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PRODUCTION OF PROTEASES BY *ASPERGILLUS NIGER*, THROUGH SOLID STATE FERMENTATION

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Abstract: A protease producing fungal strain was isolated from a sludge soil collected from Lahore. It was cultured on Potato dextrose agar (PDA) plates at 37 °C mended with 1 % casein. The strain, identified as *Aspergillus niger*, produced maximum zone of clearance and was selected for further studies. Different parameters such as different carbon sources, different ratios of carbon and nitrogen sources; incubation period, inoculum size, % moisture temperature and pH, were investigated in solid state fermentations. Maximum enzyme production was obtained at 8:2 of carbon and nitrogen (wheat bran: soybean) sources after 48 hours incubation with 5 ml inoculum size at 30 °C and 3.0 pH. After optimizing these parameters, ultraviolet mutagenesis of this strain was attempted at 254 nm. Eight mutant strains were isolated for the maximum protease production. These strains were observed under optimized conditions for the maximum production of proteases. The strain 90S1 was found as best protease producer. Partial characterization of proteases and their stability in the absence and presence of $CaCl_2$ were determined.

Keywords: Fungal protease, UV mutants and, Enzyme, effect of carbon and nitrogen source, Fermentation.

INTRODUCTION

Proteases occur naturally in all organisms and constitute 1-5% of the gene content. These enzymes are involved in a multitude of physiological reactions from simple digestion of food proteins to highly regulated cascades. They break peptide bonds in proteins. The process is called proteolytic cleavage (Barrett *et al*., 2003). Proteases are the most important industrial enzymes, representing world wide scale of

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about 60 % of total enzyme market. They all catalyze the hydrolysis of proteins but there are many differences in the modes of catalysis. Firstly, proteases are divided into two broad categories on the basis of type of attack on the protein: *i.e.* exo-proteases and endo-proteases. The two groups are further sub-divided on basis of the mechanism of action at the active site (Woods *et al*., 2001).

A large number of industrial processes in the areas of health, environment and food biotechnologies utilize enzymes at some or the other stage. The enzymes are largely produced by employing microorganisms which are exploited to ferment different substrates by different types of fermentation procedures. In this regard solid state fermentation holds tremendous potential for the production of enzymes. It can be of special interest in those processes where the crude fermented product may be used directly as enzyme source. It has been widely used for the production of proteases. Solid state fermentation (SSF) processes can be defined as the growth of microorganisms on moist solid materials in the absence of free-flowing water. SSF processes have been used for the production of food, animal feed and both pharmaceutical and agricultural products. Agro-industrial residues are generally considered best substrates for the SSF processes, and use of this technique for the production of enzymes from them is no exception to that. Wheat bran, among all agro-industrial residues, holds the has most commonly been used in development of various processes (Pandey *et al.,* 1999). The solid substrate not only supplies the nutrients to the growing microbial culture but also provides anchorage for the cells.

Ability of microorganisms for growing on a solid substrate is a function of their requirements of water activity, their capacity of adherence and penetration into the substrate and their ability to assimilate mixtures of different polysaccharides due to often complex nature, of the substrates used. Filamentous fungi are the best adapted microorganisms for SSF owing to their physiological, enzymological and biochemical properties. In addition, their ability to grow at low water activity and high osmotic pressure conditions makes fungi efficient and competitive in natural microflora for bioconversion of solid substrates (Guerra *et al.,* 2003).

Commercially, proteases are produced from fungi in highly controlled aseptic conditions for food supplementation and systemic enzyme therapy. The organisms most often used are *Aspergillus (A) niger*

and *A. oryzae*. The fungal proteases are acid and some neutral in nature (Shumi *et al*., 2004). History of safe use of *A. niger* comes primarily from its use in the food industry for the production of proteases (Bennett, 1985; Ward, 1989). In view of the accompanying peptidase activity and their specific function in hydrolyzing hydrophobic amino acid bonds, fungal neutral proteases supplement the action of plant, animal, and bacterial proteases in reducing the bitterness of food protein hydrolysates. Fungal alkaline proteases are also used in food protein modification (Rao *et al*., 1998). Besides their industrial and medicinal applications, proteases play an important role in basic research. An interesting application of alkaline is its use to decompose the gelatinous coating of X-ray films, from which silver is recovered (Rao *et al*., 1998). Mutation has improved the productivity of industrial cultures. Ultraviolet irradiation method has been very effective in obtaining mutants from *Aspergillus spp.* wild type strains (Chaouche *et al*., 2005). Above description suffice to indicate the importance of proteases. At present, in Pakistan these enzymes are imported from other countries. Therefore, the present study was undertaken to probe for their production by employing locally isolated protease producer *A. niger* while cultivating on inexpensive fermentation substrates.

MATERIALS AND METHODS

Four different types of samples were taken and used to isolate *A. niger* strain *i.e.,* a piece of cat feces, unprocessed skin of cattle from leather store, processed salty skin of cattle from a leather factory and sludge soil from Lahore. Pure culture of *A. niger* was isolated by inoculating the sludge sample on PDA with 1% casein and 2 ml of 5% ampicillin in Petri plates. Zone of clearance around the microbial growth represented the presence of protease enzymes produced by the isolated strain. To select carbon source, rice husk, defatted rice bran, wasted wheat and wheat/fatted bran were added to equal amount of soybean meal and compared to 10 g soybean meal (control). A given solid material was soaked in 4 ml of distilled water and pH adjusted at 5. Following autoclaving, 2 ml inoculums was added and incubated at 37 ºC for 5 days. After determining optimum C:N source/ratio different growth parameters were then optimized, including carbon source, incubation period, inoculum size, moisture, temperature and pH. Protease enzyme activity of culture mass

extract (filtrate) was determined by a modified Anson's method (Yang and Wang, 1999) using tyrosine as standard. One unit of protease activity was defined as the amount of enzyme that produced 1 microgram (µg) of tyrosine per min under the standard assay conditions. Specific activity was calculated as ratio of total protease activity and total protein estimation. Total Protein was estimated by Lowry *et al.* (1951).

The wild type strain was exposed to UV radiations at 254nm for 30, 60 and 90mins. The mutant strain that appeared on 90mins exposed Petri plate yielded highest protease production. The protease enzymes extracted from the wild type and mutant strains were partially characterized and assayed for their stabilities at different pH and temperatures in the absence and presence of $CaCl_2$.

RESULTS AND DISCUSSION

The strain of *A. niger*, used in the present research work was isolated from sludge soil sample and incubated at 25^{o}C for 6 days. It was identified morphologically under microscope and then studied for protease production under physico-chemical conditions. An attempt was also made to ascertain the optimum conditions for carbon source, C:N ratio, incubation period, inoculum size, moisture, temperature and pH for maximum protease production.

As can be seen from Table I, wheat bran and soybean meal (1:1) yielded highest units of protease *i.e.,* up to 22 U/g/min after 5 days of SSF. The value was even higher than that obtained when only soybean meal was employed as fermentation medium. This clearly dictates the importance of a proper carbon source along with nitrogen source for higher yield of a fermentation product. A ratio of 8:2 of the nitrogen and carbon sources yielded the highest enzymes' units after 5 days of SSF (Table II). However, when effects of different incubation periods were studied on the optimized ratio of carbon and nitrogen sources, it appeared that maximum 60.62u/g/min) enzyme production was obtained at 48 hours of incubation (Table III).

Table I: Effect of different carbon sources mixed in equal ratios with the nitrogen source (Soybean meal) on protease production by the wild strain *Aspergillus niger*.

Sr. No.	Carbon Source	Protease Content (u/g/min)
1	Soybean meal (SM)	14.55^{a} ±0.07 (3.48)
2	Wheat bran + SM (1:1)	21.82^{b} ±0.40 (3.54)
3	Wasted wheat +SM (1:1)	17.11^{c}2±0.00 (5.08)
4	Defatted Rice bran +SM (1:1)	19.59^{d} ±0.12 (5.09)
5	Fatted Rice bran +Sm (1:1)	13.31^{e} ±0.18 (3.00)
6	Rice Husk + SM (1:1)	17.36^{fg}±0.00 (4.81)

*Value are expressed as mean±SEM and represent U/g/min, while those in parentheses express specific enzymatic activity (U/mg) of the protein. Value sharing at least a common alphabet are not significantly different from each other. P<0.05.

Table II: Effect of different wheat bran: Soybean meal (C:N) ratios on protease activity by the wild strain of *Aspergillus niger*.

Sr. No.	C:N Ratio	Protease activity (u/g/min)
1	10:0	26.31a ± 0.11 (2.32)
2	9:1	29.35b ± 0.98 (2.61)
3	8:2	32.10c ± 0.11 (2.39)
4	7:3	23.70d ± 0.23 (1.85)
5	6:4	21.08el ± 0.58 (1.65)
6	5:5	21.09fl ± 0.98 (1.68)
7	4:6	18.19gm ± 0.19 (1.64)
8	3:7	18.46hm ± 0.88 (1.70)
9	2:8	16.12i (1.51)
10	1:9	13.64jn ± 0.39 (1.27)
11	0:10	12.40kn ± 0.33 (1.21)

For details of statistical analysis and values in parenthesis, see Table I.

Table III: Effect of incubation period, inoculum, size, moisture contents, pH and temperature on protease production by the wild strain of *Aspergillus niger*.

Incu-bation Period (hrs.)	Protease (u/g/min)	Inoculum Size (ml)	Protease (u/g/min)	Added water (ml)	Protease (u/g/min)	pH	Protease (u/g/min)	Temper ature °C	Protease (u/g/min)
24 hours	39.40a ± 0.79 (3.47)	1	24.66a ±0.11 (1.96)	0 ml	48.23a 1.09 (3.94)	3.0	73.02a ±1.13 (2.28)	10 °C	17.63 ±0.49 (1.60)
48 hours	60.62b ±1.10 (4.23)	2	25.49b ±0.09 (2.07)	1 ml	59.49b 1.13 (4.99)	4.0	48.22b ±1.13 (2.07)	20 °C	19.01 ±0.20 (1.68)
72 hours	38.60d ±0.57 (3.66)	3	26.04c 0.19 (2.58)	2 ml	49.60a 0.00 (4.55)	5.0	46.85c ±1.13 (1.91)	25 °C	21.91 ±0.58 (2.19)
96 hours	36.65c 0.23 (5.49)	4	30.72dg ±0.23 (2.91)	3 ml	49.60a 0.00 (4.60)	6.0	40.78dg 0.23 (1.72)	30°C	32.93c ±0.11 (2.55)
120 hours	24.66df 0.11 (3.96)	5	36.10e ±0.23 (3.25	4 ml	49.60a 0.00 (4.91)	7.0	40.23eg 0.12 (1.90)	35 °C	22.46d ±0.57 (2.10)
144 hours	24.66ef 0.11 (4.04)	6	30.72fg 0.23 (2.76)	5 ml	49.60a 0.00 (5.46)	8.0	34.72f 0.24 (2.15)	40 °C	20.53 ±0.30 (1.94)

For details of statistical analysis and values in parenthesis, see Table I.

Table IV: Protease production (u/g/min) by wild type (WT) and the mutant strain 90S1 at different pH and temperatures.

pH	WT	90S1	Temperature	WT	90S1
3.0	9.65a ±0.55	17.64 ±0.23	25 °C	13.22a ±0.34	25.08a ±0.23
4.0	10.34a ±0.29	18.74 ±0.45	30 °C	33.07b ±0.00	44.09b ±1.13
5.0	10.47a ±0.23	24.52 ±0.23	35 °C	34.45c ±1.13	44.09b ±1.13
6.0	12.68b ±0.56	34.86 ±0.23	40 °C	36.51d ±0.57	73.02c ±1.13
7.0	39.51c ±0.57	73.02 ±1.13	50 °C	38.58e ±0.57	44.09be ±1.13
8.0	15.18d ±0.56	35.82 ±0.57	60 °C	43.82f ±1.35	42.71f ±1.13

For details of statistical analysis and values in parenthesis, see Table I.

Table V: Stabilities of proteases (u/g/min) produced by wild type *A. niger* and its mutant strain (90S1) following 60(A) and 90 (B) minutes exposures at different pH in the absence and presence of $CaCl_2$.

	Absence of $CaCl_2$		In the presence of $CaCl_2$	
pH	**A**	**B**	**A**	**B**
3.0	11.71a ± 0.11 (14.19 ± 0.11)a	8.82a ± 0.11 (13.46 ± 1.12)a	39.96a ± 0.56 (57.29 ± 1.12)a	36.74a ± 1.49 (37.54 ± 0.59)a
4.0	12.67bg ± 0.11 (15.15 + 0.11)a	9.83b ± 0.08 (15.62 ± 0.37)b	39.96a ± 0.56 (56.86 ± 0.99)a	36.74a ± 1.49 (45.93 ± 1.49) b
5.0	15.57c ± 0.06 (22.73 ± 0.97)b	11.71c ± 0.00 (20.67 ± 0.00)c	67.51b ± 2.25 (88.18 ± 2.25)b	53.34b ± 0.75 (60.62 ± 0.00)d
6.0	17.57d ± 0.00 (27.56 ± 0.56)c	13.18df ± 0.04 (23.42 ± 0.00)c	99.20cf ± 0.00 (114.35 ± 1.12)c	71.64ce ± 0.00 (76.27 ± 0.75)d
7.0	18.19c ± 0.00 (39.27 ± 0.00)d	13.50ef ± 0.00 (33.98 ± 0.37)e	99.20df ± 0.00 (165.33 ± 0.00)	71.64ce ± 0.00 (110.22 ± 0.00)e
8.0	12.13fg ± 0.11 (28.25 ± 1.13)e	8.91a ± 0.04 (25.26 ± 0.75)f	82.67e ± 0.00 (70.27 ± 1.95)f	49.60d ± 0.00 (62.46 ± 1.49)fg

For details of statistical analysis and values in parenthesis, see Table I.

Table VI: Stabilities of proteases (U/g/min) produced by wild type *A. niger* and its mutant strain (90S1) after 60 and 90 minutes exposures at different temperatures in the absence and presence of $CaCl_2$.

Temperature ^oC	Absence of $CaCl_2$		Presence of $CaCl_2$	
	60 mins.	**90 mins.**	**60 mins.**	**90 mins.**
25	22.32 ± 0.67 (27.83 ± 0.11)	16.99 ± 0.07 (22.78 ± 0.30)	90.93a ± 0.00 (99.20 ± 0.00)a	66.13±0.00 (66.13±0.00)a
30	25.07 ± 0.11 (31.83 ± 0.67)	21.77 ± 0.22 (30.31 ± 0.00)	90.93a ± 0.00 (107.47 ± .00)a	66.13±0.00 (66.13±0.00)a
35	29.21 ± 0.22 (52.08 ± 0.67)	±27.56 (37.20 ± 0.23)	90.93a±0.00 (107.47±0.00)a	66.13±0.00 (66.13±0.00)a
40	51.25 ± 1.35 (74.40 ± 0.00)	42.25 ± 0.75 (47.76 ± 1.50)	90.93a±0.00 (165.33±0.00)b	66.13±0.00 (110.22±0.00)b
50	52.08 ± 0.67 (74.40 ± 0.00)	43.17 ± 0.75 (6. ± 1.50)	90.93a±0.00 (115.73±0.00)c	66.13±0.00 (77.16±0.00)c
60	66.13 ± 0.00 (74.40 ± 0.00)	49.60 ± 0.00 (47.76 ± 1.50)	107.47b±0.00 (115.73±0.00)c	66.13±0.00 (77.16±0.00)c

For details of statistical analysis and values in parenthesis, see Table I.

The *A. niger* isolate and its mutant strains appeared capable of producing protease enzyme in solid state fermentation. Similar results have been reported by Sahin and Tamer (1999). In present study, cultivation was carried out under solid state fermentation conditions to optimize different parameters. Among 5 carbon sources, wheat bran along with soybean (2:8) proved most satisfactory one for the enzyme yield. Patli and Shastri (1981) demonstrated that the production of proteases was maximum in Czapet Dox medium in which glucose was replaced by 5 % wheat bran. Ashour *et al.* (1996) have also reported comparable results. An incubation period of 48 hours and pH 3.00 were found optimum for the protease yield (Table III). Ikasari and Mitchell (1993) have also reported the same incubation period as optimum. The maximum enzyme synthesis at 30^{o}C and at pH 3.0 have been reported by Hikotaka Hashimoto *et al.* (1973) and Haq *et al.* (2004).

The increased production of extracellular protease indicated that the selected mutant strain has important potentialities for large scale enzyme production. Chaouche *et al.* (2005) have concluded similar results. The optimum protease activity for wild type strain appeared at pH 7.0 and 60 ^{o}C, while for mutant strains the corresponding conditions optimum were pH 7.0 and 40^{o}C (Table IV). The stability of proteases was also determined by using various buffers of different pH values and at different temperatures. Crude enzymes extracted by both wild type and mutant strain were stable at wide ranges of pH and temperature. The protease activity improved in the presence of $CaCl_2$ (Table V, VI).

Ca^{+2} apparently protect the enzyme against thermal denaturation and play a vital role in maintaining the active conformation of the enzyme at high pH values and at high temperatures. In this regard, Germano *et al.* (2002) reported characterization and stability of proteases from *Penicillium sp.* produced by solid state fermentation. Stability conditions revealed relatively vide ranges of temperatures and pH during this study (Tables V, VI). Adinarayana *et al.* (2003) have illustrated the purification and partial characterization of thermostable serine alkaline protease from *Bacillus subtilis* PE-11. These properties of crude enzyme extracts from wild type and mutant strains indicate the possibilities of economical provision and uses of the proteases in the food and pharmaceutical industries.

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