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Production of Proteases by a Locally Isolated Mould Culture under Lab Conditions

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Abstract: Thirteen mould cultures, capable of producing proteases by solid state fermentation, were isolated from soil. ***Rhizopus oligosporus*** IHS_{13} strain was found to be the best producer of acidic proteases. The agricultural by-products namely sunflower meal, wheat bran, soybean meal, lupin cake and cotton seed meal were evaluated as inducers for the production of enzyme. The enzyme synthesis was maximum when sunflower meal was used as substrate. The production of proteases by ***Rhizopus oligosporus*** HIS_{13} strain was studied by varying the size of inoculum and type of diluent. The optimum inoculum size and diluent were 10% and distilled water, respectively. The effect of different buffers on the extraction of enzyme was also studied and distilled water was found to be the best extractor. The maximum enzyme production, obtained during the course of study was 4.8 U ml^{-1}.

Key words: Protease, mould culture, pH, ***Rhizopus oligosporus***, fermentation

Introduction

Proteases are produced by many species of fungi such as ***Aspergillus*** (Chakraborty ***et al.***, 1995; Mulimani and Patil, 1999), ***Mucor*** (Thakur ***et al.***, 1990), ***Fusarium*** (Khan ***et al.***, 1981), ***Trichoderma*** (Dunaevsky ***et al.***, 2000), ***Cephalosporium*** (Tsuchiya ***et al.***, 1987) and ***Rhizopus*** (Ikasari and Mitchell, 1994). However, it has been investigated that the proteases produced by ***R. oligosporus*** have high proteolytic activity (Yokotsuka, 1991). Furthermore, it does not produce toxins in the fermentation broth. It has also been reported that ***R. oligosporus*** produced a satisfactory calf rennet substitute on a laboratory scale (Thakur ***et al.***, 1990).

Proteolytic enzymes are the most important industrial enzymes, representing worldwide sales of about 60% of total enzyme market (Woods ***et al.***, 2001). They find commercial applications in a number of industries like leather industry (George ***et al.***, 1995), toothpastes as antiplaque and antitartar (Hernandez and Marria, 1996), cosmetics (Ohta ***et al.***, 1996) and for the recovery of silver from used x-ray films (Ishikawa ***et al.***, 1993). But the fungal proteases are of particular importance in food industry. Solid-substrate fermentation (SSF) has the potential for higher

protease yield (Pandey ***et al.***, 1999; Dunaevsky ***et al.***, 2000). Economically this type of fermentation possesses many advantages, including superior volumetric productivity, use of simpler machinery, use of an inexpensive substrate, simpler down-stream processing, lower energy requirements and low wastewater output (Malathi and Chakraborty, 1991).

The present study was undertaken to produce proteases under lab conditions involving solid-state fermentation of ***Rhizopus oligosporus*** using sunflower meal as a substrate, which is a by-product of oil mills.

Materials and Methods

Present investigations were carried out at Biotechnology Research laboratories, Department of Botany, Government College University, Lahore during the year 2000-2001.

Microorganisms and maintenance

The mould cultures were isolated from soil samples of Lahore area by pour plate method (Clark ***et al.***, 1958). These were maintained on potato-dextrose-agar (PDA) slants (Table 1).

Inoculum development

The slants of 5-7 days old cultures were wetted by adding 10 ml of 0.005% solution of monoxol O.T. (Diacetyl ester of sodium sulpho succinic acid) to the slants. The spores were scratched by sterile wire loop to break clumps and obtain homogeneous spore suspension. One ml of spore suspension was used for inoculation.

Fermentation procedure

The 250 ml conical flasks containing 10 g of substrate moistened with 15 ml of diluent were sterilized, at 121°C (15 lbs/inch^2 pressure) cooled, inoculated and incubated at 30±2°C for 72 h. After incubation, 75 ml of distilled water was added to the flasks, which were shaken on rotary shaker (Gallenkamp, UK) for one hour at 200 rpm. The contents of flasks were then filtered and the filtrate was used for enzyme assay.

Substrates and diluents

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

D_1: (% W/V) Peptone, 1.0; dextrose, 4.0.

D_2: (% W/V) $NaNO_3$, 2.0; KH_2PO_4, 1.0; $MgSO_4.7H_2O$, 0.5; KCl, 0.5; $FeSO_4.7H_2O$ and $ZnSO_4$ (traces).

D_3: (% W/V) Glucose, 7.0; Peptone, 2.0; KH_2PO_4, 0.4; $MgSO_4.7H_2O$, 0.05; $CaCl_2.2H_2O$, 0.05; $ZnSO_4.7H_2O$, 0.01.

D_4: (% W/V) Yeast extract, 1.0; Glucose, 3.3; Peptone, 1.0; $CaCO_3$, 1.0.

D_5: (% W/V) Glucose, 1.0; Peptone, 1.0; Beef extract, 1.5; NaCl, 0.3.

D_6: (% W/V) Glucose, 2.0; Peptone, 1.0; Beef extract, 1.0; NaCl, 0.3; $CaCl_2$, 0.1; Na_2CO_3, 0.7.

D_7: (% W/V) $(NH_4)_2SO_4$, 1.4; Urea, 3.0; KH_2PO_4, 2.0; $MgSO_4.7H_2O$, 3.0; $CaCl_2$, 3.0.

D_8: Distilled water.

Assay of proteases

The method of McDonald and Chen (1965) was used for the assay of proteases. Casein (1%) was used as a substrate, to which 1.0 ml of enzyme sample was added. The mixture was incubated at 30°C for one hour. The reaction was arrested by adding 5 ml of 5% trichloroacetic acid (TCA). The mixture was centrifuged and 1.0 ml of supernatant was mixed with 5 ml of alkaline reagent. One ml of 1N NaOH was added to make the contents 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 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

Thirteen different isolates of mould culture were evaluated for protease production (Table 1). Of all the cultures tested, IHS_{13} gave maximum production of proteases (3.4 U ml^{-1}). The strain was identified as ***Rhizopus oligosporus*** and used in further studies for the production of protease using solid state fermentation. Different substrates such as sunflower meal, soybean meal, lupin cake, cotton seed meal or wheat bran were evaluated for the synthesis of proteases (Fig. 1). However, Mulimani and Patil (1999) used similar agricultural by-products for the production of proteases using ***Aspergillus flavus*** as the organism of choice. Of all the substrates examined, sunflower meal gave maximum enzyme activity (4.4 U ml^{-1}). The enzyme production decreased in the following order, soybean meal (4.0 U ml^{-1}) > lupin seed cake (3.8 U ml^{-1}) > cotton seed meal (3.6 U ml^{-1}) > wheat bran (3.5 U ml^{-1}). Sunflower meal gave maximum yield of proteases because this agricultural by-product has adequate supply of proteins, carbohydrates and minerals needed to the organism. Similar reports have also been made by Qadeer ***et al.*** (1990).

Extraction of enzyme by different buffers and water (control) showed that maximum extraction was achieved with water (4.6 U ml^{-1}), (Fig. 2). The extraction of enzymes with different buffers was less, which might be due to inhibitory reactions of chemicals present in buffers. It showed that the proteases produced were very sensitive to other chemicals. The effect of size of the spore inoculum on the production of proteases by ***Rhizopus oligosporus***

Table 1: Screening of mould cultures for enzyme production

Strains	Enzyme activity (U ml^{-1})
IHS_1 ***Mucor*** sp.	1.00
IHS_2 ***Aspergillus niger***	1.46
IHS_3 ***Aspergillus oryzae***	3.00
IHS_4 ***Aspergillus oryzae***	1.50
IHS_5 ***Mucor*** sp.	2.50
IHS_6 ***Rhizopus*** sp.	2.00
IHS_7 ***Aspergillus niger***	1.60
IHS_8 ***Aspergillus niger***	1.00
IHS_9 ***Rhizopus*** sp.	2.40
IHS_{10} ***Penecillium*** sp.	1.80
IHS_{11} ***Penecillium*** sp.	2.40
IHS_{12} ***Mucor*** sp.	2.00
IHS_{13} ***Rhizopus oligosporus***	3.40

Incubation period = 72 h Temperature= 30°C Substrate = Wheat bran

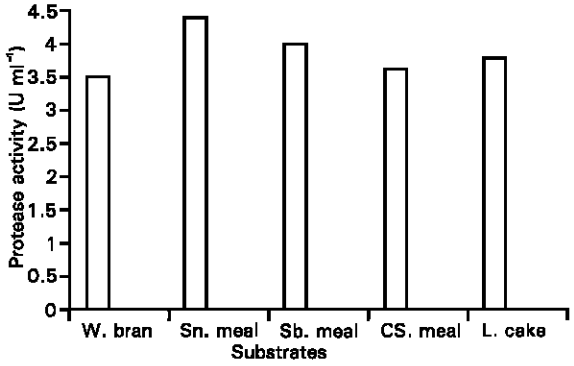

Fig. 1: Selection of substrate for protease production by ***Rhizopus oligosporus*** IHS_{13}.
W. bran = Wheat bran Sn. Meal= Sunflower meal Sb. Meal = Soybean meal
CS. Meal = Cotton seed meal L. Cake = Lupin cake

IHS_{13} showed that the size ranged from 0.5-2.0 ml. Maximum amount of enzyme (4.8 U ml^{-1}) was produced when 1.0 ml inoculum was added to the flask. Further increase in inoculum volume resulted in the decrease of protease production (Fig. 3). Our results are encouraging comparing with many those of previous workers (Kalisz, 1988; Lonsane and Ghildyal, 1992).

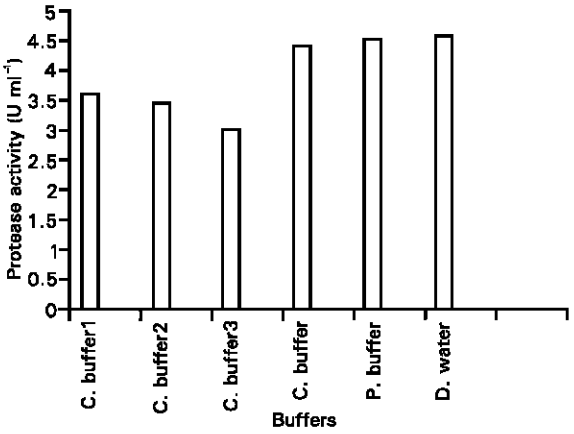

Fig. 2: Effect of different buffers on the extraction of protease produced by ***Rhizopus oligosporus*** IHS_{13}

C. buffer 1 = Citrate buffer, pH 4.0 C. buffer 2 = Citrate buffer, pH 5.0
C. buffer 3 = Citrate buffer, pH 6.0 Cp. buffer = Citrate phosphate buffer
P. buffer = Phosphate buffer D. water = Distilled water

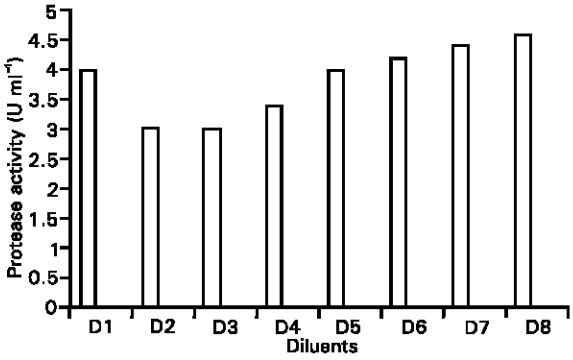

Fig. 3: Effect of size of inoculum on protease production by ***Rhizopus oligosporus*** IHS_{13}

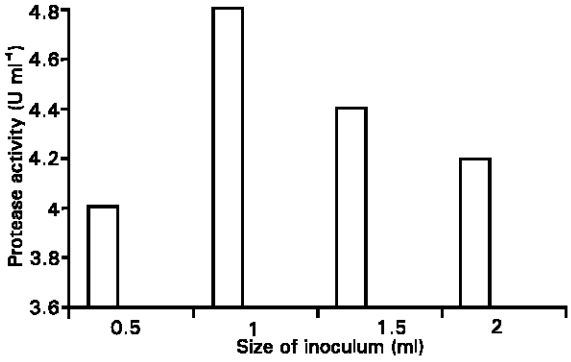

Fig. 4: Effect of different diluents on protease production by ***Rhizopus oligosporus*** IHS_{13}

The production of enzyme was maximum (4.6 U ml^{-1}) when substrate was moistened with distilled water (D_8). The synthesis of enzyme however decreased in the order of $D_7 > D_6 > D_5 > D_1 > D_4 > D_3$ and D_2, respectively. The ratio of substrate to diluent was kept at 1:1.5. The enzyme synthesis was maximum when distilled water was used as diluent which indicate that the organism did not require additional nutrients. All the nutrients were supplied by the substrate for the growth of the organism and production of the enzyme. It also seems that the nutrients present in other diluents may have an inhibitory action on the growth of the organism and subsequently the enzyme production.

The protease production was investigated in the present study and maximum enzyme productivity (4.8 U ml^{-1}) was obtained when the substrate (soybean meal) was moistened with distilled water, enzyme was extracted with water and inoculum size was kept as 10%. The results are highly significant and are of commercial level. 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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