Exploitation of Different Agro-residues for Acid protease Production by
Rhizopus sp. in Koji Fermentation

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Abstract
Two different strains of Rhizopus sp. i.e. R. oligosporus -M30 and R. arrhizus -M26 were employed
for the production of acid protease using different agro-residues such as rice husk, rice bran, wheat
bran, defatted soybean meal and sunflower meal as a sole carbon source. R. oligosporus -M30 gave
maximum enzyme level with soybean meal (15.6 U/gds) while R. arrhizus -M26 with sunflower
meal (13.6 U/gds) respectively. The optimal conditions for the enzyme production by R
oligosporus -M30 were initial pH of 5.5, temperature 30 °C with 10% of inoculum size for 72hr of
fermentation period. When R. arrhizus -M26 was cultivated that exhibit the best enzyme production
with the fermentation period of 72h, initial pH 5.0, temperature 33 °C with 5% of inoculum size

Key words: Agro-residues, acid protease, Rhizopus sp., solid state fermentation

Introduction
Fungi elaborate a wide variety of enzymes than do bacteria and proteases are among the most
important enzymes produced by fungi. Fungi produce a variety of proteolytic enzymes however;
most of these are usually acidic in nature. The filamentous fungi have a potential to grow under
varying environmental conditions such as time course, pH and temperature, utilizing a wide variety
of substrates as nutrients. Several species of Penicillium such as P. chrysogenum, P. restrictum, P.
dupontii and P. griseoroseum have been reported to produce proteases.
Microbial enzymes are produced by using either submerged liquid (SLC) or solid-state cultivation
(SSC) techniques. Solid-state fermentation processes have been developed for fungal enzyme
production (pectinases, amylases, acid-proteases, amyloglucosidases, etc.) at industrial scale. Advantages of the latter method include simplicity, lower production costs, high enzyme yields and
low wastewater output. Moreover, SSC prevents catabolic repression phenomena that adversely
influence the biosynthesis of many microbial products.
Acid proteases are synthesized by Mucor miehei, Mucor hiemalis, Mucor racemosus and
Mucor bacilliformis. The Mucor rennin proteases are derived from Mucor pusillus and other
pepsin like acid proteases are synthesized by Aspergillus species and Rhizopus species.
The present investigation was undertaken to produce the acid protease using different agro-residues
in solid state fermentation. Different growth parameters such as fermentation period, temperature,
initial pH, inoculum size and age were optimized for enhanced protease production using strains of
Rhizopus sp.
Materials and Methods

Microorganism and maintenance
The mould strains of *Rhizopus arrhizus* M-26 and *Rhizopus oligosporus* M-30 were obtained from Microbiology laboratory, PCSIR laboratories, complex Lahore. The culture was revived after fifteen days and maintained on potato-dextrose agar (Oxoid) slants at 4°C.

Inoculum development
The slants of five days old cultures were wetted by adding 10 ml of sterilized distilled water. The spores were scratched by sterile wire loop to break clumps and obtain homogeneous spore suspension. One ml of this spore suspension containing 1 x 10^8 spores was used as inoculum.

Fermentation procedure
Solid state fermentation was carried out in 250 ml Erlenmeyer flasks containing 10 g substrate moistened with 10 ml of diluent (composition: yeast extract 0.5%, KH_2PO_4 0.4%, NaCl 0.1% and MgSO_4 0.05% at initial pH 5.0) were sterilized at 121°C (15 lbs/inch^2 pressure) in an autoclave. After sterilization, the flasks were cooled, inoculated with one milliliter of spore suspension of *Rhizopus oligosporus* M-26 and *Rhizopus oligosporus* M-30 and incubated at 30±1°C for 72 h. After incubation, the enzyme was extracted and assayed for acid proteases.

Optimization of Growth Parameters
Screening of Substrates
Different agro-industrial residues such as rice husk, rice bran, wheat bran, defatted soybean meal and sunflower meal were tested for the selection of best substrate for acidic protease production. Each experiment was carried out in duplicates. Different growth parameters which influence the acid protease production include fermentation period (24, 48, 72, 96 and 120hrs.) fermentation temperature (25, 30, 33, 35 and 37°C), initial pH (3, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0), inoculum size (5, 10, 15, 20, and 25%) , and age of inoculum (2, 3, 4, 5, 6, 7, 8 and 9 days) were optimized for acid protease production from the mould cultures. All experiments were carried out in duplicates.

Analytical Procedures
Extraction of Enzyme
Enzyme from the fermented broth was extracted by the method describe by Sumantha et al. A solution of Tween-80 (0.1 %) in distilled water was added to the fermented substrate which was homogenized on a rotary shaker at 180 rpm for 1 h. The solids were removed by centrifuging at 10,000 x g at 4 °C for 10 min and the resultant clear supernatant was used for analytical studies. Different extractants such as 0.1%NaCl, 0.1% Tween-80, citrate buffer pH 5, phosphate buffer pH 7 and distilled water were also optimized for maximum proteolytic activity.

Protease Estimation
Acid protease activity was estimated by the method of McDonald & Chen. Casein (1%) was incubated with one ml of enzyme sample at 30 °C for one hour. The reaction was stopped by the addition of five ml of trichloroacetic acid (TCA) solution. The mixture was centrifuged and one ml of supernatant was mixed with five ml of alkaline reagent. To this mixture one ml of 1N NaOH was added to make the contents of the tube alkaline. After 10 min., 0.5 ml of Folin and Ciocalteau reagent was added to the test tubes and mixed. The blue colour produced was measured with UV spectrophotometer (Labomed, USA) at 700 nm after 30 min. One unit of protease activity was
defined as the amount of enzyme required to liberate 1µg of tyrosine under the standard assay conditions.

The data was subjected to statistical analysis for the determination of significance by using ANOVA.40

Results and Discussion
Screening of substrates
Among the various tested substrates for acid protease production it was observed that maximum protease production was found with soybean meal (15.6 ± 0.92 U/gds) using R. arrhizus M-26 and sunflower meal (11.7± 0.74 U/gds) using R. oligosporus M-30 (Fig. 1). Qadeer et al.33 reported that sunflower meal gave maximum proteases yield because this agricultural byproduct had adequate proteins, carbohydrates and minerals needed for the growth of organism. Ikram ul Haq et al.16 reported that sunflower meal gave maximum enzyme activity (4.4U/ml) using different mould cultures. Some workers 5,17,29 also reported the similar investigations. Macchione et al 23 worked on protease production by different thermophilic fungi and reported that wheat bran was best substrate for protease production in solid state fermentation and soybean meal extract was best substrate for protease production in submerged fermentation. These differences in findings might be due to different nature of fungus and different composition of substrate favored for protease production.

Figure 1. Screening of different substrates for acid protease production using R. arrhizus M-26 and R. oligosporus M-30. Bars represent the standard deviation among replicates which differ significantly at p≤0.05.

Optimization of the growth parameters
Recovery of the enzyme from the fermentation processes affects the enzyme activity. After the termination of the fermentation period the enzyme was extracted with different solvents and it was observed that maximum enzyme yield was obtained when distilled water (10.1 ± 0.86 U/gds in case of R. oligosporus M-30 and 12.3 ± 1.01 U/gds for R. arrhizus M-26) was used as an extractant during the solid state fermentation as shown in figure 2. Similar findings were also reported by
Ikram et al.\textsuperscript{16}, who stated that the chemical composition of the buffer might show inhibitory effect on the enzyme activity. Aikat and Bhattacharyya\textsuperscript{4} also reported highest enzyme yield when potassium phosphate buffer pH 8.0 was used as an extractant, which showed comparatively less activity than distilled water extraction.

![Different Extractants](image)

**Figure 2.** Extraction of protease from fermented mash of *R. arrhizus* M-26 and *R. oligosporus* M-30 using different extraction agent. Bars represent the standard deviation among replicates which differ significantly at \( p \leq 0.05 \).

Time course of fermentation revealed that maximum protease production was observed after 72 hr of fermentation period which was \( 13 \pm 1.12 \) U/gds of *R. oligosporus* M-30 and \( 15 \pm 1.21 \) U/gds of *R. arrhizus* M-26 as shown in the figure 3. Protease production decreased as the fermentation period increased that might be due to the depletion of the nutrients and accumulation of primary metabolites which inhibit the enzyme synthesis as reported by Romero et al.\textsuperscript{36} Similar findings were made by Karuna and Ayyana\textsuperscript{20} and Ikasari and Mitchell\textsuperscript{15} using *Rhizopus oligosporus*. Negi and Banarjee\textsuperscript{31} optimized cultural condition for protease production and reported 120hrs of incubation period was optimum for protease production. Samarntarn et al.\textsuperscript{37} observed the maximum enzyme activity after 72 hr of fermentation period using genetically engineered *A. oryzae* U1521. Chutmanop et al.\textsuperscript{4} reported 84h of fermentation period using *A. oryzae*. Ikram et al.\textsuperscript{18} also reported 72h of incubation period was optimum for protease production using *Penicillium chrysogenum*. These variations in fermentation period might be due to the different nature of the strains.

Incubation temperatures in figure 4 & 5 illustrate the effect of different in temperatures of incubation and pH on protease production. Maximum protease production was observed at temperature 33 °C pH 5 by *R. arrhizus* M-26 and 30°C, pH 5.5 by *R. oligosporus* M-30 producing \( 16.8 \pm 1.32 \) U/gds, \( 13.7 \pm 0.83 \) U/gds and \( 13.2 \pm 0.96 \) U/gds, \( 17.1 \pm 0.82 \) U/gds respectively. Nadeem et al.\textsuperscript{30} reported maximum protease production at temperature of 37 °C and pH of 5.0 in submerged fermentation using *R. arrhizus* PTCC-1. Negi and Banarjee\textsuperscript{31} reported that initial medium pH of 4.5 and incubation temperature of 35 °C by *Aspergillus awamori* was optimum for protease production. Karuna and Ayyanna\textsuperscript{20} reported that changes in pH may also cause
denaturation of enzyme resulting in the loss of catalytic activity. However, Ikram et al. reported the protease production at temperature of 30 °C, pH 7.0 using *Penicillium chrysogenum*. Tremacolidi and Eleonara reported maximum protease production at 25 °C by using *A. clavatus*. Sharma et al. reported that fungal proteases are thermolabile and showed reduce activities at higher temperatures. Tunga et al. reported that higher temperatures have some adverse effects on metabolic activities of microorganisms and causes inhibition of the growth of the fungus.

**Figure 3.** Effect of fermentation period on acid protease production using *R. arrhizus* M-26 and *R. oligosporus* M-30. Bars represent the standard error among replicates which differ significantly at *p*≤0.05.
**Figure 4.** Effect of different incubation temperature on acid protease production using *R. arrhizus* M-26 and *R. oligosporus* M-30. Bars represent the standard error among replicates which differ significantly at p≤0.05.

Enzyme was denatured at high temperature by losing its catalytic properties due to stretching and breaking of weak hydrogen bonds within enzyme structure. Protease production at low temperature has also been reported but with a lower yield.

Age and size of inoculum are also an important factor in fermentation technology. Figures 6 & 7 represent the age and different size of inoculum used for protease production. Maximum protease production was observed with 5% inoculum size of *R. arrhizus* M-26 and 10% of *R. oligosporus* M-30. Further increase in the inoculum size resulted in decreasing the protease production. According to Mitchell, spores are preferably used because of the convenience of preparation, their stability during storage and tolerance of mistreatment during harvesting. According to Sandhya et al., the maximum protease production was found by using 10% of spore suspension of *Aspergillus oryzae*. Ikram et al. showed similar findings using *Rhizopus oligosporus*. Hesseltine et al. reported that decrease in enzyme production with larger inoculum sizes could be due to the shortage of nutrients available for the larger biomass and faster growth of the culture. It was also observed that age of the inoculum has little effect on enzyme production. Malathi and Chakraborty reported 5-7 day old inoculum gave maximum protease yield in case of *A. oryzae* which is in good agreement with our findings.
Figure 6. Effect of different inoculum size on acid protease production using *R. arrhizus* M-26 and *R. oligosporus* M-30. Bars represent the standard error among replicates which differ significantly at *p*≤0.05.

Figure 7. Effect of different age of inoculum on acid protease production using *R. arrhizus* M-26 and *R. oligosporus* M-30. Bars represent the standard error among replicates which differ significantly at *p*≤0.05.

Reference


