrated in the fermentation medium. Microbial proteases are the most important industrial enzymes (Chouyyok *et al.,* 2005) and account for approximately 40% of the total worldwide enzyme sale (Godfrey & West, 1996). They are generally used in detergents (Barindra *et al.,* 2006), leather and food industries (Rao *et al.,* 1998: Haq *et al.,* 2002). They also have medical and pharmaceutical applications. Fungi elaborate a wide variety of proteolytic enzymes than bacteria. The filamentous fungi have a potential to grow under varying environmental conditions such as time course, pH and temperature, utilizing a wide variety of substrates as nutrients. Several species of *Penicillium* such as *P. chrysogenum, P. restrictum, P. dupontii* (Sharma *et al.,* 1980) and *P. griseoroseum,* (Haq *et al.,* 2004) have been reported to produce proteases.

The environmental conditions of the fermentation batch play a vital role in the growth and metabolite production of a microbial population. The most important among these are the medium pH and incubation temperature. The pH of the fermentation medium is reported to have substantial effect on the production of proteases (Al-Shehri, 2004). It can affect growth of microorganisms either indirectly by affecting the availability of nutrients or directly by action on the cell surfaces. The metabolic activities of the microorganisms are sensitive to the pH changes and the pH of the culture media has marked effect on the type and amount of enzyme produced. Changes in pH may also cause denaturation of enzyme resulting in the loss of catalytic activity (Karuna & Ayyanna, 1993). It is probable that if nutrient requirements are satisfied, most fungi will grow over a broad pH range on the acid side of neutrality, however fungal proteases are active over a wide range of pH i.e. from 3 - 13.

Another important environmental factor is the incubation temperature, which is important to the production of proteases by microorganisms. Higher temperature is found to have some adverse effects on metabolic activities of microorganisms producing proteolytic enzymes (Tunga, 1995). However, some microorganisms produce heat stable proteases, which are active at higher temperatures. The thermal stability of the enzymes may be due to the presence of some metal ions or adaptability to carry out their biological activity at higher temperature (Gaure *et al.,* 1989; Al-Shehri, 2004). Neutral proteases usually have low thermostability but there are reports of heat resistant neutral proteases, which can hydrolyze casein at fastest rate at 60 - 65°C (Balls & Weaver, 1939). Protease production at low temperature has also been reported but with a lower yields (Damare *et al.,* 2004).

The objective of the present work was the optimization of different environmental factors such as pH of the medium, incubation period and incubation temperature for protease production by *P. chrysogenum* IHH_5 as it was pre-requisite for scale up production of enzyme by the organism.

MATERIALS AND METHODS

Microorganism and maintenance. The mould culture of *Penicillium chrysogenum* IHH_5 was isolated from soil samples of different areas of Lahore using potato dextrose-casein-agar plates. The cultures showing larger zones of casein hydrolysis on the test plates were picked up and transferred to PDA slants. The culture was maintained by weekly transfer onto fresh slants of potato-dextrose-agar (PDA) and was stored in refrigerator at 4°C.

Inoculum preparation. The slants of five days old cultures were wetted by adding 10 mL of 0.005% sterilized solution of monoxal O.T. (Diacetyl ester of sodium sulphosuccinic acid) to the slants. The spores were scratched by sterile wire loop to break clumps and obtain homogeneous spore suspension.

Fermentation medium. The fermentation was carried out in 250 mL Erlenmeyer flask containing 50 mL of fermentation medium consisting of (%; w/v): soybean meal, 1.0; glucose, 1.0; polypeptone, 0.5; yeast extract, 0.1; KH_2PO_4, 0.1 and NaCl, 0.1, with pH of 6.5. The cotton-plugged flasks were then subjected to sterilization in an autoclave at a pressure of 15 lbs inch^{-2} (121°C) for 15 min. The production medium was cooled at room temperature and inoculated with 1 mL of conidial suspension as prepared earlier. The flasks after inoculation were placed in the incubator shaker (SANYO-GallenKamp PLC, UK) rotating at the speed of 200 rpm at 30°C for 72 h. Later, the contents of the flasks were filtered using Whatman 44 filter paper and the filtrate was used for the assay of protease.

Mycelial dry weight. For this purpose, the fermented broth was filtered, using preweighed Whatman 44 filter paper. It was washed with water thrice and then dried at 105°C over night in a hot air oven (Memmert, Germany) and weighed.

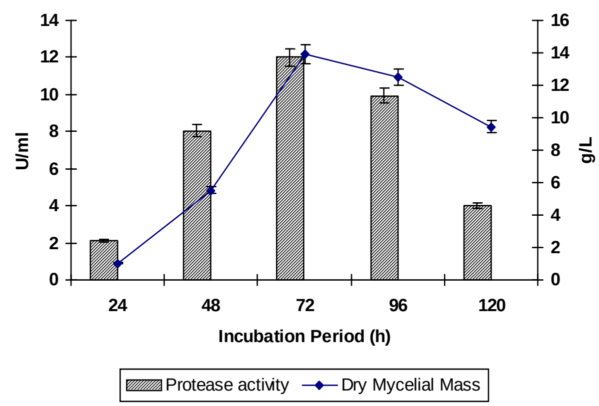
Assay of protease. The activity of protease was assayed by the method of McDonald and Chen (1965). To 1 mL of the enzyme extract in the test tube, 4.0 mL of 1.0% casein was added. The enzyme sample was incubated at 35°C for 1 h. The residual protein was precipitated by adding 5 mL of 5% TCA. The precipitates were allowed to settle for 30 min. The contents of the tube were centrifuged at 5000 rpm for 5 min. Supernatant (1 mL) was mixed with alkaline reagent (5 mL). Then 1 mL of 1N sodium hydroxide was added to make the contents of the tube alkaline. After 10 min. 0.5 mL of Folin and Ciocalteau reagent was added; as a result, blue color was produced. The tubes were left for 30 min to get maximum development of blue color. The optical density of the mixture was read at 700 nm on spectrophotometer (Cecil-CE7200, Aquarius, UK). Activity of enzyme was expressed as: One unit of protease is defined as the amount of enzyme required to produce an increase of 0.1 in optical density under optimal defined conditions.

RESULTS AND DISCUSSION

Time course fermentation. Time course for the production of protease by *P. chrysogenum* IHH_5 was studied in shake flasks. The enzyme production was gradually increased with the passage of time and highest enzyme activity (12.0 U mL^{-1}) was obtained after 72 h of incubation and the corresponding mycelial dry mass was found 13.93 g L^{-1}. It was also observed that prolonged incubation decreased the enzyme activity (Fig. 1). However the growth of the microorganism was not significantly affected. In line with the present study, Karuna and Ayyana (1993) using *Rhizopus oligosporous* and Ikasari and Mitchell (1994) using *Aspergillus* sp. obtained the highest protease yield after 72 h of incubation. The incubation period is directly related with the production of enzymes and other metabolites up to a certain extent. After that, the enzyme production and growth of the microorganism decreases, which can be attributed to the reduced availability of nutrients and the production of toxic metabolites (Romero *et al.,* 1998).

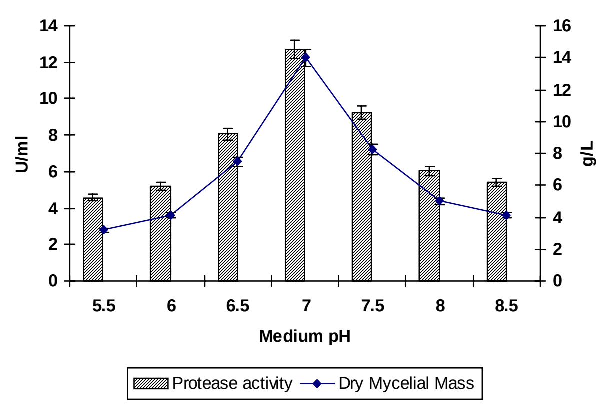
Effect of initial pH. Productivity of the enzyme by mould culture is very much dependant on pH of the fermentation medium (Kubackova *et al.,* 1975). Therefore, the effect of initial pH (5.5 - 8.5) was studied for the production of protease by *P. chrysogenum* IHH_5. There was gradual increase in the amount of protease synthesis from pH 5.5 to 6.5 and maximum production of enzyme was observed at

Fig. 1. Time course fermentation for the production of protease by *P. chrysogenum* IHH_5



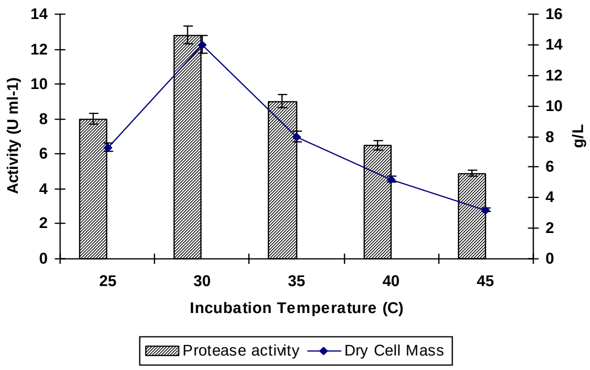
Incubation temperature = 30°C; Initial pH = 7.0
Each value is a mean of three replicates. Y- error bars indicate the standard error from mean.

Fig. 2. Effect of medium pH on the production of protease by *P. chrysogenum* IHH_5



Incubation period = 72 hrs; Incubation temperature = 30°C
Each value is a mean of three replicates. Y- error bars indicate the standard error from mean.

Fig. 3. Effect of incubation temperature on the production of protease by *P. chrysogenum* IHH$_5$



Incubation period = 72 hrs; Initial pH = 7.0
Each value is a mean of three replicates. Y- error bars indicate the standard error from mean.

pH 7.0 i.e. 12.69 U mL^{-1} (Fig. 2). However, pH of the fermentation medium beyond 7.0 resulted in a marked decrease in the production of protease. These data are in conformity to the findings of Sharma *et al.* (1980), who have also made similar results with P. *perpurogerum*, *P. funculosum* and *Paeciliomyces varioti* isolated from deteriorated finished leather, who noted the highest yield of protease at pH 7 of fermentation medium. It is likely that changes in pH cause denaturation of enzyme resulting in the loss of catalytic activity. Therefore, each enzyme has specific pH optima for its activity.

Effect of incubation temperature. The enzyme production by *P. chrysogenum* IHH$_5$ at 25 - 45°C temperature range revealed that there was a sudden increase in protease production when the incubation temperature was increased from 25°C (7.4 U mL^{-1}) to 30°C (12.79 U mL^{-1}). The enzyme production was slightly decreased up to 35°C (10.1 U mL^{-1}). However, there was sudden decrease in protease production when the incubation temperature was increased from 35°C to 45°C (Fig. 3). So the optimum incubation temperature for the production of protease was found as 30°C. Earlier studies report that different species of *Penicillium* including *P. citrinum*, *P. perpurogerum* and *P. funculosum* gave highest yield of protease when incubated at 30°C (Sharma *et al.,* 1980). Haq *et al*. (2004) have also reported that maximum production of protease by *Penicillium griseoroseum* was obtained at an incubation temperature of 30°C and the enzyme production was reduced when the incubation temperature was increased above 30°C. Fungal proteases are usually thermolabile and show reduced activities at high temperatures (Sharma *et al.*, 1980). Higher temperature is found to have some adverse effects on metabolic activities of microorganism (Tunga, 1995) 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 (Conn *et al.,* 1987).

CONCLUSION

Penicillium chrysogenum proved potent producer of protease by shake flask fermentation. The enzyme production was considerably enhanced under the set of conditions optimized in this study. These findings have great industrial implications.

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(Received 20 October 2005; Accepted 12 December 2005)