

Research Article

Production and Partial Characterization of Protease from *Aspergillus Flavus* using Rice Mill Waste as a Substrate and its Comparison with *Aspergillus Niger* Protease

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Abstract

Proteases are one of the most important groups of industrial enzymes and occur widely in plants and animals. In the present work, Aspergillus niger and Aspergillus flavus were used for the production of proteases by solid state fermentation using varieties of rice bran as a substrate and subjected to different physiological conditions to optimize the fermentation conditions. Purification of the enzyme was done by salt precipitation and ion exchange chromatography methods. Different detergents were used to check the compatibility of proteases for the application in industries. The compatibility of the protease from A.niger is quite high and hence can be further successfully exploited in the detergent industry.

Keywords: Protease, *Aspergillus niger*, *Aspergillus flavus*, Rice mill waste, Detergent Industry

1. Introduction

Proteases are one of the most important groups of industrial enzymes, account for nearly 60% of the total enzyme sale. They occur widely in plants and animals. Commercial proteases are produced exclusively from molds of the genera *Aspergillus*, *Penicillium* and *Rhizopus* as several species of these genera are generally regarded as safe.

Proteases are extracellular enzymes that can be produced by both submerged fermentation and solid-state fermentation. Solid-state fermentation is especially suited for the growth of fungi because of their lower moisture requirements compared with the bacteria. It is simple, low cost, and provides high yields of appropriate enzymes. The enzymes produced are more concentrated than those from submerged cultures. Problems with solid-state fermentation include incomplete utilization of the nutrients because of poor oxygen and heat transfer in the substrate. Solid-state fermentation is inexpensive and can be widely applied with agricultural residues as substrates.

Bran is the outer layer of cereal grains and contains fat, crude fibre, carbohydrate, and protein. Rice bran has moisture content in the range 7–13%, depending on the initial moisture content of the rice before milling. Rice bran proteins are richer in albumin and easier to digest than endosperm proteins in wheat bran. With a carbohydrate to protein ratio in the range of 2–3, it is potentially an excellent substrate for producing enzymes

by solid state fermentation. It contains minerals such as potassium, phosphorus and calcium in considerable amounts. So, rice bran potentially can be used as a substrate without further supplementation.

The major uses of free proteases are in dry cleaning, detergents, meat processing, cheese making, silver recovery from photographic film, production of digestive and certain medical treatments of inflammation and virulent wounds.

The present study was undertaken for the production of proteases from *Aspergillus niger* and *Aspergillus flavus* by Solid state fermentation using rice bran as substrate. Different varieties of rice bran like Sonamasoori, Bhavani, Basumati rice, Loknath and IR-64 were used to compare the activity of enzyme in both *A.niger* and *A.flavus*. Fermentation conditions were optimized with the effect of temperature, pH and incubation period for the enzyme production. Purification of the crude enzyme is attempted by Ammonium Sulphate precipitation and dialysis followed by DEAE column chromatography. The purified and characterized protease and the compatibility of the enzyme in the presence of commercial detergents were checked for its successful application in the industries.

2. Materials & methods

Substrate for fermentation

Different rice bran varieties – IR-64, IET-8580 (Basmati Rice), MTU-1010 (Bhavani), BPT-5204 (Sonamasoori)

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and Loknath were obtained during milling process from Gangavathi Rice mill, Koppal, Karnataka.

Maintenance of culture

Aspergillus niger, Strain 16404 and *Aspergillus flavus*, Strain 2254 were obtained from Institute of Basic Medical Sciences, Taramani, Chennai. The culture was maintained on Sabaraoud Dextrose agar slants at room temperature for one week.

Solid state fermentation

5 gm of each of the above said substrates were added separately to 250 ml Erlenmeyer flasks. Two flasks of each variety of rice broken were taken. To each of these flasks, 10 ml of the salt solution was added, autoclaved at 121.5°C for 15 minutes and Tween-80 solution was added. 1 ml each of the spore suspension of *A. niger* and *A. flavus* were added and incubated at 37°C for 72 hours.

Extraction of Crude enzyme

After 72 hours of incubation, 10 ml of 0.1% Tween-80 solution was added to 2 g of the fermented substrate from each flask. The substrate was homogenized on a rotary shaker for 1hr and kept for centrifugation at 8000 g for 15 min at 4°C. The resultant clear supernatant was used as the crude enzyme extract for further analysis.

Casein hydrolytic method for protease estimation

Enzymatic assay of protease was done using Casein as a Substrate and the amount of free tyrosine liberated was estimated by Lowry method.

Optimization of Fermentation Conditions

Effect of temperature

The effect of temperature on the production of the enzyme was determined by incubating the fermentation flasks at 27°C, 37°C and 55°C.

Effect of pH

The effect of pH on the production of the enzyme was determined by altering the pH of the solution used for fermentation by using 1N NaOH and 1N HCl.

Effect of incubation period

The effect of incubation period on the enzyme production was determined by extracting the crude enzyme after different incubation periods at 24, 48, 72, 96 and 120 hrs.

Ammonium Sulphate precipitation and Dialysis

Crude enzyme extract was saturated to 30% by Ammonium sulphate, stirred for an hour and was

centrifuged at 8000 rpm for 15 min. The pellet was suspended in 4ml of 25mM Tris HCl (pH 9.0) for the enzyme from *A.niger* and 4ml of 25mM phosphate buffer (pH 7.0) for the enzyme from *A.flavus*. The supernatant was saturated to 70% using ammonium sulphate and stirred for an hour and was again centrifuged at 8000 rpm for 15 min. The supernatant was discarded and the pellet was suspended and dialysed in the above said buffers.

DEAE Column Chromatography

For further purification of the dialysed sample, it was subjected to purification by DEAE Cellulose column chromatography. Tris-HCl buffer (pH 9.0) was used for the protease from *A.niger* and Tris-HCl buffer (pH 8.0) was used for the protease from *A.flavus*. 4g of DEAE Cellulose (Sigma) was suspended in 25mM buffer and kept overnight for equilibration. The column was carefully packed and equilibrated with the buffer at a constant flow rate of 30ml/hr. 3ml of the dialyzed enzyme extract was diluted to 15 ml and loaded onto the column. The flow through was collected as a single fraction and tested for protease activity. The column was washed with approximately 100 ml of the equilibration buffer. The washings were collected as 3ml fractions and tested for absorption at 280 nm. Fractions were collected till the absorption at 280nm is zero. The washings were checked for protease activity. The equilibration buffer with 500mM NaCl was used for elution. The column was eluted at a flow rate of 30 ml/hr and 3ml fractions were collected. The absorbance at 280 nm was recorded. The fractions with a high absorption at 280nm were tested for protease activity. The fractions with the highest protease activity were pooled followed by equilibration and elution.

Effect of activators and inhibitors

The enzyme assay was carried out in the presence of 5mM CaCl₂ and 5mM EDTA.

Compatibility with commercial detergents

Detergent solutions like Surf excel, Rin, Tide, Wheel and Nirma at a concentration of 7µg/ml were prepared in double distilled water. The solution were boiled for 10 min to destroy any protease already present and cooled. The enzymes were diluted 5 times using these detergent solutions and incubated at 35°C.

Enzyme Characterization

Effect of temperature

Enzyme activity was carried out at 25, 37, 45 and 60°C temperatures.

Effect of pH

The effect of pH on the enzyme activity was studied with different pH ranging from 6 to 10.

Effect of substrate concentration

The enzyme assay was carried out in the presence of casein at different concentrations ranging from 0.5-2%.

3. Results & discussion

The activities of the proteases extracted from various rice bran samples were compared. The sample having maximum protease activity of 24.75 U/ml for *A.niger* and 22 U/ml for *A. flavus* was found to be with Sonamasoori. The one with minimum activity was Bhavani for *A. niger* and Loknath for *A.flavus* with an activity of 8.25 U/ml (Fig1). This difference in the level of production of protease in different rice bran samples indicates the difference in the protein content of the samples. These values were different from the values of protease activity obtained by (R. Paranthaman. et al., 2009) who also studied the activity of the protease produced by *Aspergill*

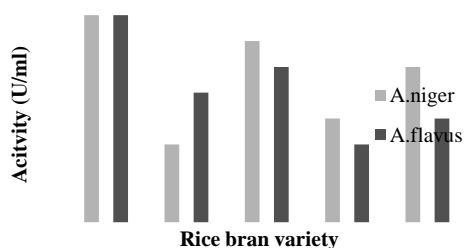


Fig.1 Activity of proteases from different rice bran varieties produced by *A.niger* & *A.flavus*

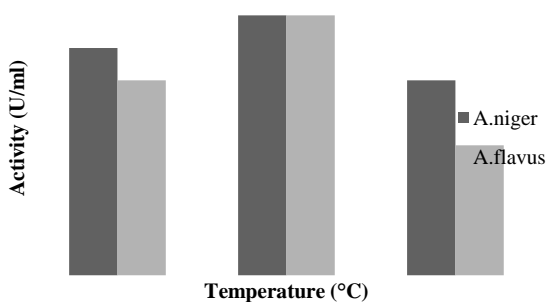


Fig.3(a) Effect of temperature on the production of protease from *A.niger* & *A.flavus*

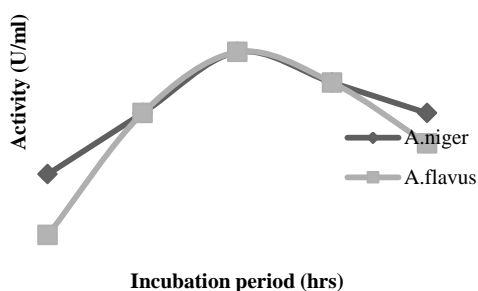


Fig.4 Effect of incubation period on the production of protease from *A.niger* & *A.flavus*

niger using rice mill waste as substrate. This difference in activities can be attributed to the difference in the variety of rice samples used. The level of protein in the samples would be quite different as the rice varieties are grown in different geographical regions.

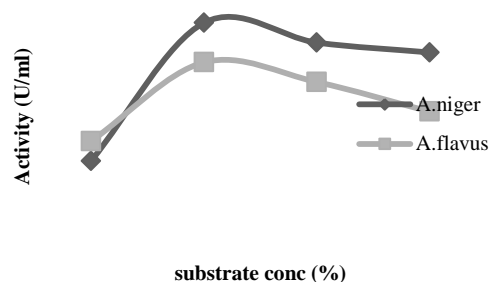


Fig.5 Effect of substrate on the production of protease

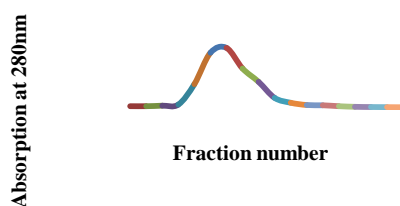


Fig.6 Absorption peak of eluted fractions from *Aspergillus niger* protease in DEAE Cellulose Chromatography

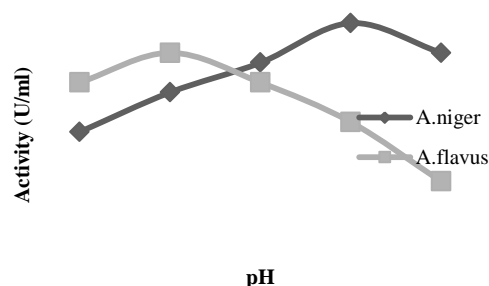


Fig.8 Effect of pH on the production of proteases

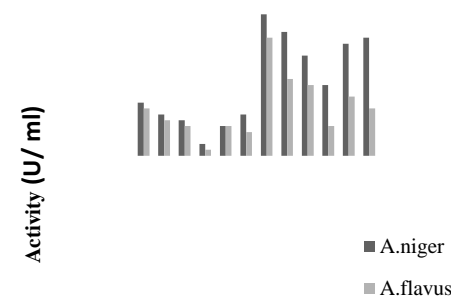


Fig. 9 Compatibility of crude and dialyzed protease extracts with commercial detergents

The conditions for the fermentation were optimized and were used for mass production of the proteases required

for the purification steps.

Effect of pH

Maximum protease production of 24.75 U/ml was seen at pH 9.0 for *A.niger* and 22.0 U/ml at pH 7.0 for *A.flavus* (Fig 2). This is in accordance with the optimum pH 7.0 reported for *A.flavus* by (I. I. Sutar et al., 1992). The results clearly indicated alkalinophilic nature of *A.niger*.

Effect of temperature

Protease production increased with increase in temperature from 27°C to 37°C. Maximum production of protease (22.0 U/ml) was obtained at 37°C (Fig 3a). Growth and protease production ceased at higher temperature (55°C) (Fig 3b). Similar observations were shown by (S. Morimura et al., 1994) for *A. usami*. It was revealed that environmental temperature not only affects growth rates of organism but also exhibit marked influence on the levels of protease production.

Effect of incubation period

Result of this study showed that protease production increased with incubation period. Maximum enzyme production was observed at 72 h of incubation (22.0 U/ml) for both the fungi (fig 4). 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 fungi for utilization of proteins present in the solid substrate. The subsequent decrease in the enzyme units could probably be due to inactivation of the enzyme by other constituent proteases. These results are in accordance with observations made by (D. R. Durham et al.1987), (A. Gessesse et al., 1997), (K. H. Yeoman et. al 1994) and (C. Edward et al 1994).

Purification of Protease enzymes

The purification of the proteases from *A.niger* and *A.flavus* by Ammonium sulphate precipitation and dialysis resulted in 11 and 9 fold purification respectively. The proteases get precipitated by ammonium sulphate at 70% saturation. The activity of the partially purified enzymes is determined to be 66.0 U/ml and 55.0 U/ml (Fig 5) for the enzymes from *A.niger* and *A.flavus* respectively. This increase in enzyme activity is attributed to the removal of other unwanted proteins by partial purification. Further purification by DEAE column chromatography resulted in 26 and 23 folds increase (Fig 6 &7) in purity respectively and it was in accordance with (M. Kalpana Devi et al.2008). CaCl₂ caused an increase of activity by about 11% in both the crude extracts and about 7-8% in the dialyzed samples. EDTA was observed to be an effective inhibitor, causing a considerable decrease in protease activity. There is a decrease of 85-92% in the activities of both the proteases in the presence of EDTA. This clearly shows that the enzymes are inhibited by the chelating

nature of EDTA, which inhibits metal ion activity which is in accordance with (M. Madan et al., 2002) and (M. Kalpana Devi et al., 2008).

The crude enzyme extract from both the fungi retains about 75-50% activity in most of the detergents except Nirma, in which only 12-22% activity is retained. This shows that the enzymes are stable in the presence of detergents. Surf excel is the detergent in which maximum protease activity is retained. In the dialyzed enzyme extracts, the activity retained is higher than in the crude enzyme extracts. This implies that the partially purified proteases are more stable in the presence of common commercial detergents and can hence be used more effectively in the detergent industry. Again, the activity retained in the presence of Surf excel is high (65-85%) and in the presence of Nirma is much lower (25-20%) (Fig 9).

4. Conclusion

The activity of the protease produced from the rice bran of Sona masoori rice was found to be the highest. Hence, the rice mill wastes from this rice sample can be used as a cheap substrate for the large scale production of proteases from *A.niger* and *A.flavus*. The activity of the protease produced from *A.niger* was found to be higher than that produced by *A. flavus*. Thus, this microorganism will prove to be more useful than the other for industrial enzyme productions. The compatibility of the protease from *A.niger* is quite high after partial purification and can therefore be successfully exploited in the detergent industry. The partially purified enzyme can be immobilized and added to detergents for breaking down protein based stains. The protease from *A.flavus* is also stable to some extent in the detergent solution.

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