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Fermentation Medium Optimization for the Biosynthesis of Protease by *Penicillium chrysogenum* in Shake Flasks

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Abstract.- Present investigation describes the optimization of fermentation media parameters for improved production of protease by *Penicillium chrysogenum* IHH_5 in shake flasks. Out of different agro-industrial byproducts used as substrates for increased production of enzyme, soybean meal was found to be the best substrate. Glucose and peptone proved to be the best carbon and nitrogen sources for the biosynthesis of protease by mould culture. Finally, a volume of 50ml of culture medium per 250 ml shake flask was found to be optimum in present studies.

Key words: Fermentation substrate, submerged fermentation, shake flask fermentation, proteases.

INTRODUCTION

Proteases, proteinases or peptidases are subclasses of hydrolases and describe the same group of enzymes that catalyze the hydrolysis of covalent peptide bonds (Raju *et al*., 1994). They vary widely in their ability to hydrolyze various peptide bonds *i.e.* each type of protease has specific kind of peptide bond to break (Sidney and Lester, 1932). Proteases occur in a wide variety of microbial species including fungi and bacteria (Watson, 1976) which produce these enzymes in their extracellular environment to digest the complex substrates present there.

Fungi are usually grown on substrates, which contain nutrient concentrations much higher than those which the organism is likely to encounter in nature. The nutrient balance provided during the period of growth may also be of importance in determining the subsequent microbial growth during fermentation (Carlile *et al*., 2001). The substrates used are usually defatted oil seed cakes and other agro-industrial byproducts. Agro-industrial byproducts such as soybean meal, sunflower meal, rice bran, wheat bran, cottonseed meal and rapeseed meal have been extensively evaluated for the biosynthesis of protease (Joo *et al*., 2002; Haq *et al*., 2003).

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Fermentation substrate is a particularly important parameter to control due to eventual associated growth inhibitions and to increase the effectiveness of the carbon flux, by reducing the amount of byproducts formed and the amount of carbon dioxide evolved. There were numerous correlations across species in physiological responses to carbohydrate and mineral nitrogen. Fungi would exhibit one of the two basic alternative strategies *i.e*., either they grow better on nitrogen source, respond little to carbohydrates and produce little in the way of protease, or they respond negatively or not at all to mineral nitrogen, grow better with more carbohydrates and produce copious protease (Eaton and Ayres, 2002).

Nitrogen is an quantitatively important bioelement which is required by many microorganisms during fermentation for their protein synthesis (Carlile *et al*., 2001). Nitrogen control in microorganisms consists of repression of the pathways of assimilation of some nitrogen sources when some other, more easily assimilated source of nitrogen is available to the cells (Herrero *et al*., 2001).

Similarly, a source of carbohydrate is needed as an energy source for respiration to release energy needed for growth. Reduction in carbohydrate supply decreases the growth of most fungi. Different carbohydrates influence the protease production by microorganisms in submerged fermentation.

Glucose, starch, fructose, lactose, and xylose act as major carbon source during fermentation process (Mabrouk *et al*., 1999; Singh *et al*., 2004). Carbon catabolite repression and availability of nucleic acid precursors are also thought to play a role in protease biosynthesis (Carlile *et al*., 2001).

The present work was undertaken for the optimization of fermentation media ingredients for production of protease by *Penicillium chrysogenum* IHH_5 in submerged fermentation. Suitable protein substrate, carbon and nitrogen sources and volume of culture medium were optimized for the production of protease during submerged fermentation in shake flasks.

MATERIALS AND METHODS

Microorganism and maintenance

The mould culture of *Penicillium chrysogenum* IHH_5 was isolated from soil samples of Lahore area using potato-dextrose-casein agar plates. The culture was maintained on potato-dextrose agar (PDA) slants and was stored in cold lab at 4°C.

Inoculum preparation

The slants of five days old cultures were wetted by adding 10ml 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. One milliliter of this suspension containing $2.4x10^7$ spores/ml was used to inoculate the fermentation flasks.

Fermentation medium

The fermentation was carried out in 250 ml Erlenmeyer flask containing 50 ml of culture 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. The cotton-plugged flasks were then subjected to sterilization in an autoclave at a pressure of 15-lbs/$inch^2$ (121°C). The production medium was then cooled at room temperature and was inoculated with 1ml of conidial suspension as prepared earlier. The flasks were then placed in the rotary type incubator shaker (Sanyo, GallenKamp PLC, UK) rotating at the speed of 200 rpm at 30°C for 72 hours. After 72 hours of incubation, the contents of the flasks were filtered using Whattman filter paper # 44 and the filtrate was used for the assay of protease.

Analytical methods

Mycelial dry weight

After fermentation, the fermented broth was filtered using pre-weighed Whattman filter paper # 44. The filtered contents were washed with water thrice and then dried at 105°C over night in a hot air oven. The dried contents were weighed again to calculate the mycelial dry weight.

Assay of protease

The activity of protease was assayed by the method of McDonald and Chen (1965). To 1ml of the enzyme extract in the test tube, 4.0 ml of 1.0% casein was added. The mixture was incubated at 35°C for one hour. The residual protein was precipitated by adding 5ml of 5% TCA (Trichloroacetic acid). The precipitates were allowed to settle for 30 minutes. The contents of the tubes were centrifuged at 5000 rpm for 5 minutes. One milliliter of the supernatant was mixed with 5ml of alkaline reagent. Then 1ml of 1N sodium hydroxide was added to make the contents of the tube alkaline. After 10 minutes, 0.5ml of Folin and Ciocalteau reagent was added as a result of which, blue color was produced. The tubes were left for 30 minutes to get maximum development of blue color. The optical density of the mixture was read at 700 nm on spectrophotometer (Cecil-CE7200, Aquarius, UK).

One unit of protease activity 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

Evaluation of different agro-industrial byproducts

Different agro-industrial byproducts such as soybean meal, sunflower meal, gluten meal, rapeseed meal, cottonseed meal and wheat flour as sources of protein, carbohydrate and minerals were evaluated for the production of protease by

Penicillium chrysogenum IHH_5 in submerged fermentation (Fig. 1). Of all the substrates examined, soybean meal supported maximum growth of the microorganism (14.01 g/L) and gave maximum production of enzyme (12.72 U/ml). Other substrate such as gluten meal, sunflower meal, rapeseed meal, wheat flour and cottonseed meal gave 7.13, 8.58, 6.23, 4.37, and 5.40 U/ml of the enzyme, respectively. Therefore, soybean meal was selected as a suitable agro-industrial by-product for the production of protease by *Penicillium chrysogenum* IHH_5.

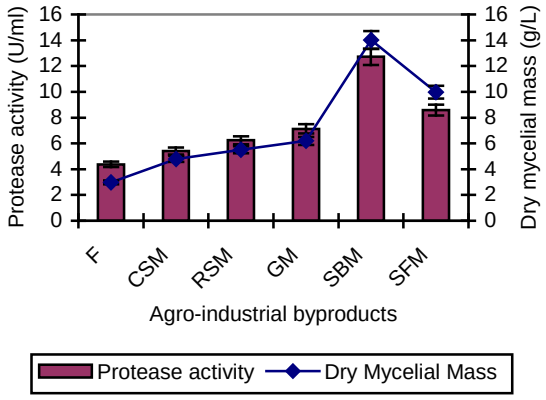

Fig. 1. Effect of different agro-industrial byproducts on the production of protease by *Penicillium chrysogenum* IHH_5. Each value is a mean of three replicates. Y- error bars indicate the standard error from mean. Incubation period, 72 h; Incubation temperature, 30°C; Initial pH, 7.0
Abbreviations: CSM, cotton seed meal; F, flour; GM, gluten meal; RSM, rape seed meal; SBM: soy bean meal; SFM, sun flower meal.

The reason of highest yield was due to the fact that soybean meal provided an adequate source of protein, carbohydrate and minerals needed by the organism for the biosynthesis of protease. It contains 42% proteins, 29.9% carbohydrates and a fairly good ratio of minerals (Traders Protein, USA). Similar results for the production of protease were reported by Heneri *et al*. (1988) who used different protein substrates for the production of proteolytic enzyme by *Aspergillus niger* and found soybean meal as the most suitable substrate.

Effect of different carbon sources

Carbon is required as an energy source and is known to greatly influence the protease production from microorganisms (Gilberto *et al*., 1999). Different carbon sources were evaluated for the production of protease by *Penicillium chrysogenum* IHH_5 in submerged fermentation (Fig. 2). Carbon sources such as starch, fructose, lactose, maltose or glucose were added to the fermentation medium at a concentration of 0.5% (w/v) which gave enzyme activity of 4.12, 5.64, 6.92, 8.54, and 12.74 U/ml, respectively. As shown in the results, highest yield of protease was observed when glucose was used as a carbon source in the fermentation medium.

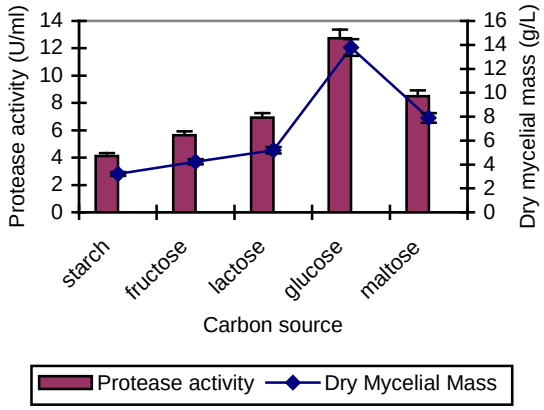

Fig. 2. Effect of different carbon sources on the production of protease by *Penicillium chrysogenum* IHH_5. Each value is a mean of three replicates. Y- error bars indicate the standard error from mean. Incubation period, 72 h; Incubation temperature, 30°C; Incubation pH, 7.0

The results were in accordance with those made by Andrade *et al.* (2002) who reported that D-glucose acted as a best carbon source for the production of extracellular proteases by *Mucor circinelloides*. It has been frequently described that in a defined medium, a protein source must be present for the enzyme to be produced. However, it has also been noticed that the absence of a proper carbohydrate (c-source) in the medium results in a dramatic decrease in enzyme production (Gajju *et al*., 1996).

The reason of obtaining highest yield of

protease by using glucose as a carbon source lies in the fact that almost all fungi can utilize glucose, which is a widespread sugar occupying a central position in metabolism. Moreover, fungi have a constitutive (permanently present) transport system for glucose, but those for other sugars this transport is inducible appearing only in the presence of certain specific inducers. Fungi require glucose for growth purposes as well as for the synthesis of a wide range of metabolites required for the structure and function of cells (Carlile *et al*., 2001).

Effect of different nitrogen sources

The protease production is directly related to the type and amount of nitrogen source present in the fermentation medium (Rao *et al*., 1998). So different nitrogen sources supplied in the fermentation medium were evaluated for the production of protease by *Penicillium chrysogenum* IHH_5 (Fig. 3). Nitrogen sources such as yeast extract, meat extract, nutrient broth or peptone were examined for the production of enzyme, which gave 7.79, 9.04, 4.15, and 12.71 U/ml of protease, respectively. So peptone was found to be the best nitrogen source for the production of protease by *P. chrysogenum.*

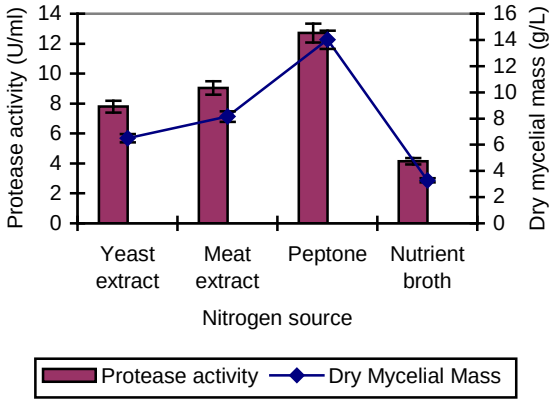

Fig. 3. Effect of different nitrogen sources on protease biosynthesis by *Penicillium chrysogenum* IHH_5. Each value is a mean of three replicates. Y- error bars indicate the standard error from mean. Incubation period, 72 h; Incubation temperature, 30°C; Initial pH, 7.0; carbon source, glucose.

Similar work has also been reported by El-Zalaki *et al*. (1974) who used peptone as a best nitrogen source for the protease production by *Aspergillus niger*. Similarly, Channe and Shewale (1998) also reported the production of extracellular protease by *A. niger* using meat peptone as a nitrogen source. Peptone is a best nitrogen source because small peptides residue in the cells of microorganisms and are hydrolyzed by intracellular peptidases. This renders the peptone as a best easily metabolizable nitrogen source (Carlile *et al*., 2001).

Effect of volume of fermentation medium

Different volumes of fermentation medium in shake flasks were evaluated for the production of protease by *Penicillium chrysogenum* IHH_5 (Fig. 4). The volume of medium ranged from 25-125ml in 250ml Erlenmeyer flasks. The amount of enzyme synthesis was considerably increased when the volume of fermentation medium was increased from 25ml of fermentation medium (8.86 U/ml) to 50ml of the fermentation medium (12.78 U/ml). It was also observed that there was a sudden decrease in the protease biosynthesis when the volume of fermentation medium was increased from 50ml to 75ml and decreased gradually by further increasing the volume of fermentation medium above 75ml reaching minimum (2.5 U/ml) when the volume of fermentation medium was kept at 125ml per 250ml Erlenmeyer flasks.

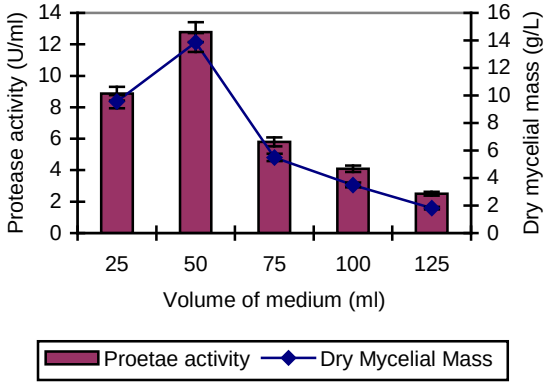

Fig 4. Effect of volume of fermentation medium on the production of protease by *Penicillium chrysogenum* IHH_5. Each value is a mean of three replicates. Y- error bars indicate

the standard error from mean.
Incubation period, 72h; Incubation temperature, 30°C; Initial pH, 7.0; carbon source = glucose; nitrogen source, peptone.

The reason lies in the fact that as the volume of medium increased, oxygen supply to the microorganism in the shake flasks was decreased which directly affected the growth of the microorganism and corresponding yield of the proteolytic enzymes. It was also due to the fact that increased volume of the medium resulted in the decreased agitation and recirculation of the media ingredients which in turn resulted in the decreased supply of nutrients to the organism and hence, less growth and less enzyme production (Carlile *et al*., 2001).

CONCLUSIONS

The production of protease from *P. chrysogenum* can be enhanced substantially by optimizing the composition of fermentation medium. In addition, the volume of the medium also has a strong bearing on the production of enzyme in shake flasks.

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