Effect of Some Growth Factors on Protease Production by *Rhizopus oryzae*

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Abstract

The main purpose of the present study was to invistgate different parameters of protease productivity and to optimize basal fermentation conditions for the production of protease enzyme under SSF where the isolated mold *Rhizopus oligosporus* was used. In this study, the effect of a number of determinants of environmental and nutritional factors in the production of the protein hydrolyzing enzyme (protease) from the isolated mold *Rhizopus oryzae* also studied. These factors which may play an influential role in improving the production of protease was to focus the source of carbon (flower without seeds) weight, the initial pH of the production medium which ingredients sunflower residue flowers without seeds was taken in conical flask as solid substrate and moistened with mineral solution 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 $CaCl_2$ per liter of distilled water. The inoculum size of the mold used in the study, the temperature of incubation and the incubation period. It was found that optimum conditions for protease production in the current study using sunflower residue as a source of carbon weighing 20g where the specific activity (2.83u/mg), optimum pH for the production of the enzyme is 5, has the resulted in the use of size 2×10^6 spores/ml inoculum to increase the production of the enzyme significantly while the optimum temperature for the production was 30ºC and enzyme production has reached to the maximum (17.41u/mg) after 144hrs of incubation.
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Keywords: Protease, *Rhizopus oryzae,* Sunflower.

Introduction

Enzymes commercially available now are not economically comparable to the chemical process. Hence, any substantial reduction in the cost of production of enzymes will be a positive stimulus for the commercialization of enzymatic depilation. Proteases are one of the most important groups of industrial enzymes and account for nearly 60% of the total enzyme sale [1,2]. They are used in pharmaceutical, food, leather and as an additive to detergent formulation in detergent industry [3]. Protease 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 [4]. A wide range of micro-organisms including *Rhizopus oligosporous* 1HS13, *Rhizopus oryzae*, *Aspergillus niger*, *Sacchromyce cerevisiae* and *Conidiobolus spp.* [5-6] have ability to produce proteases. Their biomass can be easily determined after simple drying in oven as well as in dissecator 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 spp,* also produces proteases, with a high proteolytic activity in fermentation, furthermore, it dose not produce toxins [8]. Solid-state fermentation (SSF) was chosen because it has advantages over submerged fermentation [9, 10]. Economically, SSF offers many advantages, including superior volumetric productivity, use of simpler machinery, use of inexpensive substrates, simpler downstream processing and lower energy requirements [11,12] with submerged fermentation [13].

This paper was achieved to investigate the optimum conditions for production of protease from *Rhizopus oryzae* .

Materials and Methods

Microorganisms

The isolate *Rhizopus oryzae* was used for the production of protease. It was obtained from Microbiology Laboratory of Biotechnology-Branch of Applied Science-University of Technology and maintained on potato dextrose agar (PDA) as slants, pH 5.0. It was grown on PDA in Petri dishes, incubated at 30°C for 7 days and stored at 4°C for regular sub-culturing.

Inoculum Preparation

Aliquat of 0.1% Tween-80 solution was added to a 7 days old culture slant of *Rhizopus oryzae*. Spores were dislodged using an inoculation needle under sterile conditions. Spores in suspension were collected in a sterile flask and the suspension was diluted appropriately for the required spore density. The spores count was performed on a haemocytometer slide [14].

Production of Protease

A quantity of 10g carbon source substrate sunflower residue (flowers without seeds) was taken in 250ml conical flask as solid substrate and moistened with 5ml mineral solution 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 $CaCl_2$ per liter of distilled water[5]. The pH of the medium was adjusted to 4, autoclaved at 121°C for 20min and after cooling at room temperature, inoculated with 1ml × 10^6 spores/ml of freshly prepared spore suspension of *Rhizopus oryzae*. The inoculated medium was incubated at 25°C for 96 hrs.

Extraction of Protease

To the fermented medium, 50ml distilled water containing 0.1% Tween-80 was added and agitated in shaking incubator at 180 rpm for 30min. whole contents were filtered with Whatman No.1, then the extract was centrifuged at 4000×g for 10 min at 4°C clear supernatant (crude enzyme solution) was used for analytical studies.

Enzyme Assay

The enzyme activity was determined by the method of McDonald and Chen [15]. Crude enzyme extract 200 µl of, 500 µl of casein (1%) and 300 µl of 0.2 mol/l phosphate buffer (pH 7.0) were added and incubated at 60 °C for 10 min and arrested by the addition of 1 ml of 10 % trichloroacetic acid. The reaction mixture was centrifuged at 8000 x *g* for 15 min and to the supernatant, 5 ml of 0.4 mol/l Na_2CO_3, 1 ml of 3-fold diluted Folin Ciocalteau's reagent was added. The resulting solution was incubated at room temperature for 30 min and the absorbance read at 620 nm and compared with tyrosine standard. One unit of enzyme activity was defined as the amount of enzyme that liberated 1 µg of tyrosine from substrate (casein) per minute under assay conditions and reported in terms of protease activity.

Optimization of Production Parameters

Effect of substrate concentration

Various weights of solid substrate medium (sunflower residue) ranging from 5 to 25g were moistened with 1ml distilled water (each 2g) then evaluated for optimum production of protease by *Rhizopus oryzae.*

Effect of Initial pH

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

Effect of Inoculum Size

Various sizes of inoculum ranging from 1×10^6 - 5×10^6 spores/ml were evaluated for optimum production of protease by *Rhizopus oryzae.*

Effect of Incubation Temperature

Effect of incubation temperature on production of protease was studied by incubation the medium at temperatures from 25°C and 40°C.

Effect of Incubation Time

Different incubation times ranging from 72 (hrs) to 192 (hrs) were evaluated for optimum production of protease.

Results and Discussion

Effect of substrate weight on protease production from *Rhizopus oryzae*

The effect of carbon source (sunflower residue) concentration was checked for the production of protease. The maximum enzyme production with specific activity (2.38u/mg) was found at 20g weight of sunflower as mentioned in Fig.(1). As the substrate concentration increases from 20g the enzyme activity is tended to decrease. Similar results were reported by Haq and Hamid [16] using different substrates such as sunflower meal, soybean meal, cotton seed meal and wheat bran. Ikasari and Mitchell reported slightly different results by using the fungus *Rhizopus oligosporus* on the substrate rice husk (3.9 u/mg)[17]. Different results due to the

effect of culture conditions of fungi, use on other substrates and in different concentrations other environmental factors also affects protease production.Sumantha *etal*. reported similar results by using the fungus *Rhizopus oligosporus* with the substrate mixed with casein 20g (3.6 u/mg)[18].

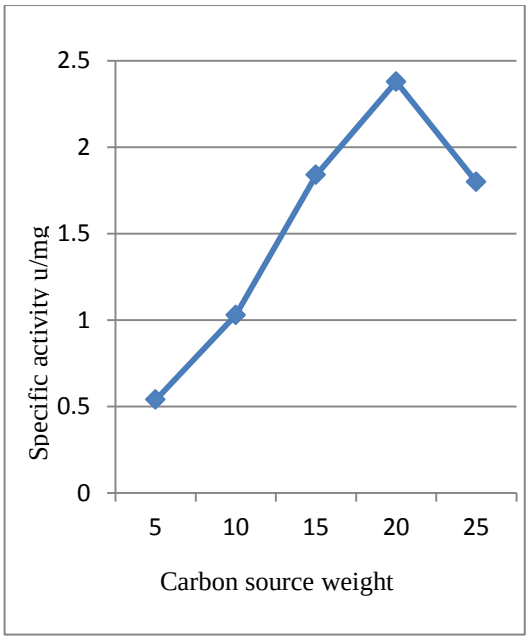

Fig.(1): Effect of carbon source weight on protease production from Rhizopus oryzae at 25°C for 96 hrs.

Effect of pH on protease production from *Rhizopus oryzae*

Protease production by microbial strains depends on the pH because medium pH strongly influences many enzymatic processes and transport of various components across the cell membranes, which in turn support the cell growth and production of metabolites [19]. The optimum pH for production of protease from *Rhizopus oryzae* was recorded at 5.0 Fig.(2).

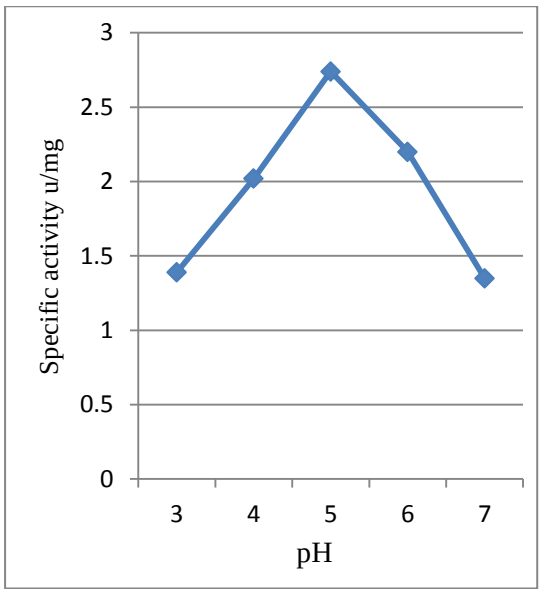

Fig.(2): Effect of pH on protease production from Rhizopus oryzae at 25°C for 96 hrs.

A notable decline in the enzyme productivity occurred at both higher and lower pH values. Maximum protease production by *Penicillium chrysogenum* cultured on sunflower was observed at pH ranged from 5-7 [20].

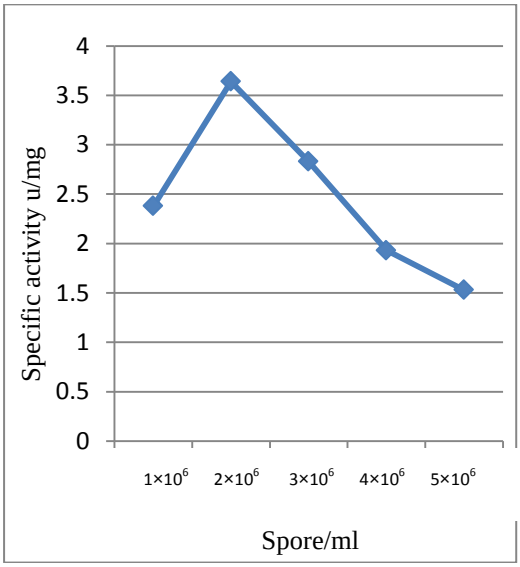

Fig.(3): Effect of Inoculum size on protease production from Rhizopus oryzae at 25°C for 96 hrs.

Effect of Inoculum size on protease production from *Rhizopus oryzae*

Inoculums' size was studied for the production of protease enzymes. The maximum enzyme production (3.64u/mg) was observed with 2×10^6 spores/ml inoculum size

Fig.(3). The effect of inoculum size on the production of protease by *Rhizopus oryzae* showed that the size ranged from (1-3ml) and maximum amount of enzyme (4.8 u/mg) was produced when 2×10^6 spores/ml was added to the flask. Further increase in inoculum volume resulted in a decrease in protease production, because much increase in inoculum volume caused overcrowding of spore that decreased the enzyme production [21]. Size of inoculum is an important biological factor, which determines biomass production in fermentation [22, 23]. Hence, a balance between the proliferating biomass and available materials will yield maximum enzyme production.

Effect of Temperature on protease production from *Rhizopus oryzae*

Fermentation carried out at 30°C was best suited for enzyme production in this study the maximum production was found at 30°C Fig.(4). Preliminary studies on growth and enzyme production at 25, 28 and 32°C indicated that although luxuriant growth occurred at all of these temperatures but the productivity was low at 25°C and higher at 28 and 32°C [24].

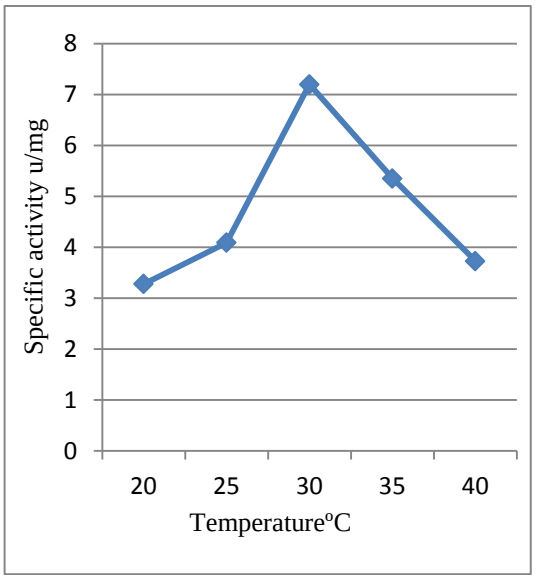

Fig.(4): Effect of Incubation temperature on protease production from Rhizopus oryzae for 96 hrs.

Effect of incubation time on protease production from *Rhizopus oryzae*

Result of this study showed that protease production is affected by incubation period. Maximum enzyme production was observed at 144hrs of incubation Fig.(5).

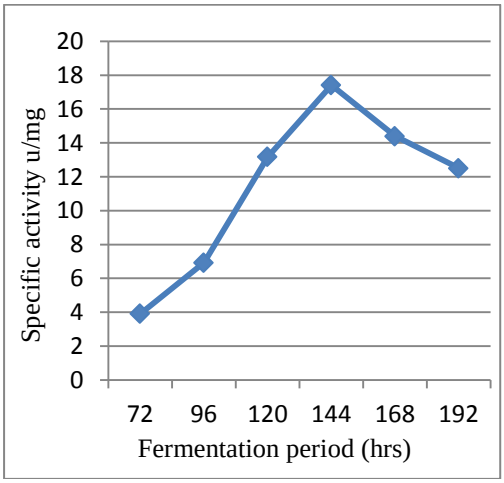

Fig.(5): Effect of Fermentation period on protease production at 30°C from Rhizopus oryzae.

A gradual decrease in enzyme units was observed with increasing incubation period clearly suggesting the enzyme's role as a primary metabolite, being produced in the log phase of the growth of the fungus for utilization of nutrients (proteins) present in the solid substrate. [25]. the subsequent decrease in the enzyme units could probably be due to inactivation of the enzyme by other constituent proteases.

Conclusion

On the light of the obtained results, it could be concluded that fermented sunflower residues (flowers without seeds) by the fungus *Rhizopus oryzae* at 30°C for 144hrs and pH 5.0 are the most suitable conditions for protease production.

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