

# Production Of Protease By Submerged Fermentation Using *Rhizopus Microsporus Var Oligospous*

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## Abstract

Production of protease via submerged fermentation using *Rhizopus microsporus var oligospous* was studied using shake flask cultures. After 96 h of growth on a BOD shaker revolving at 150 rpm, protease was partially purified using the isopropanol. Factors affecting the enzyme production viz incubation temperature, initial pH of the shake flask medium. and inoculum size were optimized. Protease having the maximum enzyme activity of 521.739 IU was obtained at an incubation temperature of 30°C, an initial pH of the basal medium as 5.5 and an inoculum size of to  $1 \times 10^5$  spores  $\text{ml}^{-1}$  of Tween-80. Protease deactivated at 80 °C.

## INTRODUCTION

Enzymes are proteins composed of 20 amino acids. They are produced by cellular anabolism, the naturally occurring biological process of making more complex molecules from simpler ones. Enzymes increase the rate of biochemical reaction and decrease the time for those reactions to reach equilibrium. They are not consumed in the chemical reaction, and thus their action is catalytic (Nielson, 1991). Enzymes used in food processing are produced by bacteria, fungi, higher plants and animals. Most of the organisms that produce enzymes are fungi. Mycolytic enzymes are primarily originated as a desire of microbiologists to understand the chemical nature and ultra structure of fungal cell wall. A number of enzymes can be classified under mycolytic enzymes viz; protease, cellulase, xylanase etc.

Protease enzyme conducts proteolysis by hydrolysis of the peptide bonds that link amino acids together in the polypeptide chain. Proteases are the most valuable commercial enzymes and account for 60% of the total enzyme market (Rao et al, 1998). They find increased application in food, pharmaceutical, detergent, leather, tanning industry and to some extent in silver recovery and peptide synthesis (Godfrey and West, 1996; Kumar and Takagi, 1999; Oberoi et al, 2001). Though plants and animals also produce extra cellular proteases, microorganisms are the preferred source because of their rapid growth, limited space required for their cultivation, longer shelf life and the ease with which they can be

genetically manipulated to generate improved enzymes (Rao et al, 1998). Extra cellular proteases help in hydrolysis of protein in the cell free environment and their cellular uptake (Kalitz, 1988).

## MATERIALS AND METHODS

### MICROORGANISM AND INOCULUM PREPARATION

*Rhizopus microsporus var oligospous* was procured from the institute of Microbial Technology, Chandigarh and was maintained at 4°C on potato dextrose agar (PDA). Spore suspension was made from five days old cultures that had been grown on PDA slants at 30°C. It was prepared by suspending the spores from one tube in 10 ml of sterilized distilled water containing 0.1ml Tween-80. The spore count was adjusted to  $10^5$  spores  $\text{ml}^{-1}$  (Smith et al, 1996). One ml of inoculum was used per flask to carry out submerged fermentation.

### SHAKE FLASK CULTIVATION MEDIA

The enzymes were produced using basal medium (Manomani et al, 1983). The basal medium contained the following ingredients ( $\text{g l}^{-1}$ ):  $(\text{NH}_4)\text{SO}_4$  - 1.4,  $\text{CO}(\text{NH}_2)_2$  - 0.3,  $\text{KH}_2\text{PO}_4$  - 4.0,  $\text{K}_2\text{HPO}_4$  - 0.84,  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  - 0.3,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  - 0.3,  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  - 0.005,  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$  - 0.00156,  $\text{ZnCl}_2$  - 0.00167, Peptone 0.25, Yeast extract - 0.10, Rice Bran - 4%. All the components of the basal medium except rice bran were dissolved in distilled water. Flasks having 250 ml capacity were taken and 50 ml of this

solution was poured in each flask. To each flask 2.0 g of rice bran was added (Thenawadjaja et al, 1990) and autoclaved at  $1.1\text{kg/cm}^2$  for 20 minutes. Flasks were cooled and inoculated with 1ml of the spore suspension. The inoculated flasks were incubated at  $30^\circ\text{C}$  on a rotary shaker cum BOD incubator revolving at 150 rpm (Seyis and Aksoz, 2005; Meenaksui et al, 2005). After 96 hours of growth, the contents of each flask were centrifuged at  $4^\circ\text{C}$  and this supernatant was used as the source of crude enzyme (Nehra et al, 2002).

### **PARTIAL PURIFICATION OF FUNGAL PROTEASE**

Protease was isolated and partially purified with isopropanol (Manomani et al, 1983). The mold filtrate (20 ml) was chilled at  $4^\circ\text{C}$  in a refrigerator and 25 ml of chilled isopropanol was added to it. The precipitates were separated by centrifugation. The precipitates were dissolved in 5 ml of 0.05 M citrate phosphate buffer having pH 5.2 (Rajinder, 1992) to obtain partially purified enzymes.

### **PROTEASE ACTIVITY**

The test tubes containing 1ml casein solution and 1ml enzyme extract were incubated at  $60^\circ\text{C}$  for 10 minutes. After 10 minutes 3 ml TCA was added to each test tube to stop the reaction. The precipitates formed were centrifuged at 5000 rpm. The protein concentration in the supernatant was determined by Lowry's method (Lowry et al, 1951). Optical density was recorded at 525 nm. Protease activity was expressed as Tyrosine equivalents using the standard curve prepared for measurement of proteins under same set of conditions as described above using standard solution of Tyrosine.

Enzyme activities of the partially purified enzyme was expressed in International Units (IU). One IU was defined as one  $\mu\text{mol}$  of tyrosine equivalents released per minute per ml under the following assay conditions using tyrosine standard curve (Silva et al, 2005). Appropriate dilution factors were used during the estimation of the enzymes.

### **FACTORS AFFECTING ENZYME PRODUCTION**

The effect of initial culture pH on the enzyme production was investigated by adjusting the initial pH of the basal medium in the range of pH 5-8. To examine the effect of temperature on enzyme production the inoculated culture flasks were incubated at different temperature varying between  $15\text{-}40^\circ\text{C}$  and then the enzyme activities were determined by using the partially purified enzymes. The effect of inoculum size based on the number of spores was

examined using the spore concentration of  $1 \times 10^3$ ,  $1 \times 10^4$ ,  $1 \times 10^5$  and  $1 \times 10^6$  spore  $\text{ml}^{-1}$  of Tween-80 (Kheng and Omar, 2005) for making the spore suspension. The flasks having the sterile basal medium were inoculated with the spore suspension and were incubated at  $30^\circ\text{C}$  on a BOD shaker cum incubator for 96 h and then the partially purified enzymes were used for the determination of the enzyme activities.

### **TESTING OF ENZYME STABILITY**

To test the stability of the enzymes, the CMCase and xylanase were incubated at different temperatures ranging from  $40\text{-}80^\circ\text{C}$  for 60 min (Silva et al, 2005). The reaction was stopped in ice cold water and the remaining activity was measured under standard assay conditions.

### **RESULTS AND DISCUSSIONS**

*Rhizopus microsporus var oligosporus* was used to produce protease via submerged fermentation. Partial purification of an enzyme improves the economics of the enzyme production process. To obtain optimum levels of the protease production, rice bran was used as substrate, which is an easily available agricultural residue. Protease having the enzyme activity of 521.739 IU was obtained under the optimized conditions. Protease deactivated at  $80^\circ\text{C}$ .

### **EFFECT OF TEMPERATURE**

The effect of different temperatures ranging from  $15\text{-}40^\circ\text{C}$  on the production of fungal enzyme protease by *Rhizopus microsporus var oligosporus* was studied. The basal medium was prepared, inoculated with the respective spore suspensions and was incubated at different temperatures ranging from  $15^\circ\text{C}\text{-}40^\circ\text{C}$ . At  $15^\circ\text{C}$ , all enzyme activities were low which showed a gradual increase with the increase in temperature at  $30^\circ\text{C}$ . Further increase in temperature resulted in decrease in enzyme production indicating that  $30^\circ\text{C}$  was the optimum temperature for the maximum production of the enzymes (Table 1). Less activity of fungal enzymes at low temperature ( $15\text{-}25^\circ\text{C}$ ) and at high temperature ( $35\text{-}40^\circ\text{C}$ ) as compared to  $30^\circ\text{C}$  might be due to slow growth at low temperature and inactivation of the enzyme at high temperature. This observation was in accordance with the observation of Tunga et al (1999) who reported  $30^\circ\text{C}$  to be the optimum temperature for protease production by *Rhizopus oryzae*.

**Figure 1**

Table 1: Effect of temperature on the production of Fungal Enzymes

Temperature (°C)	Protease (IU)
15	0.600
20	12.030
25	183.100
30	521.739
35	145.210
40	118.620

Medium – Basal Medium (Manomani et al, 1983)

Incubation time- 96 h

Temperature – 30± 2°C

pH - 5.5

The data represents the mean of three determinations each.

**EFFECT OF PH**

Effect of different pH values ranging from 4.0 to 9.0 on the production of fungal protease by Rhizopus microsporus var oligosporus was studied by altering the pH of the basal medium that was used for carrying out the submerged fermentation. Production of the enzyme protease increased with increase in pH value, reaching the maximum at pH 5.5,

followed by a gradual decrease thereafter (Table 2). The decrease or increase in activities at different pH values than the optimum pH might be due to decreased production of the mycolytic enzymes.

**Figure 2**

Table 2: Effect of pH on the production of fungal enzymes

pH	Protease (IU)
4.0	054.680
4.5	143.870
5.0	326.330
5.5	521.739
6.0	445.690
6.5	335.840
7.0	210.180
7.5	147.230
8.0	035.210
8.5	008.990
9.0	000.560

Medium – Basal Medium (Manomani et al, 1983)

Incubation time- 96 h

Temperature – 30± 2°C

pH - 5.5

The data represents the mean of three determinations each.

**EFFECT OF INOCULUM SIZE (SPORE DENSITY) ON THE PRODUCTION OF FUNGAL ENZYMES**

The effect of inoculum size based on the number of spores was examined using the spore concentration of  $1 \times 10^3$ ,  $1 \times 10^4$ ,  $1 \times 10^5$  and  $1 \times 10^6$  spores  $ml^{-1}$  on the production of fungal protease. It was found that the increase in inoculum size resulted in rapid increase in enzyme production (Table 3) due to fast degradation of the substrate. Similar observations were reported by Raimbault and Alazard (1980) who showed that maximum enzyme production and declination was achieved much faster.

**Figure 3**

Table 3: Effect of inoculum size on production of fungal enzymes

Inoculum Size (Spores $ml^{-1}$ )	Protease (IU)
$1 \times 10^3$	195
$1 \times 10^4$	256
$1 \times 10^5$	520
$1 \times 10^6$	500

Medium – Basal Medium (Manomani et al, 1983)

Incubation time- 96 h

Temperature –  $30 \pm 2^\circ C$

pH - 5.5

The data represents the mean of three determinations each.

The enzyme activity did not show a further increase at a

spore count of  $1 \times 10^6$  spores  $ml^{-1}$  because of the limitation of the substrate. Hence, based on this the inoculum size of  $1 \times 10^5$  was used for inoculation of basal medium.

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