Submerged fermentation of acidic protease by 
*Rhizopus arrhizus* PTCC-1

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**ABSTRACT**

Different agro-industrial by-products such as rice husk, rice straw, wheat bran, soybean meal, sunflower meal and cotton seed meal were screened for the production of extra-cellular protease by *Rhizopus arrhizus* PTCC-1 in submerged fermentation. Maximum enzyme synthesis was detected in soybean meal (16.23±1.11 PU/mL), followed by rice husk medium cultivated at temperature 37°C for 72 hrs. Effect of various pH values and agitation speed were studied to increase the yield of acid protease. Maximum proteolytic level was obtained at pH 5 with agitation speed of 140 rpm.

**Keywords:** Acidic protease, soybean meal, submerged fermentation and *Rhizopus arrhizus* PTCC-1

**INTRODUCTION**

Proteases execute a large variety of functions in different industries. The main sources of these enzymes are animals (e.g. calf stomach) and plants (e.g. pineapple, fig, papaya). Due to irregular production associated with the plant sources and large numbers of ethical and moral issues related to animal sources, microbial sources occupy an important place in the production of all the three major types of proteases viz: acidic, neutral and alkaline protease (Rao et al 1998). Microorganisms, especially fungi, owing to their GRAS (Generally Regarded as Safe) nature, have now become popular, especially with respect to enzyme applications in the food industries (Pandey 1992).

*Rhizopus* sp. are important major moulds in fermentation. Several reports describe the efficient protease biosynthesis by fungi belonging to the genera *Aspergillus* (Fan-Ching and Lin 1998), *Penicillium* (Chrzanowska et al, 1993), *Rhizopus* (Farley and Akasari 1992), and *Humicola* (Aleksieva and Peeva 2000). Although bacterial proteases have long been used in the industry, the main drawback of their use is that they require cost-intensive filtration methodologies to obtain a microbe-free enzyme preparation. On the other hand, proteases of fungal origin offer an advantage, that is the mycelium can easily be removed by filtration (Phadatare et al 1993).

The present work was undertaken to produce acidic protease by employing different substrates in submerged fermentation. Various process parameters such as temperature, incubation period, pH and agitation speed were optimized to get the maximum yield of enzyme from *Rhizopus arrhizus* PTCC-1.

**MATERIALS AND METHOD**

**Mould Culture**

The fungus culture of *Rhizopus arrhizus* PTCC-1 was obtained from PTCC (Pakistan Type Culture Collection), Food and Biotechnology Research Center, PCSIR Laboratories Lahore. The culture was revived on Potato Dextrose Agar (PDA) at 37°C and was maintained on PDA (Oxoid) at 4°C.

**Preparation of Spore Suspension**

A spore suspension of 1x10^6/ is was prepared by adding 10 mL sterile distilled water in 7 days old slant. The spores were scratched by sterile wire loop to break clumps to form homogenous spore suspension. Five mL of the spore suspension was used for inoculation.

**Screening of Substrates**

Different agro-industrial residues such as rice husk, rice straw, wheat bran, soybean meal, sunflower meal, cotton seed meal and rape seed cake were used for the selection of best substrate for acidic protease production.

The 2.0% quantity of each substrate was used in growth medium consisting of yeast extract (0.5%), KH₂PO₄ (0.4%), NaCl (0.1%) and MgSO₄ (0.05%). The pH of the medium was adjusted at 5.0 with 1 N HCl/ NaOH before sterilization.

Screening of substrates was carried out in triplicates in 250 mL Erlenmeyer flasks, each with 50 mL medium. The flasks were agitated at 37°C for 72 hrs on a water bath shaker (Eyela Japan) operating at 140 rpm.
Optimization of process parameters

Different process parameters influencing the production of protease such as fermentation time (24, 48, 72, 96 and 120 hrs), fermentation temperature (16, 23, 30, 37 and 42°C), pH of the growth medium (3, 4, 5, 6 and 7) and agitation speed (60, 100, 120, 140 and 160 rpm) were optimized for maximum production of proteolytic enzyme. All experiments of the process parameters were performed in triplicates.

Analytical Procedure

Proteases Assay

Proteases activity was assayed by the modified method of Anson et al. (1938) using casein as a substrate. The reaction mixture containing 2 mL 1.0% casein in 0.5 M phosphate buffer (pH 5.0) and 1.0 mL suitably diluted enzyme was incubated at 40°C for 30 minutes. The reaction was terminated by adding equal volume of 10.0% w/v of trichloroacetic acid and filtered through Whatman No. 1 filter paper. To 1 mL of the filtrate 5 mL 0.5 M Na₂CO₃ solution and 0.5 mL of three fold diluted Folin-Ciocalteau reagent were added and mixed thoroughly. The color developed after 30 min of incubation at 30°C was measured at 660 nm. One unit of the proteolytic activity was defined as the amount of enzyme required to liberate 1μg tyrosine in 30 minutes at 40°C.

Determination of Protein

Protein concentration was measured by the method of Lowery et al. (1951) using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Screening of substrates

Fermentation process is governed by a large numbers of physical, chemicals and biological factors. However, selection of the nutrient contents of both carbon and nitrogen sources have a great impact on enzyme production and play a vital role in the production processes.

The effect of different substrates on the production of acidic protease by Rhizopus arrhizus was investigated (Fig 1). Maximum yield of protease (16.23 ±1.11) was obtained from soybean meal based medium followed by sunflower meal (14.19 ±1.23) as a substrate. However, minimum enzyme activity (3.12 ±0.81) was observed in rape seed cake that might be due to the presence of some inhibitors in it. Cinthia and Rosana (2000) also found that soybean meal is the best substrate for the production of enzymes (160 U/ml) followed by wheat bran (120 U/ml). Ikram ul Haq et al. (2004) also observed that soybean meal medium produced maximum enzyme activity 58 U/g.

Figure 1. Screening of different carbon source for maximum acidic protease productin by Rhizopus arrhizus PTCC-1

Optimization of process parameters

Time course experiments (Figure 2) revealed that maximum enzyme production (17.56 PU/ mL) was obtained after 72 hrs fermentation period. Thereafter, enzyme activity decreased subsequently that could possibly be due to cessation of the production as enzymes are primarily metabolites and it also could be due to the inactivation of the enzymes. Sunantha et al. (2005) reported that maximum enzyme production after 48 hrs fermentation period by Aspergillus sp. This might be due to the difference in the culture and mode of fermentation. Our findings are in line with Samamtar et al. (1999) who observed maximum enzyme activity after 72 hrs by genetically engineered Aspergillus oryzae U1521.

Figure 2. Effect of incubation period on acidic protease production by Rhizopus arrhizus PTCC-1
Figures 3 and 4 depict the effect of temperature and pH on the protease production respectively. Maximum yield of protease was found at 37°C and pH 5.0. As the temperature increases beyond the optimized temperature, the protease production decreases. Ikram and Hamid (2004) found maximum enzyme activity at 30°C and pH 5.0 by Rhizopus oligosporus IHS13 in low cost medium. Other workers (Tremaicolli and Eleonora 2005) found maximum enzyme activity in a culture medium containing glucose and casein at 1.0 % (w/v) as a substrate at 25°C by Aspergillus clavatus.

Proper aeration is the basic need for maximum growth of aerobic microorganisms. Agitation speed plays an important role in aeration and even distribution of oxygen throughout the growth medium. Effect of different agitation speed on protease production is shown in Figure 5. Maximum yield of acidic protease (20.12 PU/ml) was recorded at 140 rpm shaking in Eyela, Japan. Dahot (1993) obtained maximum proteases at 220 rpm in 1.0% rice husk medium by Penicillium expansum. Cesar and Facundo (2003) studied the effect of agitation rate and aeration on the production of protease. They obtained maximum enzyme yield (5.28 units/mg) at 700 rpm/m and 0.5 v/v/m. All these findings indicate that hydrodynamic conditions affect the enzyme production in submerged fermentation.

REFERENCES


Fan-Ching Y and Lin IH. 1998. Production of acid protease using thin stillage from the rice spirit.


