Protease Production and Growth Characteristics of *Aspergillus sydowii*

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Abstract: The present work was aimed to evaluate the optimization of medium composition for protease production by *Aspergillus sydowii*. The fungus was isolated from the garden soil of Banasthali University and was identified as *Aspergillus sydowii* on potato dextrose agar medium. It was maintained on potato dextrose agar medium for 7 days at 28^0C. Spore suspensions was prepared, inoculated in Czapek Dox broth medium supplemented with various substrates, incubated and the mycelium was separated from the culture medium by filtration and the filtrate was used to determine the protease activity. The biomass was determined after drying the mycelium at room temperature for 24 hours. Maximum growth rate of this fungus in casein as substrate was found after 8 days of incubation ($7.11 mgmL^{-1}$) at 35^0C. But maximum protease activity was obtained after 6 days ($11.95\ Uh^{-1}mg^{-1}$dry mycelium). The highest protease production was attained with casein, peptone and mung seedlings as nitrogen sources. The extracellular protease production and mycelial growth were influenced by the concentration of casein. Other protein sources (yeast extract) supported growth but did not induce such excellent protease synthesis and ammonia as end product repressed it, indicating catabolite repression in this microorganism. Optimal protease production was obtained at final pH 5.3.

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Key words: *Aspergillus sydowii*, Casein, Nitrogen sources, Protease.

1. Introduction

Proteases are a group of enzymes that have been found in several microorganisms like bacteria and fungi which are involved in breakdown of complex protein molecules into simple polypeptide chains (Absida, 1985). They are commercially employed in many industrial processes. In foods, proteases have two main applications: in the processing of traditional food products and in the processing of new protein-based ingredients called functional foods (Nagodawithana and Reed, 1993).

Microbial protease represents about 60% of all the industrial enzyme's sales in the world due to their applications in several industrial sectors (Gupta et al. 2002). Proteolytic activity has been known and studied since the 1950's. The genus *Aspergillus* is well known for their proteolytic activity (Heldstrom, 2002). Now these proteases from any *Aspergillus* species are used in leather treatment and protease from *A.oryzae* are utilized in the breakdown of wheat gluten and in the pharmaceutical industry (Chiplonkar et al. 1985). Acid proteases are very important in beverage production and in baking (Rao et al. 1998).

The induction of protease requires a substrate having peptide bonds including substrates like peptone, casein and other proteins. The ammonia, as final product of enzymatic reaction of substrate hydrolysis, represses enzyme synthesis by a well known mechanism of catabolite repression. This extracellular protease has also been commercially exploited to assist protein degradation in various industrial processes (Srinubabu et al. 2007).). The great advantages offered by fungal enzymes are low material costs coupled with high and faster productivity and the ease with which the enzymes can be modified (Sharma et al. 2007). At present, due to high cost of substrates and mediums used, the overall cost of enzyme production is very high and therefore, development of novel processes to increase the yield of proteases with respect to their industrial requirements coupled with lowering down the production cost is highly appreciable from the commercial point of view (Kammoun et al. 2008).

Extracellular protease has high commercial value and multiple application in various industrial sectors, such as detergent, food, pharmaceutical, leather, diagnostics, waste management and silver recovery industries (Godfrey and West, 1996). The factors like composition of the culture medium, initial concentration of the protein/ substrate, cultivation condition and the microorganism itself may control the mechanism of enzymatic regulation. Besides nitrogen source, carbon source plays a vital role in survival of the microorganism. Growth conditions like pH, temperature, incubation period, humidity, substrates and the products formed influence the growth of microorganisms. Different species of *Aspergillus* show different proteolytic activity at pH and temperature. Thus, the strain showing the appreciable activity with less pathogenicity is used in different industrial field.

The present study deals with the growth of *Aspergillus sydowii* under various parameters and protease production which may contribute to understand the mechanism of action of environmental factors (incubation period, temperature and pH in the culture medium) and substrates (effect of nitrogen sources) on the growth characteristic of this fungus.

2. Materials and Methods

(1) *Microorganism and Inoculum Preparation*

The fungus *Aspergillus sydowii* was isolated from the garden soil of Department of Bioscience and Biotechnology, Banasthali University, Rajathan and was identified on the basis of its colony morphology on potato dextrose agar medium and microscopic observations. The microorganism was grown and maintained on potato dextrose agar medium for 7 days at 28^0C. Spore suspensions for inoculation were prepared by adding 3 ml of sterilized distilled water to fungal slant and vigorously shaking the culture for 1 min. The number of spores was determined with a Neubauer counting chamber and the inoculum was adjusted to $1X10^7$ spores/ml for inoculum preparation.

(2) *Growth Medium and Cultivation*

The fungus was inoculated in Czapek Dox broth medium for growth. The Czapek Dox medium was employed with various protein substrates at a concentration 2.0% (w/v) as nitrogen source and with the solution of trace elements. The pH of the medium was adjusted to 6.6 with 1N HCl. The protease production was determined at several pH values ranging from 4.0 to 10.0, at several temperatures ranging from 20^0C to 50^0C and incubation period from 2 days to 12 days using the broth medium and the pH was adjusted with HCl and/or NaOH to various values in the 2..0 to 10.0 pH range.

The medium was inoculated with 0.5 ml of spore suspension and incubated for 3 days to 12 days at optimum pH and temperature. In the completion of respective incubation periods the mycelium was separated from the culture medium by filtration and the filtrate was used to determine the protease activity. The biomass was determined after drying the mycelium at room temperature for 24 hours. After getting the optimum growth conditions like pH, temperature and incubation period, *Aspergillus sydowii* was grown in increasing concentration of casein and its effect on biomass and protease production was analyzed.

(3) *Protease Assay*

Protease assay was assayed with slight modifications. Test tubes were arranged on a test tube strand. 1ml of the Enzyme extract was pipette in to the test tubes. 1ml of 1% soluble substrate in citrate-Phosphate buffer (pH 6.5) was added. The test tubes were incubated in a water bath at 40° C for 30 minutes. A blank was set of consisting of 2ml of the enzyme extract that has been boiled for 20 minutes (boiling inactivates the enzyme), added to the substrate solution and treated with the same reagent as the experimental tubes. The reaction was stopped by adding 10 ml acetone and 100 mg Ninhydrin at 1, 3, 5, 7, 9, 11, 13 & 15 minutes. Colour variations in the test tubes were spectrometrically analyzed. One unit of enzyme activity was defined as the amount of enzyme which hydrolyzes 1mg of casein/10 minutes under the above conditions. The specific activity was expressed as units per mg dry weight mycelium per hour.

(4) *Statistical Analysis*

Each experiment was carried out in triplicate. From this, arithmetic means, standard errors of mean and graph were plotted.

3. Results and Discussion

After inoculating soil suspension of dilution 10^{-4}, a mixed colony culture was obtained. *Aspergillus sydowii* was characterized and identified by its colony morphology and microscopic characteristic as describe in Microbiology; A laboratory manual sixth edition, by “cappuccino, Sherman”, 1st Indian, Reprint, 2004. After growing the individual species in modified Czapek Dox medium with 2% substrates & incubating the plate for 2 days, the hydrolytic zones were observed and measured using Nessler’s reagent. After incubation, the halo rounds the colonies was measured and presence of degraded zone was determined using Nessler’s reagent. (More diameter of the halo the more ability of the organism is to degrade the protein.). It has been found that *Aspergillus sydowii* was having maximum proteolytic activity followed by *A. kanagawensis* and *A. stellatus*. Minimum activity was seen in *A. niger*. Among the nitrogen sources screened for maximum biomass produced using the strain *Aspergillus sydowii*, the maximum biomass was produced on casein>peptone>mung seedlings>yeast extract>gelatin. At 2% casein concentration in the culture medium, fungal biomass was enhanced from 0.72mg mL^{-1} (no casein) to 6.93mg mL^{-1} (2% casein, w/v) (Table 1).

The fungus *Aspergillus sydowii* was grown under various pH and 2% casein source. As the organism was grown in pH 6.0, the pH of the broth medium was decreased gradually to 5.3 during protease production and the highest protease activity (9.91 U $h^{-1}mg^{-1}$dry mycelium) was found at the pH 5.3. The protease activity was also recorded in the 4.0 to 10.0 pH range (Table 2). Maximum biomass was

found at final pH 6.6 with a yield of 4.44mg mL^{-1} dry weight. For pH below 4.0 or above 8.0, the fungal biomass and enzyme production were reduced gradually in relation to the optimum.

The highest protease activity (6.58U $h^{-1}mg^{-1}$dry mycelium) was obtained at 35^0C. Maximun growth of mycelium (6.38 mg mL^{-1}) was also recorded at 35^0C. So 35^0C was found optimum temperature for the growth of fungus and the enzyme production. The enzyme synthesis was also recorded in temperature range 20^0C to 50^0C (Table 3). Temperature below 25^0C and above 35^0C, the enzyme production was not obtained.

When the fungus *Aspergillus sydowii* was grown at 2% casein concentration in various incubation days, protease activity was found to be highest on the 6th day of cultivation. It was 11.95 U $h^{-1}mg^{-1}$dry mycelium, decreasing thereafter (Table 4). The mycelia production was maximum on 8th day of cultivation as it was 7.11 mg mL^{-1} and the pH values decreased to 4.7 on the 4th day of inoculation. Although highest protease activities were found on the 2nd day of inoculation, but it is not taken in consideration as the fungus didn't grow with some other nitrogen sources (substrates) within 2 days of inoculation.

When *Aspergillus sydowii* was grown in an increasing concentration of casein (1%-5%) for 6 days, maximum biomass (8.96 mg mL^{-1}) was obtained at 2% casein concentration and the final pH was found to be 4.1 with a higher protease activity (13.66 U $h^{-1}mg^{-1}$dry mycelium) (Table 5). The level of enzyme activity was markedly dependent upon the concentration of casein in the broth medium. The biomass of fungus was suddenly decreased at a casein concentration higher than 3% and the final pH enhanced to 8.2 in the culture medium without casein.

The results showed that the protease activity of *Aspergillus sydowii* was regulated by protein supply. While the mycelial growth on 2% casein reached a maximum on 8th day of incubation, maximum protease activity was produced on 6th day of incubation. The decreased activity in the later phase of growth was probably due to the catabolite repression by ammonia as it is the final product during the protein breakdown by the fungus.

Overall data imply that protease from *Aspergillus sydowii* was induced by casein, peptone, yeast extract, mung seeds and repressed by ammonia as the end product of this enzymatic reaction. Enzyme synthesis was affected by various organic nitrogen sources and maximal activity was shown attained with casein. Fungal growth and protease production were found only within a narrow pH range (4.0-7.0).

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Table 1. Effect of nitrogen sources on growth of *Aspergillus sydowii*.

Serial No.	Nitrogen sources 2% (w/v)	Final pH	Mycelium dry weight (mg mL^{-1})
1	Control	6.2	0.72
2	Casein	4.4	6.93
3	Peptone	4.1	5.4
4	Mung seedlings	3.6	5.0
5	Yeast extract	5.5	4.12
6	Gelatin	4.2	1.0

Table 2. Effect of pH on the growth and protease production by *Aspergillus sydowii*.

Serial No.	Initial pH	Final pH	Mycelium dry weight (mg mL^{-1})	Protease synthesis ($Uh^{-1}mg^{-1}$dry mycelium)
1	4.0	3.4	0.57	3.79
2	5.0	4.1	1.83	4.53
3	6.0	5.3	3.13	9.91
4	7.0	6.6	4.44	6.63
5	8.0	7.5	2.12	≤0.01
6	9.0	8.3	0.43	≤0.01
7	10.0	9.2	0.07	0.02

Table 3. Effect of temperature on the growth and protease production by *Aspergillus sydowii*.

Serial No.	Temperature (in ^{0}C)	Mycelium dry weight (mg mL^{-1})	Protease synthesis (U h^{-1}mg^{-1}dry mycelium)
1	20	1.26	0.03
2	25	4.73	2.88
3	30	4.11	4.27
4	35	6.38	6.58
5	40	0.34	0.04
6	45	0.07	0.00
7	50	0.00	0.00

Table 4. Effect of incubation period on the growth and protease production by *Aspergillus sydowii*.

Serial No.	Incubation Periods	Mycelium dry weight (mg mL^{-1})	Protease synthesis (U h^{-1}mg^{-1}dry mycelium)
1	2	0.76	2.00
2	4	3.44	8.9
3	6	5.89	11.95
4	8	7.11	5.00
5	10	5.55	3.56
6	12	2.95	0.04

Table 5. Effect of increasing concentration of casein on the growth and protease production by *Aspergillus sydowii*.

Serial No.	Concentration of Casein (%)	Final pH	Mycelium dry weight (mg mL^{-1})	Protease synthesis (Uh^{-1}mg^{-1}dry mycelium)
1	Control (0)	8.2	4.45	0.03
2	1	4.2	8.33	11.50
3	2	4.1	8.96	13.66
4	3	3.6	4.10	4.44
5	4	6.2	0.41	0.03
6	5	6.3	0.05	≤0.01

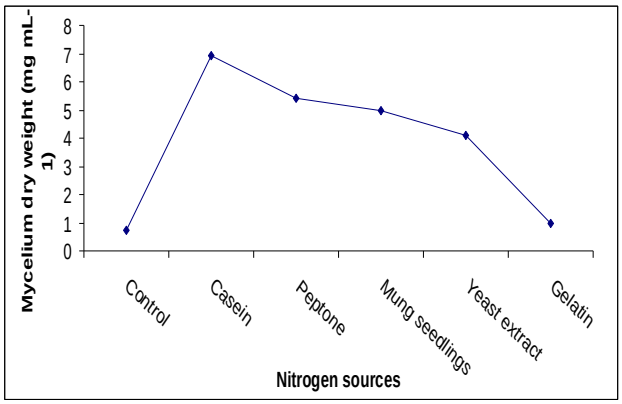

Figure 1. Effect of nitrogen sources on growth of *Aspergillus sydowii*.

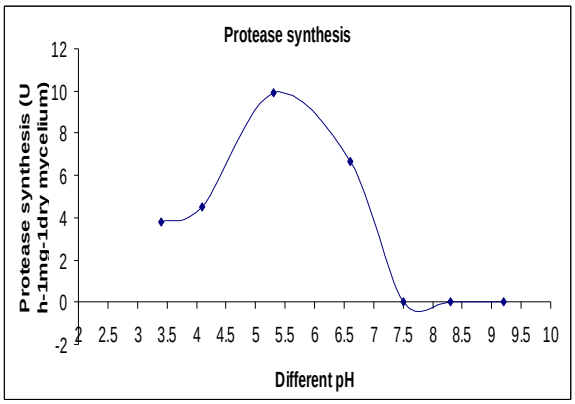

Figure 2. Effect of pH on the growth and protease production by *Aspergillus sydowii*.

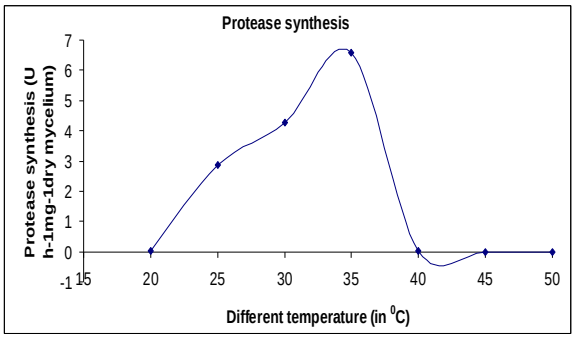

Figure 3. Effect of temperature on the growth and protease production by *Aspergillus sydowii*.

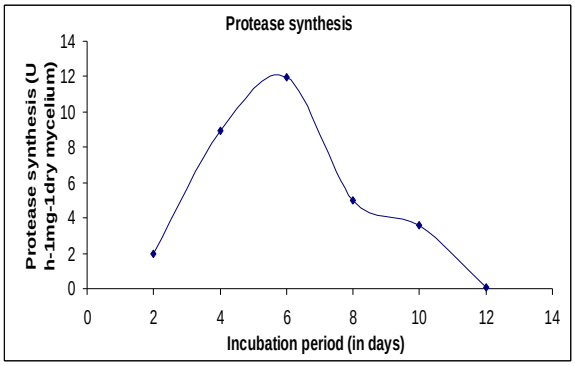

Figure 4. Effect of incubation period on the growth and protease production by *Aspergillus sydowii*.

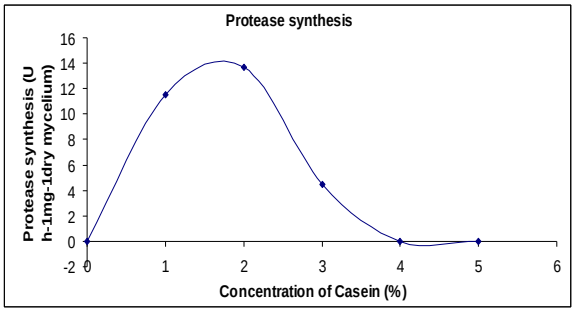

Figure 5. Effect of increasing concentration of casein on the growth and protease production by *Aspergillus sydowii*.

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