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Optimization of Growth Conditions for Acidic Protease Production from *Rhizopus oligosporus* through Solid State Fermentation of Sunflower Meal

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Abstract**—***Rhizopus oligosporus* was used in the present study for the production of protease enzyme under SSF. Sunflower meal was used as by-product of oil industry incorporated with organic salts was employed for the production of protease enzyme. The main purpose of the present was to study different parameters of protease productivity, its yields and to optimize basal fermentation conditions. The optimal conditions found for protease production using sunflower meal as a substrate in the present study were inoculum size (1%), substrate (Sunflower meal), substrate concentration (20 g), pH (3), cultivation period (72 h), incubation temperature (35^{o}C), substrate to diluent's ratio (1:2) and tween 81 (1 mL). The maximum production of protease in the presence of cheaper substrate at low concentration and stability at acidic pH, these characteristics make the strain and its enzymes useful in different industry.

Keywords**—**Acidic protease, *Rhizopus oligosporus*, Media optimization, Solid state Fermentation

I. INTRODUCTION

PROTEASES occur naturally in all organisms and constitute 1-5% of the gene content and are essential constituents of all the forms of life on earth including prokaryotes, fungi, plants and animals [1]. Proteases are one of the most important groups of industrial enzymes used in pharmaceutical, food industry, leather industry and as an additive of detergent formulation in detergent industry [2].

A wide range of micro-organisms including *Rhizopus oligosporous* 1HS13, *Aspergillus niger*, *Rhizopus oryzae*, *Saccromyce cerevisiae* and *Conidiobolus spp* [3-6] have ability to produce proteases. Their biomass can be easily determined after simple drying in oven as well as in dissector and weighing by digital balance [7]. Fungal proteases are of particular importance in the food industry *Aspergillus* and *Mucor* have been studied intensively as protease producers although *Rhizopus oligosporus* also produces proteases, has a high proteolytic activity in the tempe fermentation and furthermore, does not produce toxins [8]. This paper reports the results of a study carried out to investigate the hyper-production potential of protease enzyme from *Rhizopus oligosporus* and optimization of cultural conditions for maximum production of protease.

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II. MATERIALS AND METHODS

Micro-organism and culture condition

The strain *Rhizopus oligosporus* used for the production of protease was obtained from Microbiology Laboratory, PCSIR laboratories, complex Lahore and grown on patoato dextrose agar slants at 30^{o}C for 3-5 days and stored at 4^{o}C. The preserved culture was revived on fresh agar slants after every week for whole experiment.

Inoculum prepration

The inoculum was prepared by transferring loopful culture of *Rhizopus oligosporus* into 100 mL of inoculum medium containing contain 20g Sunflower meal, 2.0g NH_4SO_4, 0.3g K_2HPO_4, 0.5g NaCl, 0.5g $MgSO_4$, 0.2g Na_2HPO_4 and 0.1g $CaCl_2$ and sterilized. The inoculated medium was incubated in a water bath shaker (Eyela- Japan) at 140 rpm to obtain homogeneous spore suspension up to 10^{6-8} cells/mL.

Fermentation technique

20 g of sunflower meal was taken in 250 mL conical flask as solid inert support providing substrate and moistened with diluents consisting of 2.0g NH_4SO_4, 0.3g K_2HPO_4, 0.5g NaCl, 0.5g $MgSO_4$, 0.2g Na_2HPO_4 and 0.1g $CaCl_2$. It was inoculated with freshly prepared spore suspension of *Rhizopus oligosporus*. The inoculated medium was incubated at 30±1^{o}C for 72 hours.

Extraction of protease

To the fermented substrate 100 mL distilled water was added and kept in a rotary shaker at 180 rpm for half an hour to homogenize the fermented flasks. Then the fermented broth was centrifuged at 9000×g for 10 min at 4^{o}C to get clear supernatant containing enzyme solution and the resultant clear supernatant was used for analytical studies.

Optimization of Production Parameters

Effect of substrate concentration

Various conc. of substrate ranging from 5 to 25 % (w/v) were evaluated for optimum production of acid protease by *Rhizopus oligosprous.*

Effect of cultivation time periods

Different cultivation period ranging from 24 (hrs) to 120 (hrs) was evaluated for optimum production of acidic protease

by*R.oligosporus.*

Effect of initial medium pH

Effect of initial pH on production of protease was studied by changing the initial growth medium pH from 3-7 with 1N HCl/NaOH before sterilization at 121^{o}C for 15 min.

Effect of Incubation temperature

Effect of incubation temperature on production of protease was studied by changing the temperature from 25^{o}C to 45^{o}C.

Effect of Inoculums size

Various sizes of inoculum ranging from 1-5 mL (v/v) were evaluated for optimum production of acid protease by *R. oligosporus*

Effect of Tween 81

Tween 81 effect on production was studied by changing the conc. from 0.5mL to 5mL (v/v) was evaluated for optimum production of acid protease *by R .oligosporus.*

Analytical Methods

Protease enzyme assay

The enzyme activity was determined by using the method of McDonald and Chen [9]. According to this method three sets of test tubes were made one was control and the other two were experimental. In all these tubes 2mL of 1% casein in Glycine-NaOH buffer pH 10, was added in all three test tubes. In control 2mL of 1% casein solution, 1mL of enzyme and 3mL of 10% TCA was added. The rest of the two tubes contained 2mL of 1% casein solution and 1mL of enzyme .All these three test tubes were incubated at 60^{o}C for 15 minutes. After the incubation, 3mL of 10% TCA was added in the experimental and then centrifuged for three minutes. Then took 1mL of supernatant in test tube and add 5 mL of alkaline copper reagent. After 15 minutes 0.5mL Folin-ciocalteau reagent (diluted in 1:1 ratio i.e. 1 mL (Folin-ciocalteau reagent: 1mL distal water) was added in each test tubes and stands for 30 minutes. After the completion of the time the absorbance was read out at 700nm spectrophotometrically. One unit enzyme activity was defined as the amount of enzyme that releases 1µg of tyrosine per mL per min under the above assay conditions.

Statistical analysis

All the data was statistically evaluated according to Steel *et al*. [10]. The means and standard errors of means (Mean ± S.E) were calculated for each treatment.

III. RESULTS AND DISCUSSION

Effect of substrate concentration

The effect of substrate (sunflower meal) concentration was checked for the production of protease. A set of flasks (250 mL) with different substrate concentration such as 5, 10, 15, 20 and 25g was inoculated with 1 mL of inoculum and incubated at 30^{o}C for 72h. The maximum enzyme production (195.14 PU/g) was found at 20g concentration of sunflower meal as mentioned in Table I. Maximum enzyme production is found at 20g (195.14 PU/g). As the substrate concentration increases from 20g the enzyme activity is tended to decrease. Similar results were reported by Haq *et al*. [11] by using different substrates such as sunflower meal, soybean meal, cotton seed meal and wheat bran. Iksari and Mitchell [12] reported slightly different results by using the micro-organism *Rhizopus oligosporus* on the substrate rice husk (3.9 PU/g). Different results may be due to the effect of culture conditions of micro-organism, use of other substrates and in different concentrations other environmental factors also effects on the protease production. Sumantha *et al*. [13] reported similar results by using the micro-organism *Rhizopus oligosporus* with the substrate casein at 20g (3.6 PU/g).

TABLE I
EFFECT OF SUBSTRATE CONCENTRATION ON ACIDIC PROTEASE PRODUCTION FROM *RHIZOPUS OLIGOSPORUS*

Treatments	**Substrate conc. (g)**	**Enzyme activity (PU/g)**
1	5	183.83±1.25
2	10	165.64±2.21
3	15	184.64±1.65
4	20	195.14±2.36
5	25	162.01±3.11

Effect of Cultivation time periods

Table II showed the effect of cultivation period on the production of protease. A set of flasks with carbon substrates i.e. sunflower meal was inoculated with 1 mL inoculum and incubated at 30^{o}C±1^{o}C for different intervals of time such as 24h, 48h, 72h, 96h and 120 h to check that at which time period protease activity was maximum.

The results showed that the enzyme production was maximal at 72h (215.75 PU/g). A gradual decrease in enzyme units was observed with increase incubation period clearly suggesting the enzyme role as a primary metabolite, being produced in the lag phase of the growth of fungus for utilization of nutrients (proteins) present in the solid substrate.

The subsequent decrease in the enzyme units could probably be due to inactivation of the enzyme by other constituent proteases. Our results are comparable to Sumantha *et al* [13] who reported maximum enzyme activity after 3 days with *Rhizopus oligosporus* under solid state fermentation. Similar results were also reported by Ikasari and Mitchell, [12] and Haq *et al*. [11] using *Aspergillus sp.* and *P. chrysogenum* IHH5 after 72 h of incubation respectively.

Effect of Inoculum size

Inoculum size was studied by inoculating sets of flasks containing substrate (20g) with different volumes of inoculums (0.5, 1, 2, 3, 4 and 5 mL) for the production of protease enzymes.

TABLE II
EFFECT OF CULTIVATION TIME PERIODS ON PROTEASE PRODUCTION FROM *RHIZOPUS OLIGOSPORUS*

Treatments	Cultivation time periods (h)	Enzyme activity (PU/g)
1	24	173.32±1.11
2	48	190.29±1.56
3	72	215.75±2.33
4	96	202.82±3.24
5	120	197.33±1.99

The maximum enzyme production (159.99.75 PU/g) was observed with 1 mL inoculum size (Table III). The effect of inoculum size on the production of protease by *Rhizopus oligosporus* showed that the size ranged from 0.5 to 2.0 mL. Maximum amount of enzyme (4.8 U mL) was produced when 1.0 mL was added to the flask [14]. Further increase in inoculum volume resulted in the decrease of protease production. Because much increase in inoculum volume caused overcrowding of spore that decreased the enzyme activity [15]. Size of inoculum is an important biological factor, which determines biomass production in fermentation [16, 17]. Hence, a balance between the proliferating biomass andavailable materials will yield maximum enzyme production.

TABLE III
EFFECT OF INOCULUM SIZE ON PROTEASE PRODUCTION FROM *RHIZOPUS OLIGOSPORUS*

Treatments	Inoculum size (mL)	Enzyme activity (PU/g)
1	0.5	135.12±1.15
2	1	159.99±1.25
3	2	128.37±2.11
4	3	108.35±1.06
5	4	98.28±1.12
6	5	88.02±0.99

Effect of initial medium pH

To obtain the optimum pH different experiments was undertaken to find the suitable pH for maximum production of an acidic protease. For this purposes different pH i.e. 3, 4, 5, 6 and 7 were kept of the cultural media for fermentation process. The results showed that pH 3 gave the highest protease production (149.08 PU/g) (Table IV). Microbial strains depend on the extra-cellular pH because culture pH strongly influences many enzymatic processes and transport of various components across the cell membranes, which in turn support the cell growth and product formation [18]. Djamel *et al.* [19] reported protease synthesis by the mutant strain S08M10 under optimum pH of 3 and proteolytic activity of 1400 U/mL in the case where the substrate was the skimmed milk.

TABLE IV
EFFECT OF INITIAL PH ON PROTEASE PRODUCTION FROM *RHIZOPUS OLIGOSPORUS*

Treatments	Initial pH	Enzyme activity (PU/g)
1	3	149.08±1.36
2	4	143.83±1.55
3	5	119.99±2.22
4	6	129.28±2.31
5	7	136.96±1.33

Effect of incubation temperature

Table V showed the effect of incubation temperature on the production of protease. A set of flasks with carbon substrates i.e. sunflower meal was inoculated with 1 mL inoculum and incubated at different temperatures such as 25 ^{o}C, 30^{o}C, 35 ^{o}C, 40 ^{o}C and 45 ^{o}C to check that at which temperature maximum enzyme production achieved. The results showed that maximum enzyme production was achieved at a temperature of 30^{o}C (103.68 PU/g). Higher temperature is found to have some adverse effects on metabolic activities of micro-organism and cause inhibition of the fungus growth. The enzymes become denatured by losing its catalytic properties at high temperature [11]. However, there was sudden decrease in protease production when the incubation temperature was increased from 35°C to 45°C [14]. Sumantha *et al.* [13] reported similar results by using the micro-organism *R. microsporus* NRRL 3671, being a thermophilic culture was found to be highly sensitive to temperature changes below and above its optimum for both enzyme production and growth. Paranthaman *et al.* [18] reported slightly different result this may be due to the change of micro-organisms, substrate variation and some other environmental factors.

Effect of Tween-81

The effect of tween 81 on acid protease was studied and maximum production was observed at I mL of inoculum as shown in table VI. The study reveals that the enzyme secretion is greatly influenced by the change in the concentration of tween 81.

TABLE V
EFFECT OF INCUBATION TEMPERATURE ON PROTEASE PRODUCTION FROM *RHIZOPUS OLIGOSPORUS*

Treatments	Incubation temperature (^oC)	Enzyme activity (PU/g)
1	25	93.56±1.10
2	30	103.68±1.08
3	35	92.52±1.01
4	40	88.30±0.99
5	45	73.56±1.10

Higher concentration of tween 81 effects the enzyme production because the substrates also have adequate supply of nutrients. Our results are highly correlates with Haq *et al* [11], Sumantha *et al.* [13]. Similar results were reported by Haq *et al.* [14] by using the micro-organism *Penicillium griseoroseum* with solid state fermentation at 4 mL (220.45 PU/g).

TABLE VI
EFFECT OF TWEEN-81 ON PROTEASE PRODUCTION FROM *RHIZOPUS OLIGOSPORUS*

Treatments	Tween-81 (mL)	Enzyme activity (PU/g)
1	0.5	110.52±1.12
2	1	211.56±2.26
3	2	105.39±3.65
4	3	153.55±5.33
5	4	216.45±2.33
6	5	118.82±1.33

IV. CONCLUSION

The acidic protease from *R. oligosporus* exhibit maximum activity at pH 3 and was found stable over pH range of 5 and 6. In conclusion attempt was made towards finding the best growth conditions for successful cultivation of *R. oligosporus*, and production of acidic protease enzyme. However, the suitability of acidic protease for biotechnological applications can be investigated through its purification and kinetic characterization.

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