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High yield production of industrially important multi enzymes by *Aspergillus foetidus* using solid state fermentation

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Abstract

Isolation and screening of *Aspergillus foetidus* strains for the biosynthesis of high yield multi enzymes such as amylase, protease, xylanase and pectinase was conducted using solid state fermentation wherein agro-waste rice and wheat bran was used as a fermentation substrate. Different fermentation parameters were evaluated for maximum enzymes production. The results revealed that highly significant enzyme secretion was observed in rice bran substrate with optimum temperature 28 °C, pH 4-6, 40% moisture content, 0.2ml inoculum size (24 million spores/gram) and 48 hrs. of incubation period. Under these fermentation parameters maximum enzyme activity for amylase (9,145 U/g), protease (2,013 U/g), xylanase (14,641U/g) and pectinase (390 U/g) was achieved. The present study is successful in isolating a high yield multi enzyme producing *A. foetidus* strain and standardizing SSF parameters, which can be exploited industrially to achieve maximum enzyme yield.

Key words : Rice bran, Wheat bran, Solid state fermentation, Aspergillus foetidus.

INTRODUCTION

Enzymes are protein catalyst that enhances the rate of biochemical reactions. The use of microorganisms as a source of industrially important enzymes is increasing due to wider application of microbial enzymes in various industrial processes and also ease of fermentation processes [1-3].

The Solid State Fermentation (SSF) has gained a renewed interest in recent years for the production of many enzymes. Industrially important enzymes have traditionally been obtained from SSF because of the ease of handling and greater control of environmental factors such as temperature and pH. However, SSF technique is also proved to improve the yield and reduces the cost of enzyme production [4-5]. Filamentous fungi are the most commonly used microorganisms in SSF because they are able to grow on solid materials with low water contents [6]. SSF is particularly advantageous for enzyme production, since it simulates the natural habitat of the microorganisms. From the environmental point of view, the main benefit of SSF is the ability to use agro industrial waste (sugarcane bagasse, wheat bran, rice bran, soybean meal, etc.) as a solid substrate that acts as a source of both carbon and energy [7].

Value addition to agro waste using SSF is the best biological and ecofriendly method to reduce the waste problem than unscientific waste management strategies generally employed in the agricultural fields leading to environmental pollution, transmission of the pathogens and also reduction in the soil fertility. Hence in this study rice and wheat bran, which is easily and cheaply available in the study area, are used as a SSF substrate to produce industrially important enzymes.

Amylase [8-9], xylanase[10], protease [11-12], pectinase [13-14] are proved to be industrially important and screening for efficient strain with high yield producing ability is still in progress [15].

Considering these, the present study is aimed at isolation of industrially important high yield multi-enzymes producing *Aspergillus* sp. from different sources such as decaying wood, different soil samples, agricultural wastes and other selected polluted zones. The isolated strains were further screened for the biosynthesis of multi enzymes on rice and wheat bran. Different fermentation parameters were also evaluated to achieve maximum enzyme production.

MATERIALS AND METHOD

Isolation and screening of *Aspergillus* species

Large number of samples were aseptically collected from different sources like soil, air, food samples, water and agro wastes in and around Mysore, Srirangapatna and Pandavapura town. Serially diluted sample prepared from the different sources were spread on surface of Czapeck dox agar media (Hi Media) and incubated for seven days at 28 ºC. After incubation colonies were sub-cultured to obtain the pure culture. Stock cultures were maintained on the same media at 4 ºC for further evaluation.

SSF for enzyme production

The fermentation medium was prepared by adding 30g of rice bran and 30g of wheat bran in two separate 250ml Erlenmeyer flasks and then mixed with 16 ml of acetate buffer (pH 4.2) and 2 ml of mineral solution ($FeSO_4$, $CuSO_4$ and $ZnSO_4$) to achieve 40% moisture. After sterilization by autoclaving at 121 °C for 30 min, the medium in the flask was cooled, inoculated with 0.2 ml spores suspension (24 million spore /gram) and then incubated at 28 °C for 48 hr.

This experiment was also carried out in different fermentation parameters such as different temperature i.e., 25°C, 26°C, 27°C, 28°C, 29°C and 30°C, different pH- 2, 4, 6, 8 ,9 ,10 and 12, with different moisture content i.e., 10%, 20%, 30%, 40%, 50% and 60% and with different volume of inoculum i.e., 0.05ml (5 million spore/gram), 0.1ml (11 million spore/gram), 0.2ml (24 million spore/gram) and 0.3ml (39 million spore/gram).

Crude enzyme extraction

After 48 hr. incubation the SSF samples were transferred to drier at 50 °C to attain 10% moisture content and then it was powdered. One gram of powdered sample was soaked in 30 ml water and kept in sonicator for 30 min. The enzyme extract was

Table 1: Screening of various samples for isolation of Aspergillus species

Source	Number of Samples Tested	No. of *Aspergillus* strains isolated
Food Samples (Onion, Rice, coconut , vegetables, garlic , Potato, pickle , spoiled fruits and peanuts)	18	12
Agro wastes (Paddy straw, sugarcane wastes)	32	28
Soil	47	43
Air and water	38	19
Total	**135**	**102**

filtered by a Whatman #1 analytical filter paper and the suitable dilution was prepared for further analysis.

Quantification of enzymes

Assay for Xylanase

The xylanase activity was determined using beeach wood xylan as substrate. The reducing sugars produced were quantified by the dinitrosalicylic acid method using D-xylose as standard. In brief, the reaction containing 0.05 ml of appropriate enzyme solution and 1 ml of 1% (w/v) beeach wood xylan (pH 4.2 acetate buffer) was incubated in a water bath at 50 °C for 1 hr, then enzyme was quantified by adding 3 ml of dinitrosalicylic acid solution and kept for incubation in a boiling bath for 5 min [15]. The liberated reducing sugars were measured at 540nm in spectrophotometer. One unit (U) of xylanase activity was defined as the amount of enzyme that released 1 µmol of xylose per minute under the assay conditions. Xylanase production of fermentation was expressed as U/g dry substrate.

Assay for Protease

The activity of protease was assayed by the method of McDonald and Chen [16] using casein as a substrate. To 1 ml of the enzyme extract in the test tube, 2.0 ml of 1.0% casein was added. The enzyme sample was incubated at 37 °C for 20 min. The residual protein was precipitated by adding 3ml of 10% trichloroacetic acid. The precipitate was allowed to settle for 10 min and then centrifuged at 5000 rpm for 5 minutes. One milliliter of supernatant was mixed with 5 ml of 1M sodium carbonate. After 10 min, 1ml of 1:1 Folin and Ciocaltaue reagent was added. The optical density of the mixture was read at 620 nm in spectrophotometer.

Assay for Amylase

Amylase activity was determined using the modified method of Xiao *et al*[17]. The reaction mixtures consisted of 0.5ml of 0.1% (w/v) starch (Sigma) in 0.1M acetate buffer, (pH 5.0) as substrate and 0.1ml of enzyme extract. 0.5ml of the prepared substrate served as control. The contents of both experimental and control tubes were incubated at 60 °C for 10 mins. The reactions were terminated with 1 ml of 1N HCl, later 0.1ml of iodine solution and 22.9 ml of distilled water was added to all the test tubes. Optical density was measured spectrophotometrically at 620nm. Enzyme activity was defined in units. One unit of enzyme activity was defined as the amount of enzyme which produced 0.1% reduction in the blue colour of the starch-iodine complex.

Figure 2: Selected *Aspergillus* species

Isolates name	Number of *Aspergillus* species
Aspergillus niger	16
Aspergillus foetidus	9
Aspergillus tubingensis	8
Aspergillus oryzae	5
Total	**38**

Assay for Pectinase

Pectinase was measured as follows: 2ml of 1% pectin solution, 1 ml distilled water, 1 ml acetate buffer (0.05 M, pH 4.0) and 0.1ml of enzyme extract and then contents were incubated at 50 °C in water bath for 20 minutes. After incubation 1ml of enzyme mixture was transferred in to another sets of test tubes containing 3ml of DNS and boiled for 5min. The increase in reducing sugar was estimated by Stiles *et al.,* [18] method. One unit of pectinase is defined as 1µ mol reducing sugar liberated per minute under assay condition.

RESULT

Isolation and identification of *Aspergillus* species

More than 102 strains of *Aspergillus* were isolated (Table. 1), *A. niger, A. foetidus, A. tubingensis* and *A. oryzae* were identified (Table. 2) based on morphological characteristics such as color of the colony and growth pattern studies, as well as their vegetative and reproductive structures observed under the microscope (Figure. 1 & Figure. 2). In the present study, out of 38 strains, only *A. foetidus* was selected for further studies due to its ability to produce highly significant amount of industrial grade multi enzymes on the selected substrate.

All the experiments were carried out in triplicate and the results were analyzed statistically. The enzyme production by *A. foetidus* is remarkably influenced by conditions like substrate, inoculum size, pH, temperature, incubation period and successfully achieved production of the high yield multi enzyme and the results observed was as follows.

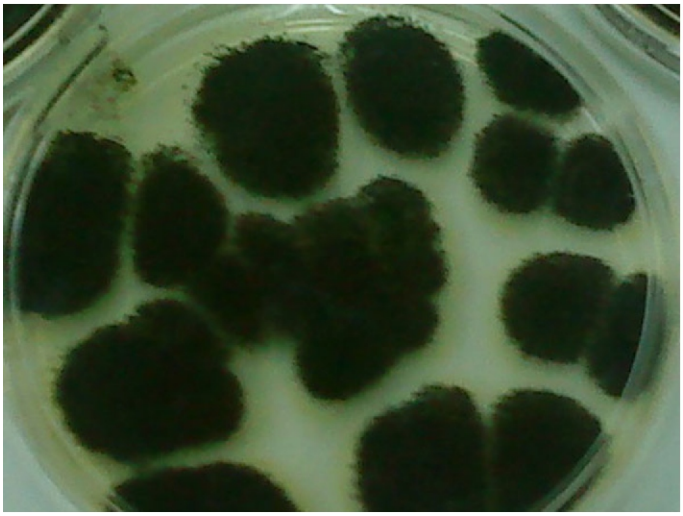

Figure 1: Colony of the *Aspergillus foetidus*

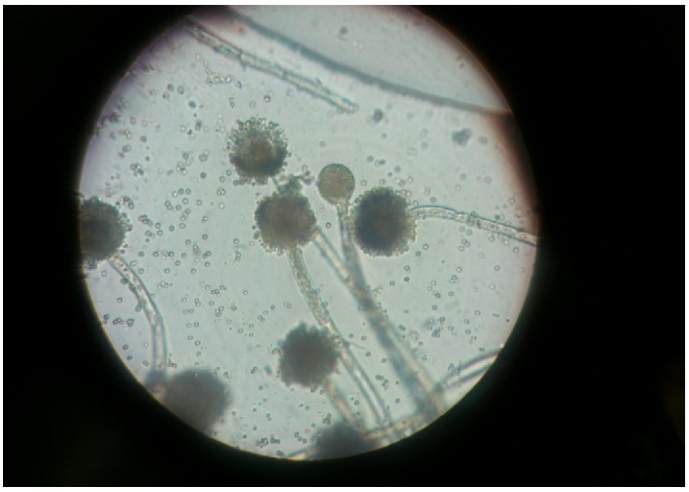

Figure 2: Microscopic observation of *Aspergillus foetidus*

Effect of substrate

Substrate played a very important role in enzyme production. Isolated strains showed maximum production of xylanase (14, 841U/g) and amylase (390 U/g) on rice bran whereas maximum production of amylase (12,000 U/g) and xylanase (9,100 U/g) was observed on wheat bran as a substrate (Figure. 3).

Effect of inoculum size

Among the different inoculum size tested for its efficiency to produce more amount of multi enzymes, 0.2 ml of inoculums with 24 million spore/gram recorded maximum production of all the enzymes on both the substrates. It was observed that enzyme production gradually increased with increase in the inoculum load. But amount of enzyme production with the same inoculum size varied among the substrates used in the present study. There is significant production of all the enzymes using rice bran than the wheat bran. It is interesting to note that further increase in inoculums size revealed the decreased production of all the enzymes on both the substrates **(**Figure. 4).

Effect of incubation period

Studies on the effect of incubation period on enzyme production revealed varied production of enzymes with different incubation periods. It was observed that at 48hr. incubation, there is significantly high production of all the enzymes on both the substrates. The production was drastically decreased with increasing hours of incubation at 72 and 96 hr. Among the different enzymes quantified, rice bran served as a good substrate for production of xylanase **(**Figure. 5).

Effect of temperature

The isolated strain was grown at various temperatures revealed that optimum temperature with high yield of multi enzyme production was found at 28 °C on both the substrates, **(**Figure. 6).

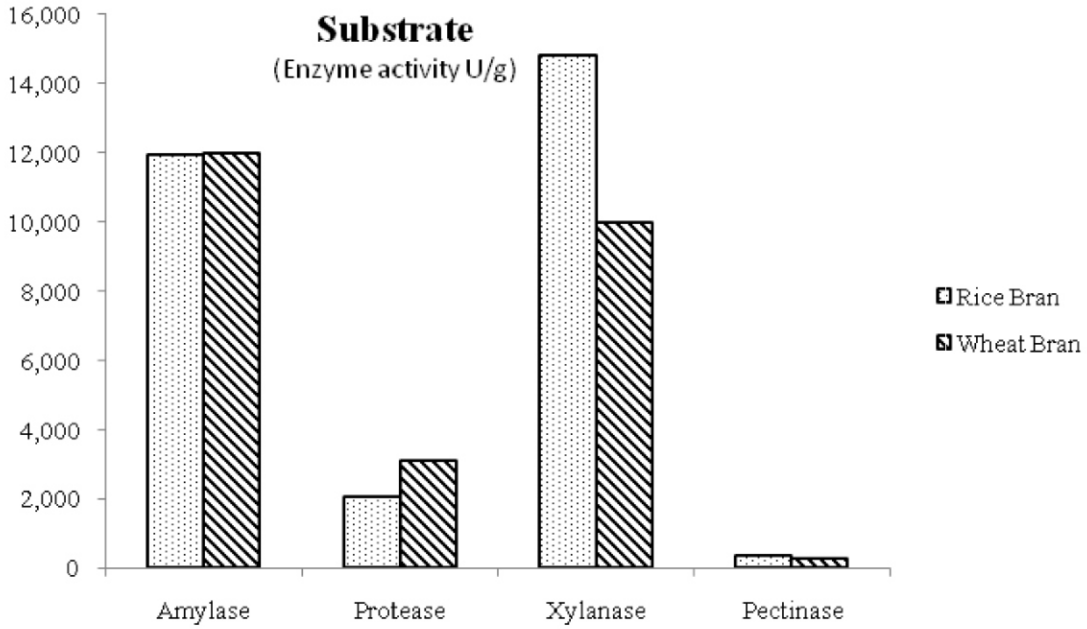

Figure 3: Effect of substrate for maximum enzyme production

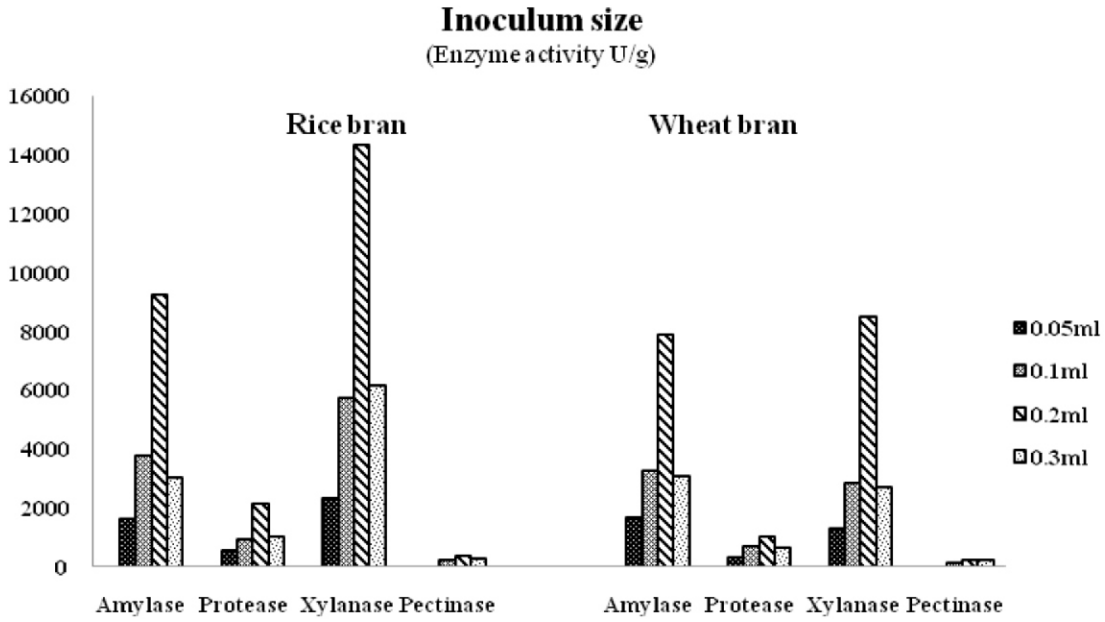

Figure 4: Effect of Inoculum size maximum enzyme production

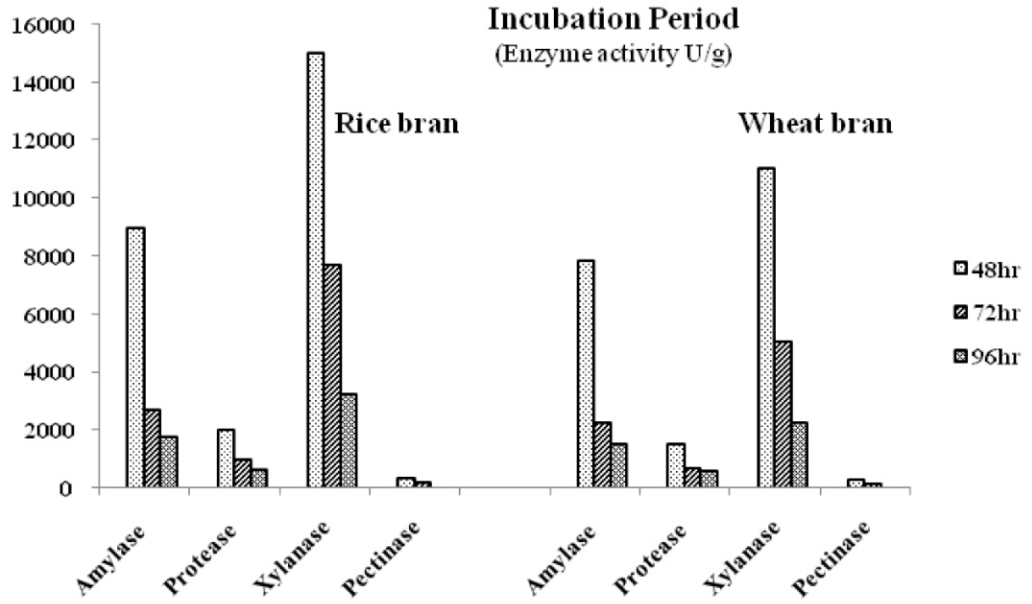

Figure 5: Effect of Incubation Period for Maximum Growth and Enzyme production

Effect of pH

SSF tested at different pH of the media revealed that pH 6 is optimum to promote more production of all the enzymes except protease and xylanase on rice bran. For significant production of these enzymes pH 4 was found to be optimum. Even in this variable also xylanase production was highly significant on rice bran compared to all other enzymes (Figure. 7).

Effect of moisture content

Production of enzymes was found to vary significantly with variation in moisture content. Highest production of all the enzymes were observed at 40% moisture content on both the substrates except for amylase, which was found significantly produced in higher amount at 50% moisture content than at other percentages. The amount of enzymes produced was found to follow the same result as with other parameters (Figure. 8).

Average value of enzyme activity with both the substrate

Based on average value of enzyme activity with different parameters it is inferred that maximum enzyme activity was obtained with rice bran than compared to wheat bran (Figure. 9). The present study revealed that, maximum enzyme production obtained with rice bran than compared to wheat bran at optimum conditions such as temperature 28 °C, pH 4-6, incubation period 48hrs, inoculum size 0.2 ml (24 million spore/gram) and moisture

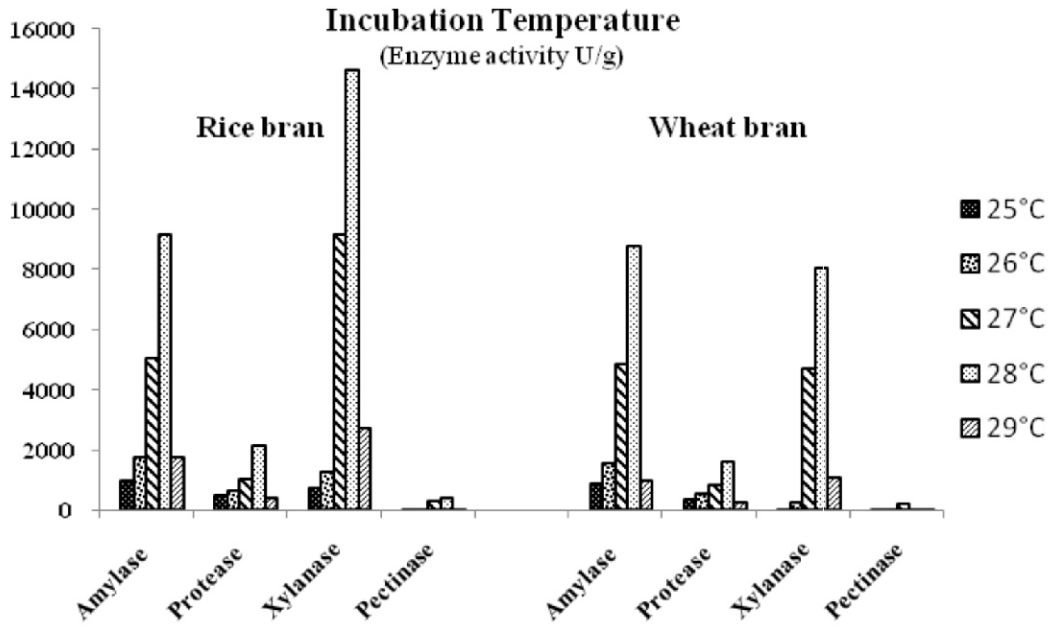

Figure 6: Effect of Temperature for Maximum Growth and Enzyme production

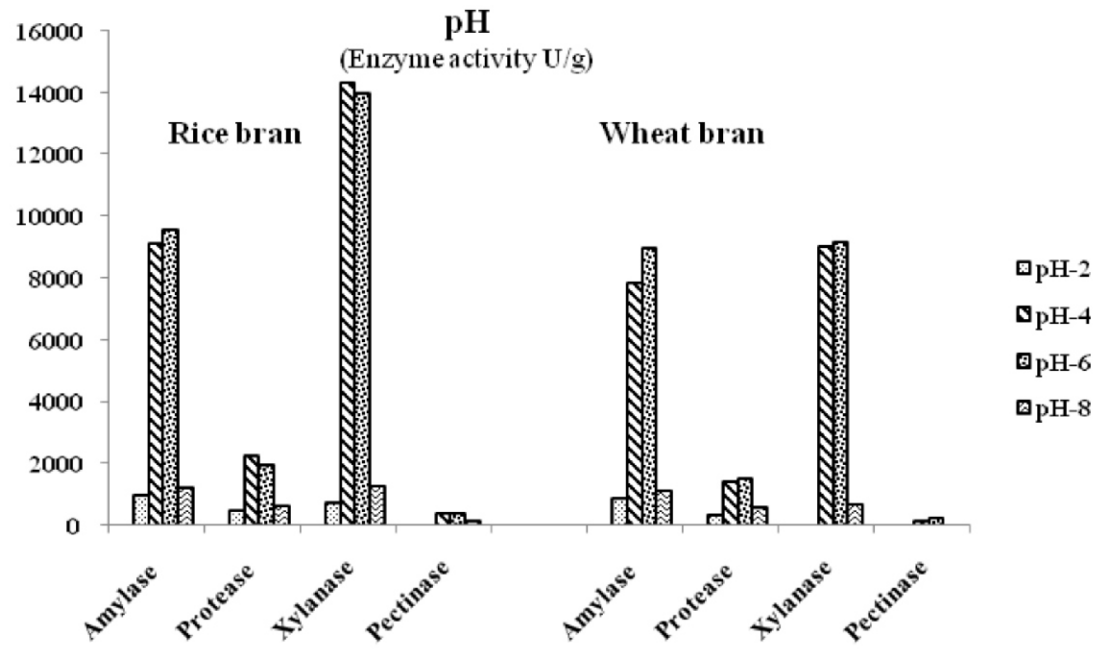

Figure 7: Effect of pH for Maximum Growth and Enzyme production

content 40%.

DISCUSSION

The demand for industrially important enzymes particularly of microbial origin is increasing owing to their low production cost. Since the diversity of microbial enzymes is great, they present a wide spectrum of characteristics that make them utilizable for quite specific applications. A number of microbial enzymes like protease, amylase, xylanase, cellulase, tannase, lipase etc. are being used in the field of food, agriculture, pharmaceuticals, cosmetics, and other biotech industries in our country and also abroad [19]. These enzymes have wider applications even in bioleaching of paper pulp, bread making, improvement of animal feed, application in solid waste treatment, preparation of juice from fruits or vegetables; to improve retting of flax fibers and production of biofuels [20].

Due to wider application of these enzymes, several works on different industrially important enzymes have already been reported but still research is going on for the screening and identification of efficient strains. The present study revealed that *A. foetidus* has the ability to produce industrially important high yield multi enzymes such as amylase, protease, xylanase and pectinase.

The selection of a substrate for enzyme production in a SSF process depends upon several factors, mainly related with cost

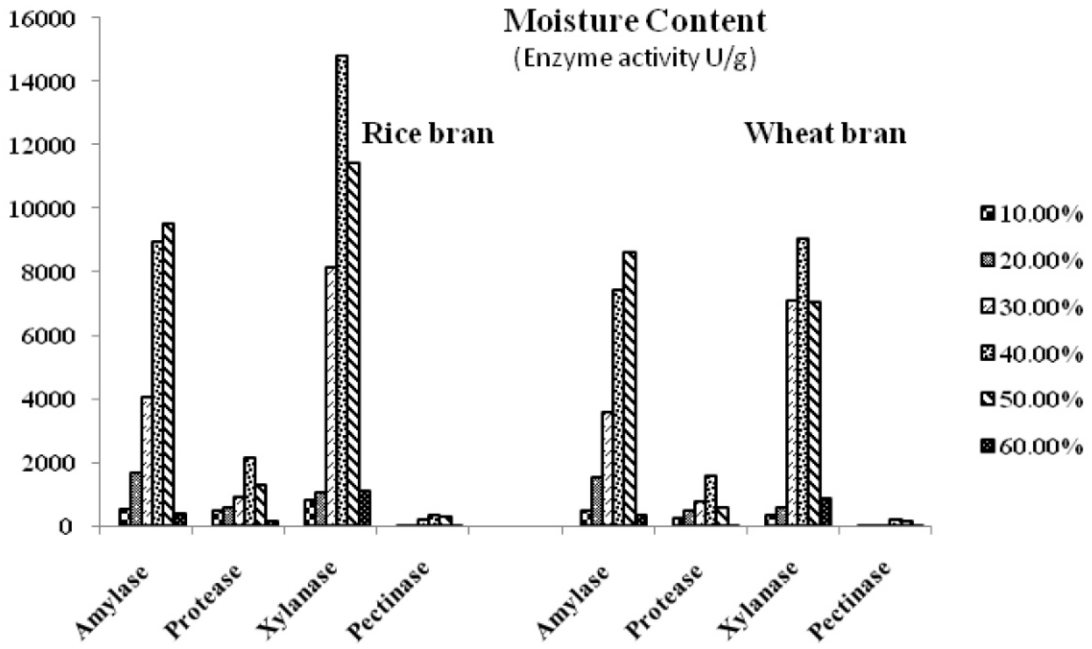

Figure 8 : Effect of Moisture content for Maximum Growth and Enzyme production

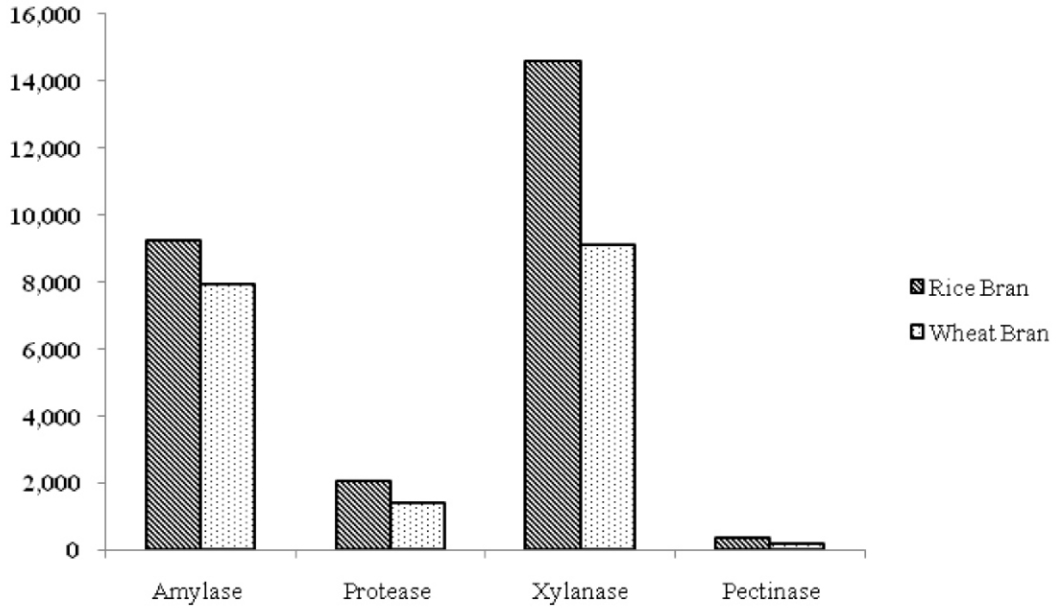

Figure 9 : Average value of enzyme activity with rice bran and wheat bran

and availability of the substrate, and thus may involve screening of several agro-industrial residues [21]. In a SSF process, the solid substrate not only supplies the nutrients to the microbial culture growing in it but also serves as an anchorage for the cells.

Agricultural wastes for example wheat and rice bran, sugar cane bagasse, corn cobs, citrus and mango peels are one of the major pollutants in the environment, their biological conversion could serve as a remedy for environmental problems as well as a source of suitable microbial byproducts like food, fuel and chemicals [22]. Considering these along with its easy availability in the study area, rice and wheat bran is selected as a substrate for the present study. It is a fact that during the processing of whole rice, grain's outer layers is accumulated in large quantity which will impart nutritional value to the rice bran and hence can be used in food industry and human [23]. Wheat, which is another major cereal grain all over the world, and the wheat-based industry, is a multi-billion dollar market. The milling process of wheat produces large amount of wheat bran as a by-product [24] and hence wheat bran was used in the present study.

CONCLUSION

*High yield production of industrially important multi enzymes was successfully achieved by isolated strain of A. foetidus. M*aximum enzyme production was observed at optimum

temperature 28 °C, pH 4-6, moisture content 40%, incubation period 48 hrs and inoculums size 24 million spores/gram (0.2ml) with rice bran as substrate. The present study is successful in isolating indust,rially important high yield multi enzyme producing strain of *Aspergillus* sp. from different sources including waste. These strains can be exploited further for strain improvement studies and finally can be employed for other industrial applications. The strain can also be employed in addressing the agricultural waste conversion into animal feed.

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