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Production of Protease from Sesame Oil Cake by *Penicillium Chrysogenum* under Solid State Fermentation

B. Nagamani, M.V.V Chandana Lakshmi*, V. Sridevi and P. Rajani
Centre for Biotechnology, Department of Chemical Engineering, Andhra University,
Visakhapatnam-03, Andhra Pradesh, India.
*E-mail: mahantilakshmi@yahoo.com

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ABSTRACT
The present work describes the biosynthesis of neutral protease by *Penicillium chrysogenum* (NCIM 737) through solid state fermentation. Environmental parameters such as fermentation time, fermentation temperature and pH were optimized for the production of protease. It was found that the maximum production of neutral protease by the mold culture was obtained for 7 days of fermentation, Similarly, pH of 7.0 and incubation temperature of 25^0C.
Keywords: Solid state fermentation, sesame oil cake, *Penicillium chrysogenum* NCIM 737, Neutral protease, fermentation time, temperature, pH.

INTRODUCTION

Proteases are the degradative enzymes, which catalyze the total hydrolysis of proteins. The total molecular weight of proteases ranges from 18-90 kDA[1]. Proteases are the most important industrial enzymes accounting for about 60% of the total enzyme market [2,3]. According to Enzyme Commission (EC) classification, proteases belong to group 3 (hydrolases), and subgroup 4 (which hydrolyse peptide bond) [4]. Commercial application of microbial proteases is attractive due to the relative ease of large scale production as compared to proteases from plants and animals [5]. Molds of the genera *Aspergillus, Penicillium* and *Rhizopus* are especially useful for producing proteases, as several species of these genera are generally regarded as safe [6]. Microbial proteases account for approximately 40% of the total worldwide enzyme sales, since they possess almost all the characteristics desired for biotechnological applications [7,8].

Among microbes, fungi as enzyme producers have many advantages, since they are normally GRAS (generally regarded as safe) strains and the produced enzymes are extra cellular which makes its easy recuperation from fermentation broth [9].The major uses of free proteases occur in dry cleaning, detergents, meat processing, cheese making, silver recovery from photographic film, production of digestive and certain medical treatments of inflammation and virulent wounds [10].

Proteases are mainly classified on the basis of their pH optimum as acidic, neutral or alkaline proteases [11]. Neutral proteases are mainly used in food processing such as baking, brewing, and also in the healthcare sector. One of the more recent applications of these proteases exploit their eco-friendly nature and hence their suitability to act as food processing aids, wherein these enzymes can be used for the extraction of plant oils [11,12].

Proteases are extra cellular enzymes that can be produced by both submerged fermentation and solid state fermentation [6] (SSF). SSF offers the greatest possibilities when fungi are used. Because, unlike other microorganisms, these typically grow in solid substrates [13]. Some of the advantages of SSF over conventional submerged cultures for work involving fungi are: (a) simplicity of equipment, (b)low moisture content, which prevents bacterial contamination, (c)superior volumetric productivity, (d)use of inexpensive substrates, (d)simpler downstream processing, (e)lower energy requirement and flow wastewater output [13,14].

The present study was undertaken to produce the neutral protease under SSF by *P. chrysogenum* NCIM 737 using sesame oil cake as substrate, and to determine the optimum value of fermentation time, temperature and pH for maximum production of protease.

MATERIALS AND METHODS

Substrate

The substrates used in this study namely Green gram husk, Black gram husk, Rice bran, Coconut oil cake, Sesame oil cake (SOC) and Paddy straw + Rice bran (7:3) were obtained from local grocery shop in Visakhapatnam.

Microorganism and maintenance of culture
The organism used in the present study, *P. chrysogenum* NCIM 737 which was obtained from National Collection of Industrial Microorganisms (NCIM), Pune, India. The culture was routinely maintained on potato dextrose agar slants. The organism was subcultured for every month.

Inoculum preparation
The inoculum was prepared by dispersing the spores from a week-old fungal slant culture in 0.1 % Tween-80 solution with a sterile inoculation loop.

Solid-State fermentation
Five grams of substrate was taken in 250 ml Erlenmeyer flask separately, moistened with salt solution [composition (%w/v) (g/100ml): ammonium nitrate 0.5, potassium dihydrogen orthophosphate 0.2, sodium chloride 0.1 and magnesium sulfate 0.1], sterilized at 121.5ºC for 15 min, cooled inoculated with 1 ml of fungal spore suspension and incubated at 25ºC for 7 days.

Extraction of crude enzyme
A solution of Tween 80 (0.1%) was added in 100 ml of distilled water. 25 ml of water was added to the 5 gm of fermented substrate and the substrate was homogenized on a rotary shaker at 180 rpm for 1 hr and then filtered the solution. The solids were removed by centrifuging the homogenate at 8000 x g at 4ºC for 15 min and the resultant clear supernatant was used for analytical studies.

Assay for neutral protease
To 200 µL of crude enzyme extract, 500 µL of casein (1%) and 300 µL of 0.2 mol/L phosphate buffer (pH 7.0) were added. The reaction mixture was incubated at 60°C for 10 min and arrested by the addition of 1mL of 10% trichloro acetic acid [16]. The reaction mixture was centrifuged at 8000 x g at 4^0C for 15 min and to the supernatant, 5 mL of 0.4 mol/L Na_2CO_3, 1 mL of 3-fold diluted Folin and Ciocalteau's phenol reagent were added. The resulting solution was incubated at room temperature for 30 min and the absorbance of the blue color developed was read at 660 nm and its concentration was determined using tyrosine standard curve. One unit of enzyme activity was defined as the amount of enzyme that liberated one microgram of tyrosine from substrate (casein) per minute under assay conditions.

Standard graph for tyrosine
To a series of test tubes, 0.1mL, 0.2mL, 0.4mL, 0.6mL, 0.8mL, 1mL of standard solution of tyrosine (100 µg/mL) was taken and water is added to each test tube to make the solution up to 1mL. Each test tube contains 10, 20, 40, 60, 80, 100 µg/mL of tyrosine. To each test tube 5 mL of 0.5 M Na_2CO_3 and 1 mL of 3-fold diluted Folin and Ciocalteau's Phenol reagent were added and incubated for 30 min. The optical density of above solutions was measured at 660 nm (Fig. 1). Blank was prepared with 1mL of water instead of tyrosine solution.

Screening of substrates and fungal neutral protease producers
Seven different substrates like green gram husk, black gram husk, rice bran, coconut oil cake, sesame oil cake, paddy straw rice bran (7:3) were screened using three different fungal species namely *Penicillium chrysogenum* NCIM 737, *Rhizopus oligosporus* NCIM 1215 and *Acremonium chrysogenum* NCIM 893 for neutral protease production using SSF.

Optimization
Optimization of process parameters for neutral protease production
The protocol adopted for the optimization of process parameters was to evaluate the effect of an individual parameter at a time and to incorporate it at the standard level before optimizing the next parameter.

Optimization of fermentation time
The production profile of neutral protease was studied by conducting the fermentation at different time intervals 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 days.

Optimization of fermentation temperature

The inoculated substrates were incubated at different temperatures ranging from 20 to 45°C, to determine the optimum fermentation temperature for neutral protease production.

Optimization of medium pH

Optimum pH for neutral protease production was determined by conducting the fermentation at different pH 4, 5, 6, 7, 8, 9 and 10.

Table-1: Screening of microorganisms and substrates for the protease production.

Substrate	*P.chrysogenum* protease activity(U/gds)	*R.oligosporous* protease activity(U/gds)	*A. chrysogenum* protease activity(U/gds)
Green gram husk	50	47.5	5.0
Black gram husk	17.5	7.5	10.0
Rice bran	50.0	20.0	82.5
Coconut Oil cake	17.5	10.0	11.25
Sesame oil cake	130.0	102.5	2.5
Paddy straw + Rice bran(7:3)	10.0	15.0	5.0

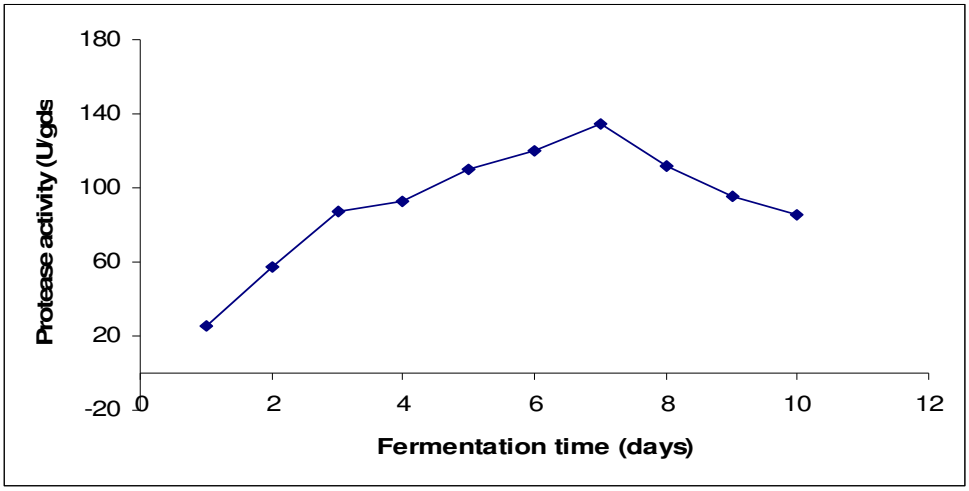

Fig.-1: Effect of fermentation time on the production of neutral protease by *P.chrysogenum* NCIM 737.

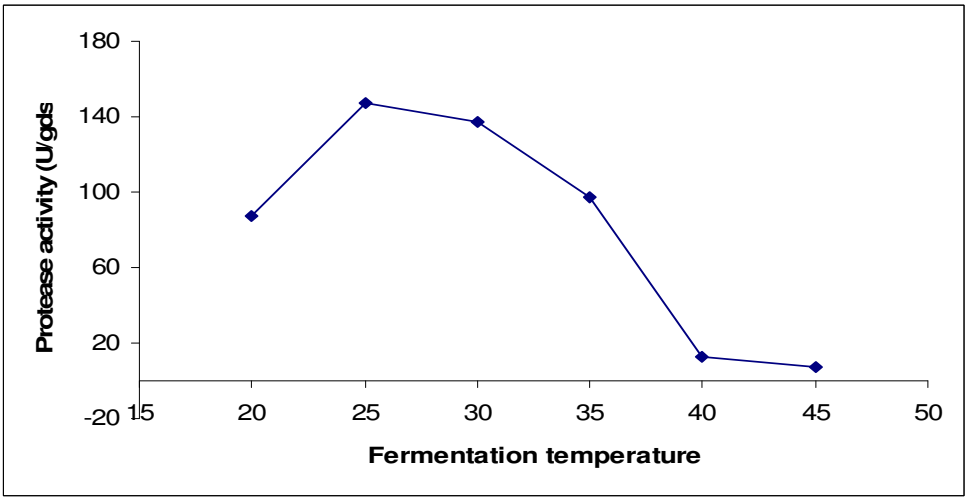

Fig.-2:Optimization of fermentation temperature for neutral protease production by *P.chrysogenum* NCIM 737.

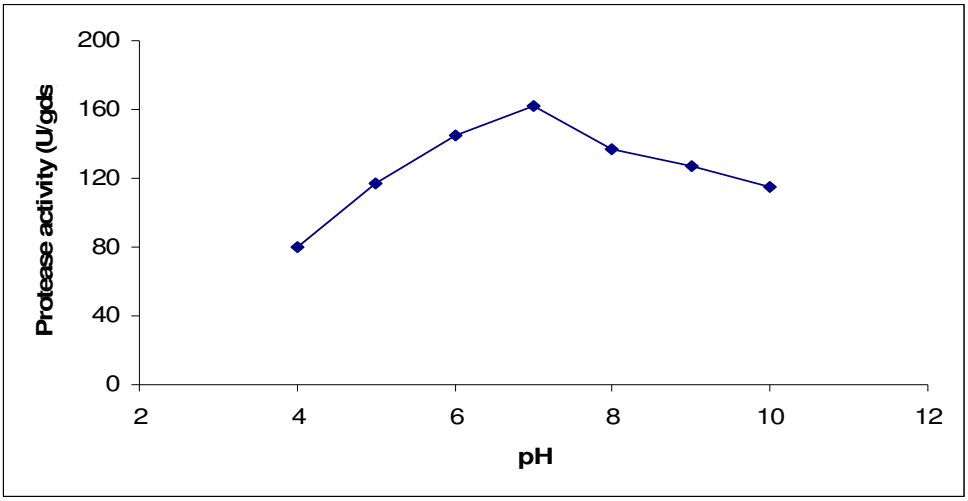

Fig.-3: Effect of pH on the production of protease by *P.chrysogenum* NCIM 737.

RESULTS AND DISCUSSION

Screening of microorganisms and substrates

The selection of an ideal agro industrial waste for the enzyme production in a SSF process depends upon several factors, mainly related with cost and availability of the substrate material. The results in the present study indicated that protease production pattern varied with the type of agro waste. This could be attributed to solid materials dual role supply of nutrients to the microbial culture and anchorage for the growing cells[8]. Maximum enzyme production was observed with sesame oil cake.

P.chrysogenum NCIM 737 proved to be the best strain for neutral protease production on sesame oilcake substrate giving 130 U/gds of enzyme activity (Table 1). This *Penicillium* strain was selected to optimize the process parameters for the enzyme production on sesame oilcake substrate under the SSF.

Effect of fermentation time

Time course for the production of protease by *P.chrysogenum* NCIM 737 was studied in SSF. The enzyme production was gradually increased with the passage of time and highest enzyme activity (135.0 U/gds) was obtained after 7 days of incubation (Fig1). The gradual decrease in the enzyme activity was observed with increasing incubation time clearly suggesting the enzyme being produced in the log phase of the growth of the fungus for the utilization of nutrients present in the solid substrate [11]. The subsequent decrease in the enzyme units could probably due to the inactivation of the enzyme by other constituent proteases, the reduced availability of nutrients and production of toxic metabolites [1]. Tremacoldi and Carmona 2005 reported that the highest protease activity was obtained by *Aspergillus clavatus* after 6 days of incubation for culture medium containing glucose and casein at 1% (w/v) as substrates [15].

Effect of fermentation temperature

The protease activity was assayed at different temperatures ranging from 20 to 45^{0}C. Enzyme activity was increased up to 25^{0}C and further increase in temperature resulted in decrease of enzyme activity (Fig 2). Hence, the optimum temperature was found to be 25^{0}C. The reduction in enzyme activity may be of enzyme denaturation by losing its catalytic properties at high temperature due to stretching, breaking of weak hydrogen bonds within the enzyme structure[1]. In earlier reports, Pushpa and Madhava Naidu 2010 reported the maximum production of protease from coffee by products by *Aspergillus oryzae* was obtained at temperature 30^{0}C [9].

Effect of pH

Protease production by microbial strains depends on the extra cellular pH because culture pH strongly influences many enzymatic processes and transport of various components across the cell membranes, which in turn support cell growth and product production [10,8]. Extremely high or low pH values generally result in complete loss of activity of enzymes. pH range of 4-10 was varied to estimate maximum protease activity. The enzyme synthesis was increased with increase of medium pH towards neutrality, maximum (162.5 U/gds) being at pH 7.0 and further

increase resulted in decrease of enzyme activity (Fig 3). The decrease may be due to the enzyme instability at any pH other than its optimum, viz., 7.0. Similar results were also reported by Paranthaman *et al*., 2009 that the maximum production of neutral protease from rice mill waste by *Aspergillus niger* was obtained at pH 7.0 [10].

CONCLUSIONS

Optimization is an important aspect to be considered in the development of fermentation technology for minimizing the amount of unutilized components at the end of fermentation. However, particularly the *Penicillium* spp.are known for their ability to produce proteolytic enzymes with potential use in industry. In the present study, sesame oil cake with *P.chrysogenum* NCIM 737 was found to be potential producer of neutral protease. Fermentation time, temperature and pH are the key factors which influenced the outcome of SSF. The optimized conditions at which the protease production was enhanced were fermentation time 7 days, temperature 25^0C, pH 7.0. From the results, it could be inferred that neutral protease produced through SSF of the sesame oil cake by *P.chrysogenum* NCIM 737 could possibly find useful application in food industries.

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