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BIOSYNTHESIS OF ACID PROTEASES BY *PENICILLIUM GRISEOROSEUM* IH-02 IN SOLID-STATE FERMENTATION

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Abstract

The present investigation describes the production of acid proteases by *Penicillium griseoroseum* IH-02 using solid-state fermentation technique. The enzyme biosynthesis was maximum (8.2 U/ml) when fermentation was carried out on a substrate containing soybean meal and wheat bran mixed at a ratio of 3:1 and moistened with D_4 diluent. The substrate to diluent ratio was found to be optimum at 1:2. The extraction of the enzyme from fermented broth was carried out with different extractant and distilled water having pH 5.0 was found to be the best extractant of the enzyme.

Introduction

Fungi elaborate a wide variety of enzymes than do bacteria and proteases are among the most important enzymes produced by fungi. Fungi produce a variety of proteolytic enzymes however, most of these are usually acidic in nature (Fernandez *et al*., 1998; Wu & Hang, 2000). Acidic proteases are characterized by maximum activity and stability at pH 4.0-4.5 and 2.0-6.0 respectively (Yongquan *et al*., 1998) and are rapidly inactivated at higher pH values. They exhibit broad substrate specificity and have a molecular weight around 35,000 (Rao *et al*., 1998). They are low in basic amino acid content, have low isoelectric point and clinical esterase activity but can split a wide range of peptide bonds. They are generally used in detergents and leather industries (Rao *et al*., 1998; Kumar &Takagi, 1999) and particularly in the food industries due to their narrow pH optima and temperature specificities. They have also important medical and pharmaceutical applications (Koeabiyik & Erdem, 2002). However, their main drawbacks are lower reaction rate and worse heat tolerance than do the bacterial enzymes (Rao *et al*., 1998).

A great number of fungal strains have been used to produce proteases such as *Aspergillus flavus* (Mulimani & Patil, 1999), *Aspergillus niger* (Godinez *et al*., 2001), *Aspergillus oryzae* (Battaglinio *et al*., 1991), *Rhizopus oligosporus* (Arroteia *et al*., 2001, Haq *et al*., 2003), *Mucor meihi* (Thaker *et al*., 1990; Ottensen & Rickert, 1970.), *Mucor pusillius* (Arima *et al.,* 1970; Khan *et al.,* 1981), *Mucor bacilliformis* (Fernandez *et al*., 1997), *Trichoderma harzianum* (Dunaevsky *et al*., 2000), *Verticillium lecanii* (Barranco *et al.*, 2002) and *Fusarium subglutinans* (Hamdy, 2003). But *Penicillium species* have a great biotechnological potential for the production of proteases and other enzymes. These include *Penicillium expansum* (Fernandessalomae *et al*., 1996), *P. citrinum* (Zhu *et al*., 1996), *P. occitanis* (Choabouni *et al*., 1995), *P. notatum* (Szczodreak *et al*., 1994), *P. verrucosum* (Roussos *et al*., 1991), *P. janthinelum* (Mikhailov *et al*., 1994), *P. purpurogenum* (Steiner *et al*., 1994), *P. candium* (Ortizvazquez *et al*., 1993) and *P. duponti* (Hashimoto *et al.,* 1972; Hashimoto *et al.,* 1973).

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The filamentous fungi have a potential to grow under varying environmental conditions utilizing a wide variety of substrates as nutrients. So the present work was undertaken for the production of acid proteases by *Penicillium griseoroseum* IH-02 in solid-state fermentation. Solid-state fermentation involves the utilization of water insoluble material for microbial growth and metabolism and is usually carried out in solid or semi solid systems in the shortage of available water. Such a system provides nearly same condition to the fungi as in their natural habitats. In addition, SSF has a number of advantages over submerged fermentation that is why it was selected in the present studies.

Materials and Methods

Microorganism and maintenance: The mould culture of *Penicillium griseoroseum* IH-02 was taken from the culture collection of our Institute. The present strain was previously isolated from soil samples of Lahore area. The culture was maintained on potato-dextrose-agar (PDA) slants at 4^oC.

Inoculum development: The slants of five days old cultures were wetted by adding 10 ml of 0.005% sterilized solution of monoxol O.T. (Diocetyl 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 ml of this spore suspension containing 2.8×10^6 spores was used as inoculum.

Fermentation procedure: 250 ml conical flasks containing 10 g substrate moistened with 20 ml of diluent (pH 5.0) were sterilized at 121°C (15 lbs/inch^2 pressure) in an autoclave. After sterilization, the flasks were cooled, inoculated with the organism and incubated at 30±1 degrees C for 72 h. After incubation, 70 ml of distilled water was added to the flasks, which were shaken on rotary shaker for one hour at 200 rpm. The contents of flasks were then filtered and filtrate was used for the assay of acid proteases.

Substrates and diluents: Different agricultural by-products such as sunflower meal, soybean meal, wheat bran, rice husk and cottonseed meal were evaluated for the production of acid proteases. Following eight diluents (pH adjusted to 5.0) were used to moisten the substrate:-

D_1: Glass distilled water.
D_2: Tap water.
D_3: (g/l) $NaNO_3$, 2.0; KH_2PO_4, 1.0; $MgSO_4.7H_2O$, 0.5; KCl, 0.5; $ZnSO_4.7H_2O$, 0.1 and $FeSO_4.7H_2O$, 0.1.
D_4: (% w/v) Yeast extract, 1.0; Glucose, 1.0; Peptone, 1.0; $CaCO_3$, 0.5.
D_5: (%w/v) Glucose, 1.0; Peptone, 1.0; Beef extract, 1.5; NaCl, 0.5.
D_6: (g/l) $(NH_4)_2SO_4$, 1.5; Urea, 3.0; KH_2PO_4, 2.0; $MgSO_4.7H_2O$, 3.0; $CaCl_2$, 3.0.

Extractants: Different extractants were used for the recovery of acid proteases from the fermented broth. These include citrate buffers (pH adjusted to 4.0, 5.0 and 6.0), citrate phosphate buffer (pH 7.0), phosphate buffer (pH 8.0) and distilled water (pH 5.0).

Assay of proteases: The method of McDonald & Chen (1965) was used for the assay of proteases. Casein (1%) was incubated with one ml of enzyme sample at 30 degrees C for one hour. The reaction was arrested by the addition of five ml of trichloroacetic acid (TCA) solution. The mixture was centrifuged and one ml of supernatant was mixed with five ml of alkaline reagent. To this mixture one ml of 1N NaOH was added to make the contents of the tube alkaline. After 10 min., 0.5 ml of Folin and Ciocalteau reagent was added to the test tubes and mixed. The blue colour produced was measured with UV spectrophotometer (CECIL, CE 7200, Cambridge, England) at 700 nm after 30 min. One unit of protease activity is defined as the amount of enzyme required to produce an increase of 0.1 in optical density at 700 nm under defined conditions.

Results and Discussion

Selection of substrate: Different substrates such as sunflower meal, soybean meal, cottonseed meal, wheat bran and rice husk were evaluated for the synthesis of acid proteases (Fig. 1). Of all the substrates examined, soybean meal gave maximum enzyme activity (5.6 U/ml). The enzyme production decreased in the following order, sunflower meal (4.8 U/ml) > wheat bran (3.6 U/ml) > cotton seed meal (3.5 U/ml) > rice husk (3.0 U/ml). Soybean meal gave maximum yield of proteases because this agricultural by-product had adequate supply of proteins, carbohydrates and minerals needed for the growth of organism. Similar reports have also been made by Qadeer *et al.,* (1990) while Mulimani & Patil (1999) used similar agricultural by-products for the production of proteases using *Aspergillus flavus* as the organism of choice.

Selection of diluent: The effect of different diluents (moistening agents) on the production of acid proteases by *P. griseoroseum* were studied and it was found that the production of enzyme was maximum (5.6 U/ml) when substrate was moistened with D_4. The synthesis of enzyme however decreased in the order of $D_2 > D_5 > D_1 > D_3$ and D_6 respectively (Fig. 2). The different ratios of substrate to diluent were also investigated and it was found that optimum ratio of substrate to diluent was 1:2 (Fig. 3). The enzyme synthesis was maximum when D_4 was used as diluent, which indicated that the organism required additional nutrients for its growth. All the deficient nutrients from the substrate were supplied by the diluent D_4 for growth of the organism and production of the enzyme. It also seems that the nutrients present in other diluents may not be sufficient or may have an inhibitory action on the growth of the organism and subsequently on the enzyme production so gave less production of the enzyme.

Partial replacement of soybean meal with wheat bran: Figure 4 shows the effect of partial replacement of soybean meal with wheat bran. Soybean meal was replaced with different ratios of wheat bran and maximum enzyme synthesis (8.2 U/ml) was achieved when soybean meal was replaced with wheat bran at a ratio of 3:1. Other ratios of replacement did not show appreciable results.

It is evident from the above results that replacement of soybean meal with wheat bran showed better results because it increased the aeration of the growth medium and prevented the formation of a compact scum with very less interspaces, as was the case when only soybean meal was used. Wheat bran also provided sufficient nutrients to the fungus for growth because reports have shown the production of acid proteases from *Mucor bacilliformis* and *Neosartorya fisheri* using wheat bran as a sole substrate (Fernandez *et al*., 1997; Wu & Hang, 2000).

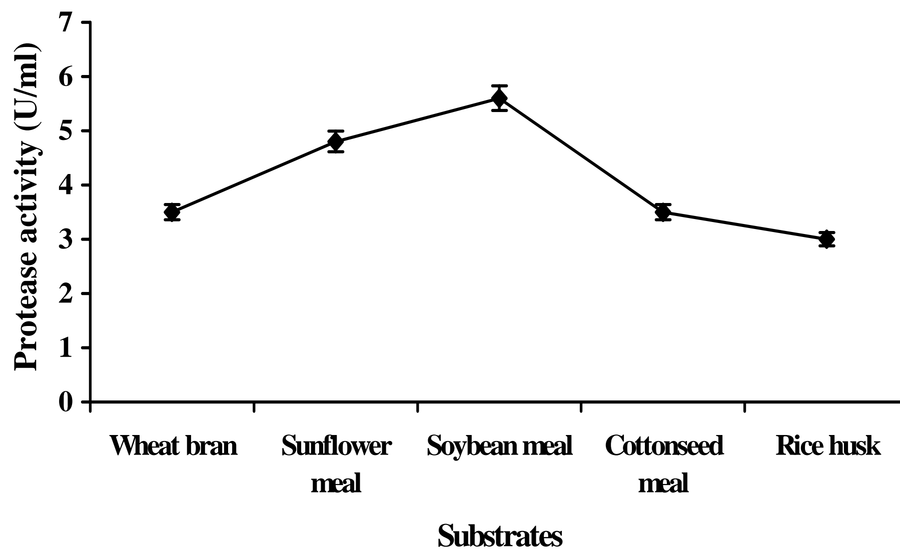

Fig. 1. Selection of substrate for acid proteases production by *P.griseoroseum* IH-02. (Temperature: 30 Cº; Diluent: D_4; Inc. period: 72 h)

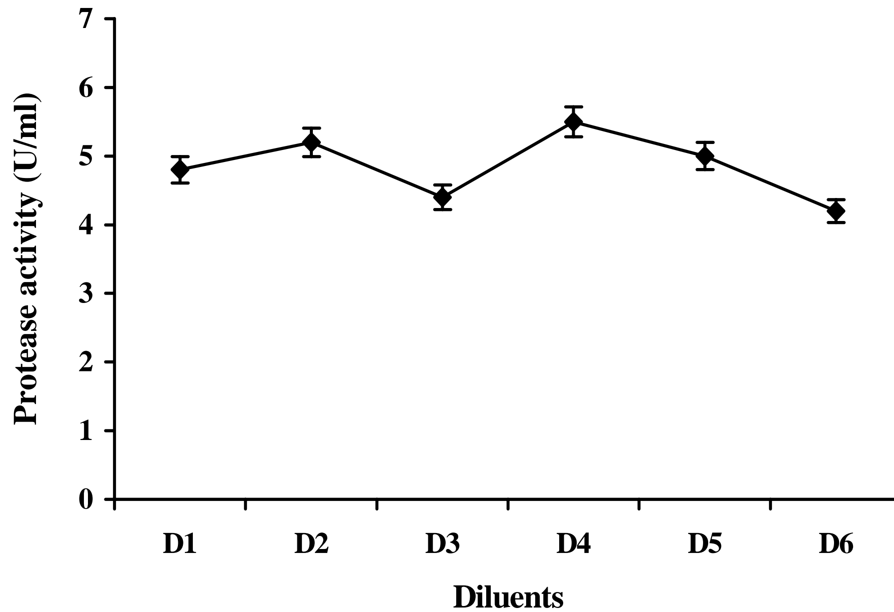

Fig. 2. Effect of different diluents on the production of acid proteases by *P.griseoroseum* IH-02. (Inc. temperature: 30 Cº; Substrate: Soybean meal; Inc. period: 72 h)

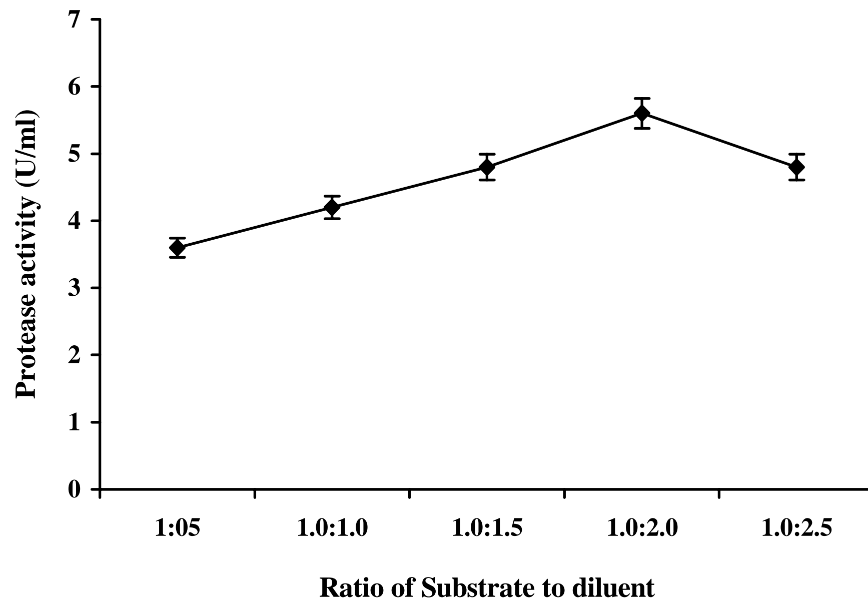

Fig. 3. Effect of different ratios of substrate to diluent on the production of acid proteases by *P.griseoroseum* IH-02.
Inc. temperature: 30 Cº; Substrate: Soybean meal; Diluent: D_4; Inc. period: 72 h

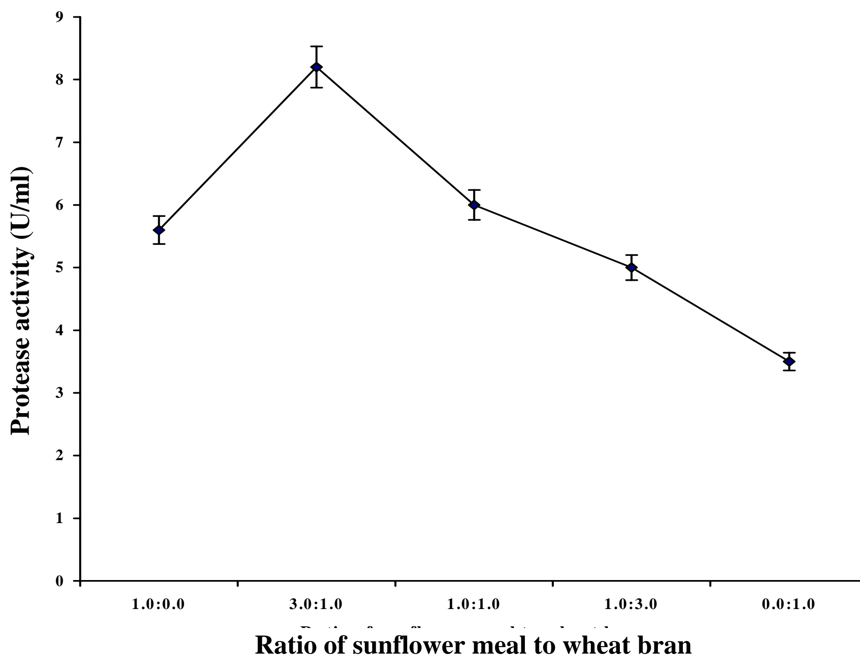

Fig. 4. Effect of partial replacement of soybean meal with wheat bran on the production of acid proteases by *P.griseoroseum* IH-02.
Inc. temperature: 30 Cº; Substrate: Soybean meal; Diluent: D_4; Inc. period: 72 h

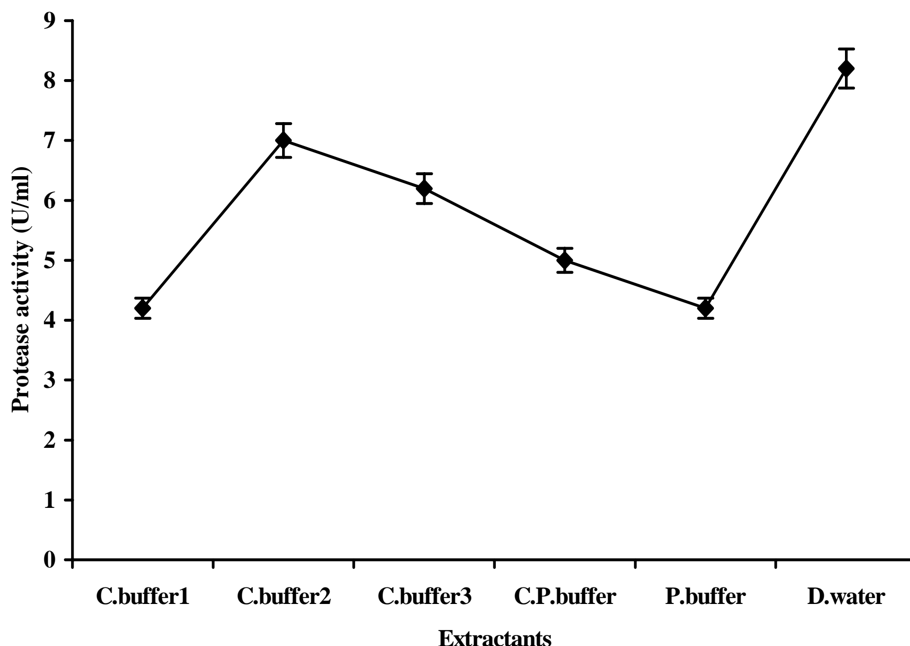

Fig. 5. Effect of different extractants on the production of acid proteases by *P.griseoroseum* IH-02. Inc. temperature:30 C°; Substrate: Soybean meal and wheat bran; Diluent: D_4; Inc. period: 72 h

Abbreviations: C.buffer1 = Citrate buffer, pH 4.0; C.buffer2 = Citrate buffer, pH 5.0; C.buffer3 = Citrate buffer, pH 6.0; C.P. buffer = Citrate phosphate buffer, pH 7.0; P. buffer = Phosphate buffer, pH 8.0; D. water = Distilled water, pH 5.0

Extraction of enzyme: Extraction of enzyme from the fermented medium with different buffers and water was carried out and it was found that maximum extraction (8.2 U/ml) was achieved with distilled water (pH 5.0). The extraction of enzymes with different buffers showed less recovery of the enzyme (Fig. 5) which might be due to inhibitory actions of chemicals present in the buffers. It showed that the acid proteases produced were very sensitive to other chemicals. Some other workers (Ammar *et al*., 1997) have also reported that distilled water gave maximum recovery of proteases than any other extractant.

Conclusion

The protease production was investigated in the present study and maximum enzyme productivity (8.2 U/ml) was obtained when the substrate (soybean meal) was partially replaced with wheat bran at a ratio of 3:1, moistened with diluent D_4 and enzyme was extracted with distilled water. The results are highly significant and are of commercial potential. Further work on the application of proteases in different fields like bating of leather, in detergents and for the recovery of silver from used x-ray films is in progress.

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