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Protease Production by *Fusarium oxysporum* in Solid-State Fermentation Using Rice Bran

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Abstract Agroindustrial wastes constitute valuable source for microbial cultivation and enzyme production. An attempt was made to study protease production by *Fusarium oxysporum* on agroindustrial waste rice bran under solid-state fermentation. Rice bran obtained from rice mill has proved to be efficient substrate for enzyme production by *F. oxysporum*. To study the maximum enzyme production the process parameters of fermentation where optimized. Maximum protease production i.e. (70.5U/g) was obtained with an initial moisture content of 50% (w/w) on incubation period of 72h at 35℃ temperature with initial pH of substrate 7.0. The above data revealed that agroindustrial waste rice bran and optimized condition of solid-state fermentation can be successfully employed for protease production by *Fusarium oxysporum*.

Keywords: *agroindustrial waste, protease, Fusarium oxysporum, solid -state fermentation*

1. Introduction

Protease is one of the most important commercial enzymes used in food processing, cheese making, detergents, dairy industry and leather making [12]. The protease constitute two third of total enzymes used in various industries [8]. Protease occurs widely in plant and animals, but commercial proteases are produced exclusively from microorganisms. Molds of the genera *Aspergillus, Penicillium, Rhizopus and Fusarium oxysporum* are especially used for production of protease [15]. Protease is extracellular enzyme that can be produced by both submerged and solid-state fermentation. Solid-state fermentation is especially suited for the growth of fungi and it is simple, low cost, and provides high yields of appropriate enzymes [16]. Solid-state fermentation can use inexpensive and widely available agricultural residues as substrates. Agroindustrial wastes constitute spent grain, cocoa husk, rice bran, wheat bran, chickpea bran, sawdust and other agricultural waste Among various solid substrates reported, commercial wheat bran has been found to be a suitable substrate for producing mold proteases by solid-state fermentation but wheat bran is not easily available in many regions. In many Asian countries rice bran is less expensive than wheat bran [15]. Rice bran is a byproduct of the milling of rice and is a good source of proteins. Utilization of agroindustrial wastes clean environment and increase the economical value of agroindustrial waste. The present study was undertaken to produce protease on rice bran by using *F. oxysporum* in solid-state fermentation and to determine the effect of moisture, pH, temperature and incubation period on protease production.

2. Materials and Methods

2.1. Microorganism and Preparation of Inoculums

The organism used in present study was *F.oxysporum* isolated from diseased seed of groundnut by standard blotter method [4]. The sample of groundnut seeds were placed on 3 layered moistened blotter discs in 9 cm glass petriplate @ four seeds per plate and incubated for 7 days at 22 ± ℃ temperature in incubator. Cultural characters were assessed by eye slight and by microscopic examination. Colony morphology was recorded from cultures grown on potato dextrose agar. The morphology of macro and microconidia was studied by using the criteria of Alexopoulos [3] and Gerlach and Nirenberg [7]. The inoculum was prepared by dispersing the spores from a week-old fungal slant culture in 0.1% Tween -80 solution with a sterile inoculation loop under aseptic condition.

2.2. Substrate Preparation

The rice bran was obtained from rice mill of Parbhani (India). Rice bran was cleaned, and washed and dried. It was milled with an electric wearing blender and sieved through 20-40mm mesh sieve. The powder with 0.42 and 0.85mm mesh particle size were collected and used as solid substrate for solid-state fermentation.

2.3. Preparation of Fermentation Media

Ten gram of rice bran powder with 0.42 and 0.85mm mesh particle size was supplemented with 0.166g K_2HPO_4 and 0.032g MgSO4 in 250mL Erlenmeyer flasks and

moistened with 5ml of distilled water. After through mixing with glass rod the moist media was autoclaved for 20min at 121 ℃.

2.4. Fermentation Process and Extraction of Crud Enzyme

After cooling to room temperature the moist fermentation media was inoculated with 2ml of spore suspension (10^{12} spore/ml) and mixed carefully with sterile glass rod under strictly aseptic conditions to achieve a uniform distribution of inoculums throughout the solid medium. The inoculated flask having initial moisture content of 50% (w/w) was incubated at 30 ℃ for 7 days of incubation. After every 12h of interval 1 g substrate was harvested from flask and transferred to a test tube containing 5ml phosphate buffer (pH 7.4). The content was homogenized and centrifuges at 2000rpm for 30min to remove all particulate matter. The cultural filtrate was filtered through Whatman filter No. 1 paper and used for enzyme assay.

2.5. Assay for Protease

The activity of protease was assayed as suggested by Agrawal *et al*. [1]. To 1ml of supernatant 5ml solution of 2% casein dissolved in 0.5mL^{-1} carbonate buffer (pH 10) were mixed. The obtained solution was incubated at 40 ℃ on a rotator shaker at 300rpm for 30min. A 0.5ml of the reaction was withdrawn and the reaction was terminated by adding 1.5ml of pre-chilled trichloroacetic acid (10%). The mixture tube was then immersed in an ice bath for five minute to precipitate all the protein. Precipitate was recovered by centrifugation of mixture at 10,000rpm for 10min. Tyrosine liberated during casein hydrolysis was measured in the supernatant using the method of Lowry *et al.* [11]. A unit of protease activity was defined as the amount of enzyme liberating 1µg tyrosine min^{-1} at the incubation temperature of 40 ℃. The protease activity is reported per g of dry solids used in initial extraction.

2.6. Effect of Incubation Period

The effect of incubation period on protease was determined by incubating fermentation medium for different incubation periods in hrs. i.e. 24,48,72,96, 120, 144 and 168h at 35 ℃ with initial substrate pH 7.0.

2.7. Effect of Initial Moisture Content

Initial moisture of substrate plays important role in mold growth and enzyme production in solid-state fermentation. To determine the effect of moisture, substrate was moistened at 40, 45, 50, 55, 60, and 65% with distilled water. The fermentation was carried out for 120h at 35 ℃.

2.8. Effect of Incubation Temperature

The inoculated substrate was incubated at different temperatures i.e. 25, 30, 35, 40, 45, and 50 ℃ to observe the effect of temperature, by keeping all other conditions at their optimum level.

2.9. Effect of Initial pH

In general practice, the pH of solid-state fermentation is almost never controlled during fermentation, only the initial pH of the substrate was adjusted before inoculation. To evaluate production of protease initial pH of substrate was adjusted at 5, 5.5, 6, 6.5, 7 and 7.5 pH with 1N HCl or 1N NaOH. The fermentation was carried out at 35 ℃ to study their effect on enzyme production.

3. Result and Discussion

3.1. Effect of Incubation Time

Protease production by *F. oxysporum* in solid-state fermentation was studied over a 7- day of incubation period. It is notable that in other studies, enzyme production was studied over an incubation time of 48h for bacteria and 8-9 days for fungi [10]**.** The study of protease production versus incubation time showed that protease production increased with incubation time. Maximum enzyme production was observed at 72h of incubation (70.5U/g) shown in Figure 1. Similar finding were reported by Paranthaman *et al*. [14]. 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 log phase of the growth of the fungi for utilization of nutrients i.e. protein present in the solid substrate[2]. The subsequent decrease in the enzyme unit could probably be due to inactivation of the enzyme by synthesis of other metabolites in the medium. These results are in accordance with observation made by Yeoman and Edwards [17].

3.2. Effect of Initial Moisture Level

Initial moisture content of solid substrate is important parameter for mold growth and enzyme production in solid-state fermentation [5]. Water play important role in physico-chemical properties of the substrate, which affect enzyme production [13]. Increase in moisture level is believed to reduce the porosity of the rice bran, thus limiting oxygen transfer. While lower moisture content causes reduction in the solubility of nutrients of the substrate and lower degree of swelling. Therefore the amount of protease production varies as per the initial moisture content of substrate. The effect of initial moisture on protease production is shown in Figure 1 was maximum (70.4U/g) at initial moisture level of 50% (w/w). Similar finding were reported by Jarun *et al*. [9] in the case of *Aspergillus oryzae*. Substrate moistened at 50% (w/w) level shown maximum protease activity while moisture level above 50% decreased enzyme production as the substrate become less pores and adversely affect on oxygen supply to the mold [5,15].

3.3. Effect of Incubation Temperature

The effect of different incubation temperature on enzyme production is shown in Figure 1. The optimum temperature for maximum protease production was found at 35 ℃ which was also optimum temperature for *A. niger [14]*. A decrease or increase in incubation temperature reduced the productivity of enzymes. Temperature is an essential factor affecting solid-state fermentation performance because of its importance in microorganisms' growth and metabolite production [13].

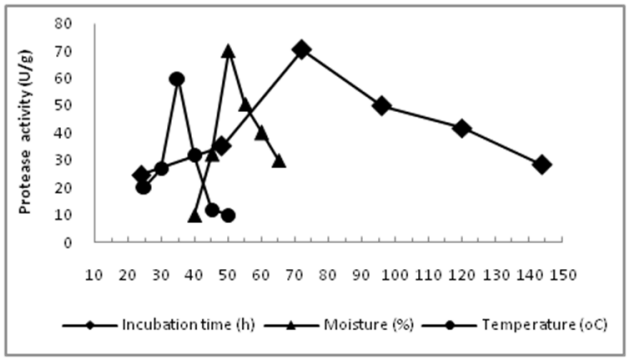

Figure 1. Effect of incubation time, moisture, temperature on protease production by *F.oxysporum* on rice bran in SSF

3.4. Effect of Initial pH

The pH in solid-state fermentation is almost never controlled during fermentation; only the initial pH of the substrate is adjusted before incubation. Protease production by microorganism depends on the extracellular pH because culture pH strongly influences many enzymatic processes and transport of various components across the cell membranes, which in turn support the cell growth and product production [6]**.** Optimum pH for protease production by *F. oxysporum* was recorded at 7.0 as shown in Figure 2. Similar finding were also reported by Paranthaman *et al*. [14].

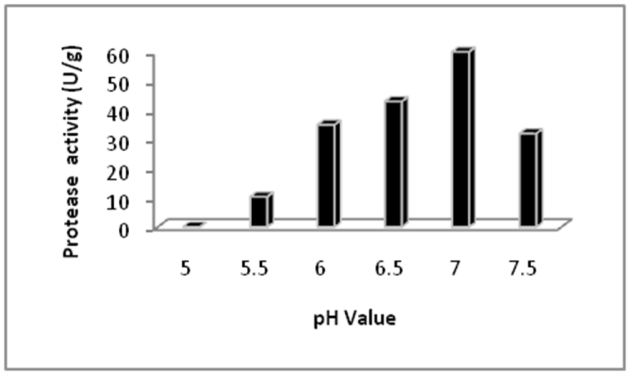

Figure 2. The effect of Initial pH on protease production by *F. oxysporum* on rice bran in SSF

4. Conclusion

The utilization of agroindustrial waste is important factor for cleaning environment and reducing environmental pollution. There are several reports on use of agroindustrial wastes for energy production, biofuel and renewable organic matter. The purpose of present study was to increase the protease production and reduce the cost of enzyme production process. Considering the economic value of other process and substrate, utilization of rice bran as a substrate proved economical. The results of above study revealed that rice bran can be successfully utilized as substrate for protease production by *Fusarium oxysporum*. Maximum protease production can be achieved at 35 ℃ on incubation period of 72h at initial pH 7.0 of substrate.

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