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Effect of Fermentation Conditions for the Production of Protease from Rice Mill Wastes Using *Aspergillus flavus*

R. Paranthaman, K. Alagusundaram and J. Indhumathi

Indian Institute of Crop Processing Technology, Thanjavur-613 005, Tamil Nadu, India

Abstract: The fungus *Aspergillus flavus* was isolated from the soil and tested for the production of Protease when grown on different varieties of Rice brokens (PONNI, IR-20, CR-1009, ADT-36 and ADT-66). Among the five substrates tested PONNI was the satisfactory one for Protease production. The optimum condition for the enzyme production was 35°C, 72 hrs of incubation and pH 7.

Key words: Protease · Rice mill wastes · Rice brokens · Purification

INTRODUCTION

Proteases, also known as proteinases or proteolytic enzymes, are a large group of enzymes. Proteases belong to the class of enzymes known as hydrolases, which catalyse the reaction of hydrolysis of various bonds with the participation of a water molecule. Filamentous fungi are used in many industrial processes for the production of enzymes and metabolites [1]. Proteases represent an important group of enzymes produced industrially and account for 60% of the worldwide sales value of the total industrial enzymes [2]. Proteases are capable of cleaving proteins into peptides and amino acids, they are characterized by their optimal pH (acid, neutral or alkaline), their temperature, their ability to hydrolyze specific proteins (collagenase, keratinase, etc...), their homology to well characterized enzymes as chymosine, chymotrypsin, pepsin and trypsin (trypsin-like, pepsin-like, etc.) and their stability. The major uses of proteases are in the biotechnological production of detergents (pepsin) [2], in dairy industries as milk-clotting agents (calf rennet composed mainly of chymosine and pepsin) [3] and as an agent for meat tenderization [4]. Proteases have also clinical and medical application (reduction of tissue inflammation) [1, 5]. Proteases of microbial origin have long been used in industry; they are replaced by fungal proteases [6,7], easily extracted and separated from mycelium [8]. *Aspergillus proteases* have been used in many fields, especially in food processing.

By-products from the growing and processing of rice create many valuable new products. Rice husks, rice stubble, rice bran, broken rice and rice straw are used as common ingredients in horticultural, livestock, industrial, household, building and food products. Unfortunately, during the rice milling process some of the rice grains break. Broken rice is the rice kernel that does not survive the milling process. They have length dimensions smaller than ¾ of the whole grains. Rice kernels can develop cracks in the field during drying or during milling, quick drying, oven drying (below moisture content levels of12%) or rehydrating oven dried kernels as the major causes for the breakage of kernels during milling. There are no reports for the production of protease from Rice brokens.

This present study was undertaken for the production of protease from Ricebrokens by *Apergillus flavus* and optimization of culture conditions for protease production in solid state fermentation.

MATERIALS AND METHODS

Substrate: The substrate Rice broken (PONNI, IR-20, CR-1009, ADT-36 and ADT-66) were obtained during milling process of rice milles in Indian Institute of Crop Processing Technology, Thanjavur.

Microorganism and Inoculum Preparation: The organism used in the present study *Aspergillus flavus* was isolated from soil. The culture was maintained on potato dextrose agar slants. The spore suspension for inoculation was prepared by adding 10 ml of sterile distilled water with 0.1% Tween 80 to each slant and dislodged using a sterile inoculation loop under aseptic conditions. The volume of 1 ml of spore suspension was used as the inoculums.

Corresponding Author: R. Paranthaman, Indian Institute of Crop Processing Technology, Thanjavur-613 005, Tamil Nadu, India

Substrates and Solid State Fermentation: Five grams of rice broken (PONNI, IR-20, CR-1009, ADT-36 and ADT-66) was taken in a 250 ml Erlenmeyer flask seperately, moistened with salt solution [composition (% w/v): ammonium nitrate 0.5, potassium dihydrogen orthophosphate 0.2, sodium chloride 0.1 and magnesium sulphate 0.1] to achieve the desired moisture content, sterilized at 121.5°C for 15 min, cooled, inoculated with 1 ml of fungal spore suspension (106 spores/ml) and incubated at 30°C for 120 h.

Extraction of Crude Enzyme: A solution of Tween-80 (0.1 %) in distilled water was added to the fermented substrate and the substrate was homogenized on a rotary shaker at 180 rpm for 1 h. The solids were removed by centrifuging the homogenate at 8000 x g at 4°C for 15 min and the resultant clear supernatant was used for analytical studies.

Purification of Protease:

Ammonium Sulfate Precipitation: The crude enzyme from the culture filtrate was precipitated with solid ammonium sulphate (80%) at 4°C for overnight. The precipitate was collected by centrifugation (8000 rpm, 20 min), dissolved in citrate buffer (0.05 M, pH 5.0) and dialyzed against the same buffer for overnight

Assay for Protease: To 200 µl of crude enzyme extract, 500 µl of casein (1 %) and 300 µl of 0.2 mol/l phosphate buffer (pH 7.0) were added. The reaction mixture was incubated at 60°C for 10 min and arrested by the addition of 1 ml of 10 % trichloroacetic acid [9]. 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 and 1 ml of 3-fold diluted Folin and Ciocalteau's phenol reagent, was added. The resulting solution was incubated at room temperature for 30 min and the absorbance of the blue color developed was read at 660 nm using a tyrosine standard [10]. 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 per gram dry fermented substrate.

Effect of Incubation Period: The inoculated medium was incubated for different incubation periods ranging from 24 h 120 h.

Effect of Incubation Temperature: The inoculated medium was incubated at different temperature (20°C, 25°C, 30°C, 35°C and 40°C) for the production of protease.

Effect of pH: Production of protease on different pH (5.0, 6.0.7.0, 8.0) were studied.

RESULTS AND DISCUSSION

The extracellular protease production by *Aspergillus flavus* on different varieties of rice broken (PONNI, IR-20, CR-1009, ADT-36 and ADT-66) was studied and the results were tabulated in Table 1. The activity was increased after dialysis. Among the all varieties the maximum protease production was found in PONNI (41 U/g) which was incubated at 35°C, 72 h and pH 7 and the minimum protease production was observed in ADT-66 (25 U/g).

Effect of Incubation Period: In our study the maximum protease production was observed in 72 h of incubation in all varieties of rice broken (Fig. 1). The similar type of results was observed by Ikram-ul-haq [11] who obtained the maximum enzyme activity after 72 h of incubation and also he found that prolonged incubation period decreased the enzyme activity. Karuna and Ayyana [12] using Rhizopus oligosporous and Ikasari and Mitchell [13] using *Aspergillus* sp. obtained the highest protease yield after 72 h of incubation. Further increase the incubation period the activity would be decrease. The incubation period is directly related with the production of enzymes and other metabolites up to a certain extent. After that, the enzyme production and growth of the microorganism decreases, this can be attributed to the reduced availability of nutrients and the production of toxic metabolites [14].

Effect of Temperature: Effect of various temperature on protease production in different varieties of rice broken was studied. It was observed that production of protease

Table 1: Protease activity on different varieties of Rice brokens

	Protease activity (unit/gm)		
Rice broken	Crude	Ammonium sulphate precipitation	Dialysis
PONNI	22	31	41
IR-20	19	27	35
CR-1009	13	20	28
ADT-36	15	26	32
ADT-66	12	20	25

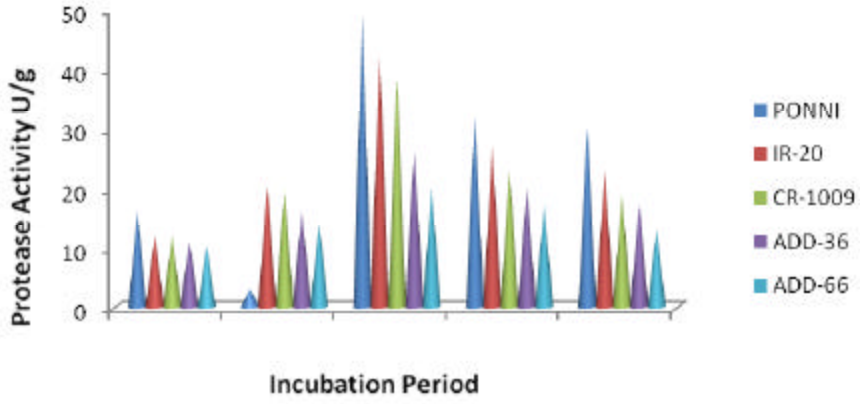

Fig. 1: Effect of incubation period for Protease production on different varieties of Rice brokens

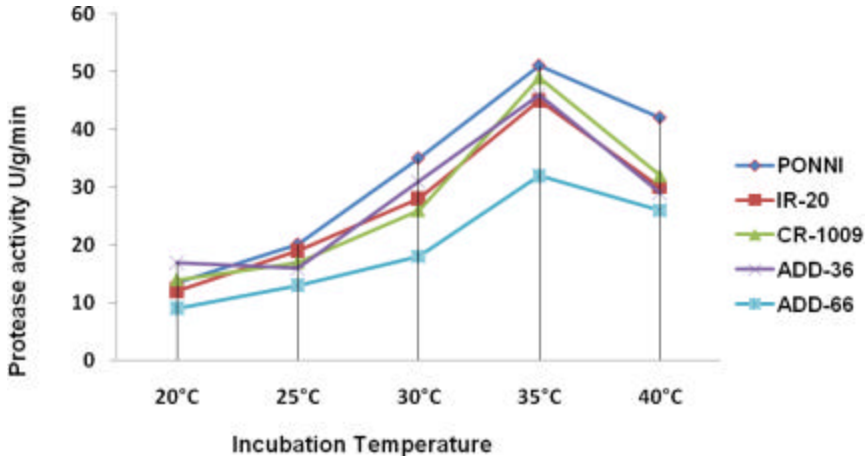

Fig. 2: Effect of Temperature for Protease production on different varieties of Rice brokens

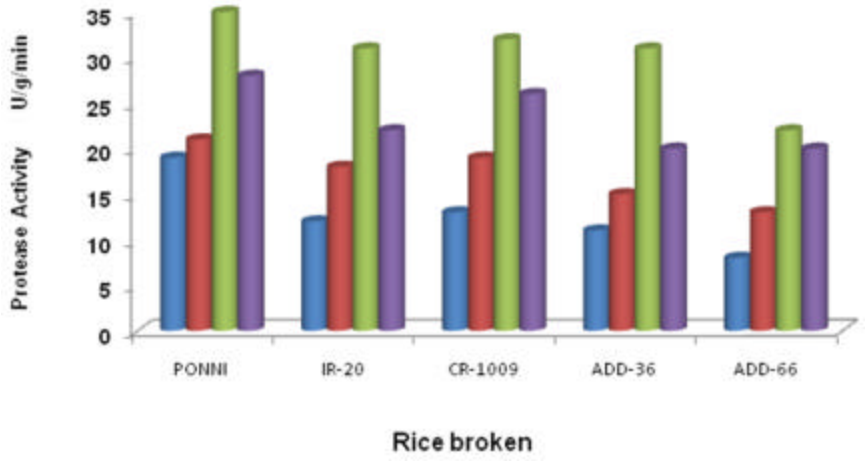

Fig-3: Effect of pH for protease production

maximum at 35°C in all varieties (Fig. 2). 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. Higher temperature is found to have some adverse effects on metabolic activities of microorganism [15] and cause inhibition of the growth of the fungus. The enzyme is denatured by losing its catalytic properties at high temperature due to stretching and breaking of weak hydrogen bonds within enzyme structure [16].

Effect of pH: Productivity of the enzyme by mould culture is very much dependant on pH of the fermentation medium [17]. In this study Production of protease was done at various pH values ranging from (5.0,6.0,7.0 and 8.0). The maximum production was found in pH.7.0 in all varieties of rice broken (Fig. 3). Sharma *et al*. [18], who have also made similar results with *P. perpurogerum*, *P. funculosum* and *Paeciliomyces varioti* isolated from deteriorated finished leather, who noted the highest yield of protease at pH 7 of fermentation medium. It is likely that changes in pH cause denaturation of enzyme resulting in the loss of catalytic activity. Therefore, each enzyme has specific pH optima for its activity.

CONCLUSION

Aspergillus flavus proved potent producer of protease by solid state fermentation on Rice brokens. The enzyme production was considerably enhanced under the set of conditions optimized in this study.

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