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Research article

OPTIMIZATION OF PROTEASE PRODUCTION FROM FUNGI ISOLATED FROM SOIL

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ABSTRACT: Fungal strains isolated from soil by serial dilution method were screened for alkaline protease production. Isolate *Penicillium chrysogenum* the most potent producer of alkaline protease was identified. The isolate showed highest activity in the optimized medium at pH 9.0, temperature 35ºC, with 1% soycake and peptone incubated for 7 days. Proteases represent one of the largest groups of industrial enzymes and find application in detergents, leather industry, food industry, pharmaceutical industry and bioremediation processes.
Key words: Protease, Fungi, Soil

INTRODUCTION

For the microbial and biological activities soil is a dynamic medium and the number and kind of microorganisms present in soil depends on environmental factors (Prescott *et al*., 1993). Microorganisms are the most common sources of commercial enzymes due to their physiological and biochemical properties, facile culture conditions and ease of cell manipulation (Dias *et al*., 2010). 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 (Mohanasrinivasan *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). 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 largest use in house hold laundry with a worldwide annual production of detergents of approximately 13 billion tons (Nehra *et al*., 2002). Plant, animal and microbial sources are employed in enzyme production. Microbial proteases are preferred to plant and animal sources to various advantages. A variety of microorganisms such as bacteria, fungi, yeast and actinomycetes are known to produce these enzymes (Madan *et al*., 2002). Even though most commercial proteases originated from microorganisms belonging to the genus Bacillus, fungi exhibit a wider variety of proteases than bacteria. Furthermore, fungi are normally generally regarded as safe strains and they produce extracellular enzymes, which are easier to be recovered from fermentation broth 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 (Sandhya *et al*., 2005). In the present investigation, protease producing fungi were isolated from soil sample, screened and identified. The enzyme was then produced in shake flask and the critical production parameters like pH, incubation time and inducer concentrations were optimised.

MATERIALS AND METHODS

Collection of Sample

Soil samples were collected from the Dr. B. Lal Institute of Biotechnology campus by the means of sterilized spatulas and collected in sterile polythene bags. The samples were then brought to the laboratory for microbiological study.

Isolation of Fungi

Soil samples were collected for the isolation of fungi. One gram was transferred to aliquots of 9 mL sterile distilled water in test tube. It was shaken vigorously at constant speed for 15 min. The soil suspension was then subjected to serial dilutions from the appropriate plate in duplicate. The plates were incubated for 5 days at 28°C. The well-grown spread single colonies were picked up and further sub-cultured on potato dextrose agar slants. (Cappuccino and Sherman, 2002).

Screening for Protease enzyme production

Soil associated fungi were tested for their ability to produce protease enzyme by casein hydrolysis on agar plates containing 0.5% casein, 0.5% of glucose and 2% of agar (w/v), pH 7.0 (Larsen *et al*., 1998). The plates were then incubated at 28ºC for 1-2 days. Enzyme activity was indicated by the formation of a clear zone around colonies after precipitation with 1 M HCl solution.

Production of Protease enzyme

The culture medium used for protease production was mineral medium containing (g L^{-1}): $MgSO_4$, 0.52; KCl, 0.52; KH_2PO_4, 1.52; $FeSO_4$ 7H2O, 0.01; $ZnSO_4$.7H2O, 0.01; sodium caseinate, 5. A suspension (1 mL) containing 10^8 spores mL-1 from 8-day-old colonies was inoculated into Erlenmeyer flasks containing 100 mL of culture medium. Flasks were kept at 28ºC, under agitation at 150 rpm, for 5 days. The mycelial mass was obtained by filtering the contents of each flask and then drying the mass at 60ºC until it reached a constant weight. The flasks were sampled at intervals and the culture supernatant used as the enzymatic source. All microbial cultivation was performed in triplicate for each selected isolate

Protease enzyme activity assay

A proteolytic activity assay, using casein as the substrate, was performed according to described by Ramakrishna and Pandit (1988) with some modifications. Enzyme activity was determined by incubating 250 µL of the culture supernatant with 500 µL 1% (w v-1) casein sodium salt in 50 mM buffer (pH 5.0, 7.0 and 9.0) for two hours at 30ºC. The reaction was stopped by the addition of 375 µL 20% (w v-1) trichloroacetic acid. The tubes were placed in an ice bath for 30 minutes and then centrifuged at 5000 x g for 15 min. at 4ºC. Proteolytic activity was determined by the absorbance reading of the supernatant at 280 nm versus an appropriate blank. Tyrosine was used as standard. One unit of protease activity is defined as the amount of enzyme which liberates one micromole of tyrosine per minute per gram dry substrate under experimental conditions. Protein was estimated by the method of Lowry *et al*., (1951).

Optimization of culture conditions for Protease enzyme production

Effect of pH on Protease enzyme production:

To determine optimal pH, fungus cultures were cultivated in a 150 mL flask containing 50 mL optimized medium with different pH ranges from 6.0 to 11.0. The pH of the medium was adjusted by using 1 N HCl or 1 N NaOH. The flasks were kept in stationary stage at 28°C for 5 days of cultivation.

Effect of temperature on Protease enzyme production:

In order to determine the effective temperature for protease production by the fungal species, fermentation was carried out at 10°C intervals in the range of 35, 40, 45, 50 , 55 and 60 ± 2°C.

Effect of carbon sources on Protease enzyme production:

Effects of various carbon compounds namely, fructose, glucose, sucrose, lactose and maltose were used for studying. The broth was distributed into different flasks and 1.0% of each carbon sources were then added before inoculation of the strain and after culture inoculation, the flasks were incubated for 5 days at 28°C.

Effect of nitrogen sources on Protease enzyme production:

The fermentation medium was supplemented with organic and inorganic compounds (ammonium sulphate, urea, yeast extract and peptone) replacing the prescribed nitrogen source of the fermentation medium.

Effect of agricultural waste on Protease enzyme production:

In the present study, we aim to determine the appropriate concentration of municipal solid waste residue for protease production by the fungal sp. The fermentation medium was supplemented with agricultural waste residue such as Groundnut Cake, Coconut Cake, wheatbran and Soya cake, replacing the prescribed carbon source of the fermentation medium.

Statistical Analysis

Data presented on the average of three replicates (±SE) obtained from their independent experiments.

RESULTS AND DISCUSSION

Fungi occurring in natural habitats with changing environmental conditions are important from the industrial point of views. They can be found producing novel metabolites or enzymes with hyper catalytic properties.

Screening of fungi for Protease enzyme activity

Screening of fungi for their protease activity was carried out by the hydrolysis of substrate incorporating in the basal salt medium. After an incubation period, enzyme activities were detected by the appearance of zones around the fungal colonies. Four fungal isolates i.e. *Aspergillus sp, Penicillium sp, Fusarium sp and Microsporium sp* showed the highest zone around the colony, were used for further study. Among fungi species of certain genera, *Aspergillus, Penicillium, Paecilomyces, Rhizopus* and *Rhizomucor* are well-known producers of proteases (Sindhu, 2009; Vamsi Krishna, 2009) and are reported active over a wide pH range (Rao, 1998).

Optimization of culture conditions for cellulase enzyme production

Effect of pH on protease enzyme production

Among physical parameters, pH of the growth medium plays an important role by inducing morphological changes in microbes and in enzyme secretion. The pH change observed during the growth of microbes also affects product stability in the medium. The optimal pH varies with different microorganisms and enzymes. All the four isolates were allowed to grow in media of different pH ranging from 6.0 to 11.0. Maximum enzyme activity was observed in medium of pH 9.0 in case of *Penicillium chrysogenum* (1.39 U/ml) followed by *Aspergillus niger* (0.28 U/ml) (Fig 1).

It has been reported widely that protease production from microbial source can be acidic or alkaline proteases as reported by many researchers depending on the organisms and source of the isolation. Pagre *et al*., (2009) obtained an optimum pH for protease activity of 8 for .but relatively high pH between 3.0 and 9.0 for extracellular protease from *Aspergillus niger* and *Bacillus subtilis*. Nascimento and Martins (1996) and Sookkheo *et al.*, (2000) also reported the optimum pH for protease activity to be between 7.0 and 8.5. According to Borris (1987) alkaline protease production is found to be higher at pH 9-13.

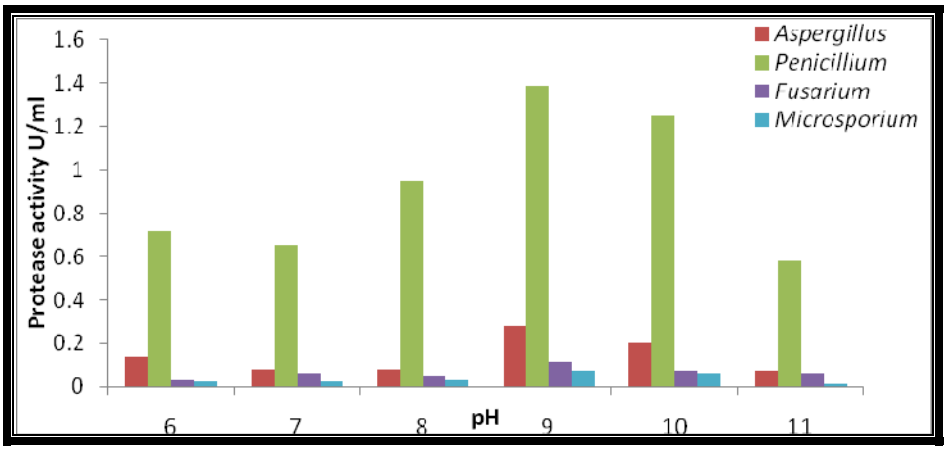

Fig 1: Effect of pH on Enzyme Production

Effect of temperature on protease enzyme production

Incubation temperature plays an important role in the metabolic activities of a microorganism. Even slight changes in temperature can affect enzymes production. Presently, the optimal temperature for maximum protease production was at 28ºC with production decreasing at higher temperature. Since enzyme is a secondary metabolite produced during exponential growth phase, the incubation at high temperature could lead to poor growth and thus a reduction in enzyme yield (Sabu *et al*., 2012). Assay mixture was incubated at different temperature ranging from 35-60°C and enzyme activity was found to be highest at 35°C. However, the enzyme was completely inactivated at 60°C. The results of the test made at different temperatures value showed that the optimal temperature for protease activity (0.28 U/mL) produced by *Penicillium chrysogenum* at 35°C (Fig 2)**.** Similarly, Ganesh Kumar *et al.*, (2008) reported the optimum temperature for protease production for by was in mesophilic fungi *Synergistes* species at 35^0C. Growth and protease production ceased at higher temperature (50°C) similar observation were shown by Morimura *et al*., (1994) for *Aspergillus usami*.

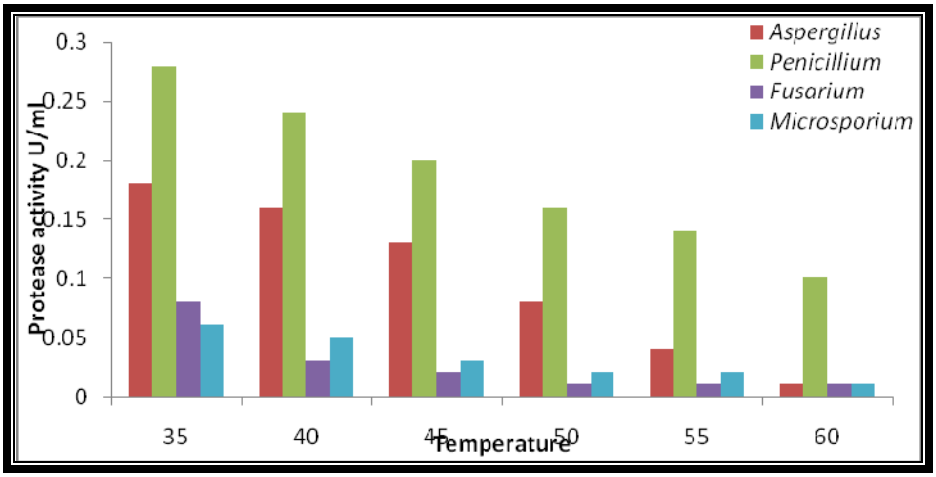

Fig 2: Effect of Temperature on Enzyme Production

Effect of carbon sources on Protease enzyme production

Various sources of carbon such as fructose, sucrose, lactose and maltose were used to replace glucose which was the original carbon source in growth media. Results obtained showed that *Penicillium chrysogenum* in presence of sucrose, fructose and lactose also brought about the protease production compared to other carbon sources (Fig 3). There are general reports showing that different carbon sources have different influences on extracellular enzyme production by different strains (Nehra, 2002). Sinha, (2009) compared the effect of carbon sources such as glucose for protease production; among these lactose induced the protease production with 0.118 U ml-1 on 4th day of fermentation. Similarly, Narayana (2008) was reported that carbon sources like glucose, maltose, starch were indispensable components for protease production by *S. albidoflavus* in submerged fermentation.

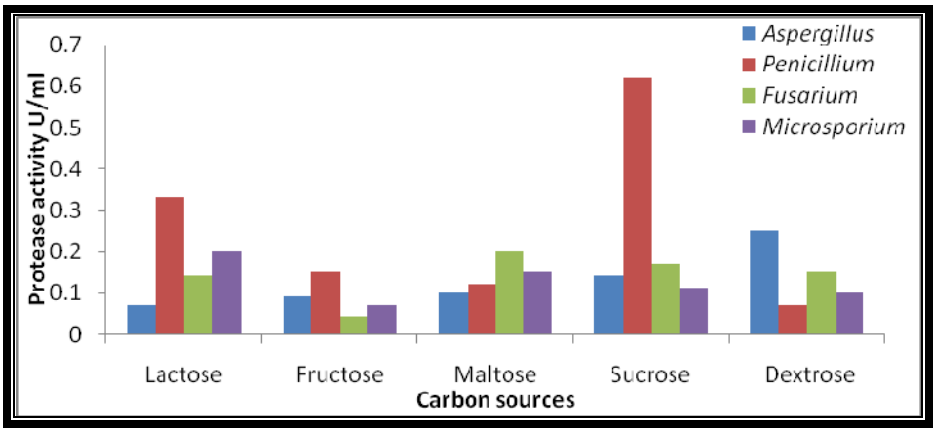

Fig 3: Effect of Carbon sources on Enzyme Production

Effect of nitrogen sources on Protease enzyme production:

Results indicate that the sources of nitrogen greatly affected the production of protease enzyme. Ammonium sulphate (AS) was the best nitrogen source for *Penicillium chrysogenum* (Fig 4). It was reported that good protease yield can be obtained with ammonium compound as the nitrogen source. Our results are in accordance with the work of Kalpana Devi *et al.*, (2008) who reported that good protease production can be obtained with mannitol and ammonium sulphate. The mechanism that shows the formation of extracellular enzymes is influenced by the availability of precursors for protein synthesis. Many papers have reported that ammonium compounds are the most favourable nitrogen sources for protein and cellulase synthesis.

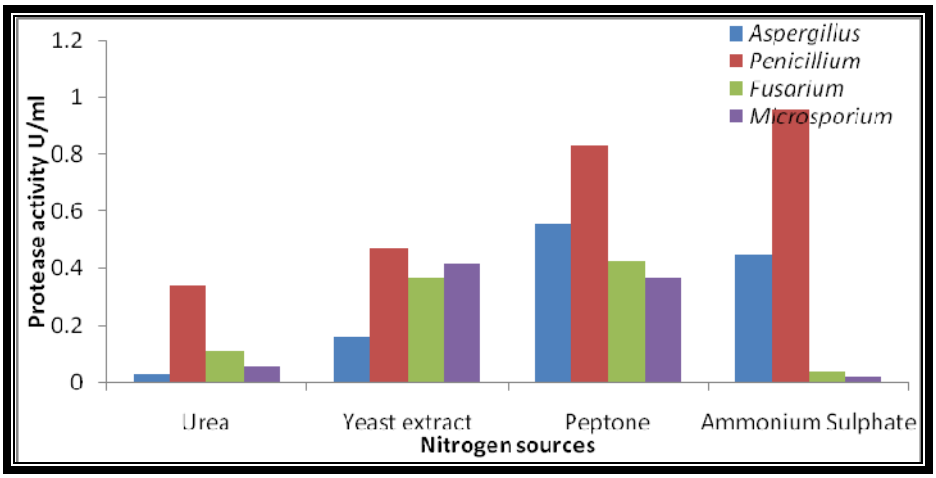

Fig 4: Effect of Nitrogen sources on Enzyme Production

Effect of agricultural waste on Protease enzyme production:

The bioconversion of agro waste based lignocellulosic material to energy has gained much interest during the recent past. Low cost of enzyme production improves the economics, as the cost of enzymes constitutes a major part of the total cost of hydrolysis (Depaula *et al.*, 1999). The enzymatic degradation of waste by fungal enzymes has been suggested as a feasible alternative for the conversion of lingo cellulosic material in to fermentable sugars and ethanol (Shin *et al.*, 2000).

The degree of saccharification was assayed on the basis of release of reducing group. The amount of reducing sugar increased with time of incubation in the presence of enzyme. The maximum amount of percent saccharifaction was found to be 0.67 U/ml by *A. Niger* for soya cake (Fig 5). Enzymatic conversion of protease to food, fuel and chemical feedstock is a well-established process. However, high cost of protease production has hindered use of this enzyme in industry. This source of raw material is available in abundance and generally free of cost.

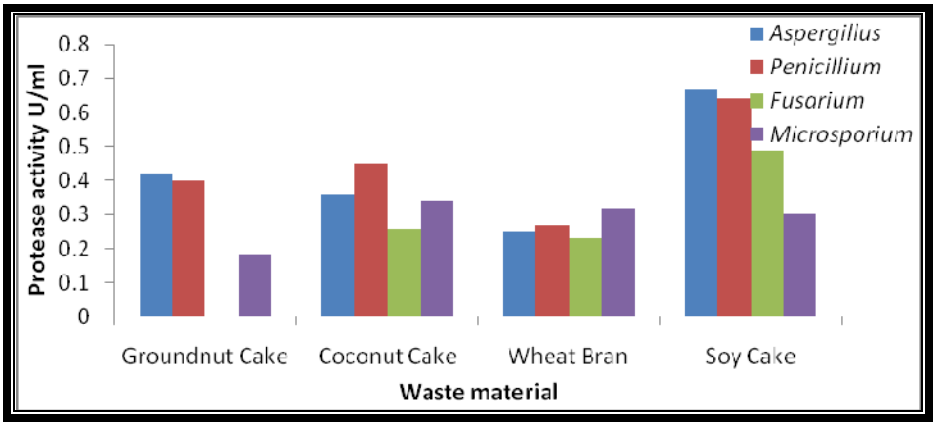

Fig 5: Effect of Agricultural waste on Enzyme production

CONCLUSION

Based on the results from this study, we finally concluded that the Soy coke and coconut cake were proved to be suitable substrates for protease production in submerged fermentation by *Penicillium* spp. Optimum pH, temperature, carbon, nitrogen source and Agricultural waste for alkaline protease production were determined as 9, 35°C, Sucrose, Peptone and Soyacake respectively.

The cost-effective technologies are needed for economical production of proteases using Agrobased waste residues as substrate. Major parameters affecting the fermentation process for enzyme production were studied and optimal levels were identified. Enzyme production is closely controlled in microorganisms and for improving its productivity, these controls can be ameliorated. Proteases yields appear to depend on a complex relationship involving a variety of factors like inoculum size, pH value, temperature, presence of inducers, medium additives, aeration, growth time, and so forth. This study has given a hint that microbial wealth of alkaline protease producing fungi isolated from soil can be harnessed for biotechnological processes. The appreciable high enzyme activity at alkaline pH (pH 9.0) suggested that *Penicillium chrysogenum* is a potential producer of alkaline proteases which can find application in detergent and textile industries.

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