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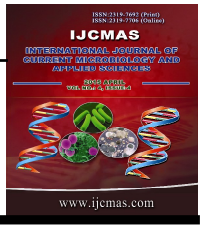

Original Research Article

Cellulase production by *Aspergillus niger* on different natural lignocellulosic substrates

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A B S T R A C T

Keywords

Lignocellulosic materials, SmF, SSF, FPase, CMCase and β – Glucosidase

Cellulases are the group of hydrolytic enzymes–Filter paperase (FPase), Carboxymethylcellulase (CMCase) and β-glucosidase (BGL) and are responsible for release of sugars in the bioconversion of the lignocellulosic biomass into a variety of value - added products. The present study was aimed to examine cellulase production by *Aspergillus niger* on individual lignocellulosic substrates in both submerged (SmF) and solid State (SSF) Fermentations. Rice bran supported maximum enzyme yields followed by wheat bran in both fermentations. Among different combinations with rice bran at equal ratio (1:1w/w) tested, combination of rice bran and wheat bran served the best combination for production of cellulolytic enzymes. Maximum titers of FPase, CMCase and BGL obtained on this combination were 2.632, 2.478 and 2.984 U/mL in SmF and 29.81, 25.2 and 32.18 U/gDS in SSF respectively.

Introduction

Plant biomass in the form of lignocellulose is one of the most abundant renewable resources on the earth. Lignocellulosic biomass includes agricultural residues, forest residues, municipal solid wastes and other industrial wastes. The lignocellulosic biomass is mainly composed of cellulose, hemicellulose, and lignin that are strongly intermeshed and chemically bonded by non-covalent interactions and by covalent cross-linkages (Margeot *et al*., 2009). Depositing/leaving of lignocellullosic biomasses in nature is one of main causes of environmental pollution. Their conversion into useful products may mitigate the problems of environmental pollution but also generate wealth out of waste. Every part of plant such as leaves, straws, stems, stalks, cereals, corncobs, wheat bran etc., comes under plant biomass. A huge amount of these materials are left on agricultural lands which have to be decomposed by microorganisms such as bacteria and fungi (Jadhav *et al*., 2013). The enzymatic hydrolysis of cellulosic materials involves synergistic actions of the three components of cellulase enzyme complex (Lynd *et al*., 2002). Even though these enzymes are

produced by several microorganisms-bacteria, fungi and actinomycetes, but fungi are known for secretion of cellulase in copious amounts. Among fungi *Aspergillus* and *Trichoderma spp*. are being exploited for commercial production of cellulases (Shin *et al*., 2000; Immanuel *et al*., 2007). The enzymes of Industrial importance have traditionally been produced in submerged fermentation (SmF) because of the ease of handling and good control of environmental factors such as temperature, aeration, agitation and pH (Singh *et al*., 2007). However, solid state fermentation (SSF) techniques are better adapted to enhance the yield, which reduces the cost of enzyme production because of the ability of filamentous fungi to grow well on solid substrates (Ghildyal *et al*., 1985; Pandey, 1992; Hui *et al*., 2010) The other advantages of SSF include maximum productivity; ease of technique; low capital investment, low energy requirement and less water output, better product recovery and lack of foam build up and reported to be most appropriate process for developing countries (Zeng and Chen, 2009; Souza and Magalhaes, 2010) There are several reports describing the use of lignocellulosic agro industrial wastes for the production of cellulases (Alegre *et al*., 2009; Singhania *et al*., 2010), Cost of enzyme production is one of factors that control the process of bioconversion of lignocellulosic masses into value added products (Klein- Marscuschamer *et al*., 2011). Cost of enzyme production can be reduced with use of cheap and locally available raw materials for cellulase production. Availability of lignocellulosic masses varies from one region to another region in our country because of specific patterns of cultivation of crops in different regions. The present study was to examine production of cellulase enzyme using *Aspergillus niger* grown on lignocellulosic substances of local and national relevance in both fermentation process. Further production of cellulase by the same fungal culture was compared on the best substrate - rice bran in combination with other lignocelluloses.

Materials and Methods

Microorganism:

A local isolate of *Aspergillus niger* used in the study was isolated from soil contaminants with effluents of cotton ginning mills (Narasimha *et al*., 1999) This culture was maintained on potato dextrose agar slants.

Substrates

Lignocellulosic substrates used in the present study -Rice bran, saw dust, Sugarcane bagasse, Ground nut shells, Wheat bran and Corn cobs were procured from local sources.

Experimental design of submerged fermentation (SmF)

Submerged fermentation was conducted by culturing *A. niger* in Czapek-Dox liquid medium that had been amended with individual natural lignocelluloses. The liquid medium (pH 5.0) was dispensed into 250 ml Erlenmeyer flasks at a rate of 50 mL per flask. Only one of the different natural lignocelluloses such as rice bran, saw dust, sugar cane bagasse, ground net shells, wheat bran and corncobs was added to each flask at 1% W/V. Flasks containing Czapek-Dox liquid medium amended with the lignocellulosic materials were sterilized in an autoclave and the flasks were inoculated with 1×10^8spores of *A. niger* at a density from spore suspension prepared by flooding the 7-day old slant with 2 mL of sterile distilled water. The flasks were incubated at

30^0C on a rotary shaker (180 rpm) for 7days. In view of secretion of maximum titres of cellulase enzymes by the same culture in the previous study (Narasimha et al, 2006), culture broth of the flasks after 7-days of growth was filtered through pre-weighed Whatman No. 1 filter to separate mycelia mat and culture filtrate. Activities of cellulase enzymes in the culture filtrate were measured and dry weight of mycelia mat on filter was determined. Another experiment was designed to determine the effect of combination of the best substrate – rice bran along with different natural lignocellulosic substrates at equal proportion at final concentration of 1% w/v in the same fashion.

Experimental design of solid state fermentation (SSF)

Ten gram sample of lignocellulosic substrates (rice bran, saw dust, sugar cane bagasse, ground net shells, wheat bran and corncobs) each in alone was taken in 250 mL Erlenmeyer flasks. The substrates in the flasks were moistened with mineral salt solution (pH maintained at 5.0) to final moisture content of 70 % and sterilized. The flasks were inoculated with spores of *A. niger* at a density of 1×10^8 from spore suspension prepared by flooding the 7-day old slant with 2 ml of sterile distilled water. The flasks were incubated at 30^0C an incubator for 7-days. Enzyme extraction was carried out by mixing fermented bran with 100mL sodium acetate buffer (0.2 M, pH–4.8, 1:10 w/v) for 1 hour in an orbital shaker at 180 rpm. The contents of the flask were filtered through muslin cloth and the filtrate was centrifuged at 6000 rpm in cooling centrifuge for 10 minutes. Activities of cellulase enzymes in the supernatant obtained were measured. Another experiment was conducted with *A. niger* grown in the same way on 10gram sample of rice bran in combination with other lignocelluloses in equal proportions (5grams each) for production of cellulose.

Enzyme assays

Filter paper activity (FPA) for total cellulase activity in the cultural filtrate was determined according to the method of Mandels (Mandels and Roche, 1976). Aliquots of appropriately diluted culture filtrate/extract as enzyme source was added to Whatman No.1 filter paper strip (1 X 6 cm; 50 mg) immersed in 1 mL of 0.05 M sodium citrate buffer of pH 5.0. The reducing sugar released was estimated by dinitrosalicylic acid (DNSA) by method Miller (Miller, 1959), after incubation at 50^0C. One unit of filter paper (FPU) activity was defined as the amount of enzyme releasing 1 µ mole of reducing sugar from filter paper per mL per min. Endoglucanase activity of carboxymethylcellulase (CMCase) was measured as described previously by Ghosh method (Ghosh, 1987).The reaction mixture contains 1 mL of 1% carboxymethyl cellulose (CMC) in 0.05 M citrate acetate buffer (pH 5.0) and aliquots of suitably diluted filtrate. The reaction mixture was incubated at 50°C for 1 hour and the reducing sugar produced was determined by DNS method. One unit (IU) of endoglucanase activity was defined as the amount of enzyme releasing 1 µmole of reducing sugar per min. β- glucosidase activity was assayed by method of Herr (Herr, 1979). β- Glucosidase activity was measured in 1mL of 5 mM *p*–nitrophenyl-β-D-glucopyranoside (PNPG) in 0.05 M citrate buffer (pH 5.0) and aliquots of appropriately diluted culture filtrate and incubated at 50°C for 30 min. The reaction was stopped by addition of 4 mL of 0.1 M NaOH-glycine buffer solution and the released *p*-nitrophenol was read at 410 nm in a spectrophotometer (ELICO-SL164).

The activity was expressed in terms of liberation of *p*-nitrophenol from *p*–nitrophenyl-β-D-glucopyranoside (PNPG). One unit of the enzyme activity was defined as the amount of enzyme producing 1 µmole of *p*-nitrophenol per min.

Protein estimation

An aliquot of this culture filtrate with appropriate dilution was used for estimation of soluble protein content according to the method of Lowry (Lowry *el al.,* 1951) Bovine Serum albumin was used as protein standard. Suitable aliquots of filtrate were mixed with 5mL of alkaline solution. After 10 minutes, 0.5mL of appropriately diluted Folin-Ciocalteu reagent was added. After 30 minutes, extinction was read at 550 nm in a spectrophotometer (ELICO-SL164).

Results and Discussion

The secretary capacity of *A. niger* on different lignocelluloses in terms of release of extracellular protein after growth for 7-days in SmF was assessed (Fig-1). The content of extracellular protein on rice bran was maximum with 1400 µg/mL as against minimum of 700µg of extracellular protein in the broth with corn cob. Wheat bran was the second best substrate in supporting secretion of extracellular protein in to the broth by *A. niger* in SmF. Activities of cellulolytic enzymes were detected in culture broth of *A.niger* grown on different lignicelluloses (Fig-2) Secretion of cellulolytic enzymes by *A. niger* into broth of different lignocelluloses followed the similar trend as observed with extracellular protein content (Fig-3). Culture broth of *A. niger* derived after 7-day growth on lignocelluloses contained all three cellulolytic enzymes – FPase, β-glucosidase and CMCase. There was a pattern of yields of enzymes topped with FPase. However, rice bran served the best substrate followed by wheat bran in supporting maximum production of FPase (2.5U/mL), β - glucosidase (2.5 U/mL) and CMCase (1.0 U/mL). In view of rice bran as the best substrate, cellulase production by *A. niger* on rice bran in combination with other linocelluloses were tested (Fig-4). Combination of rice bran and wheat bran served the best combination not only in supporting maximum secretion of extracellular protein (Fig-5) but also secretion of cellulolytic enzymes (Fig-6) by *A. niger* in SmF.

Performance of *A. niger* on the same lignocelluloses for secretion of extracellular protein into fermented bran in SSF after 7-day growth was examined (Fig-7). Like in SmF, growth of *A. niger* on rice bran in SSF resulted in maximum secretion of extracellular protein. Least secretion of extracellular protein was recorded on corn cob and saw dust in SSF. The increasing order of lignocelluloses in supporting production of cellulolytic enzymes by *A.niger* in SSF in the present study was as follows: corn cobs < Saw dust < Ground nut shells < Sugar cane bagasse < wheat bran < rice bran. The same pattern of secretion of cellulolytic enzymes – FPase, β-glucosidase and CMCase on lignocelluloses by *A. niger*, observed in SmF was also noticed in SSF. Maximum of about 30 U of FPase and β-glucosidase was recorded on one gram of rice bran as against of minimum of 10 U/g on corn cobs and saw dust. Combination of rice bran and wheat bran among different combinations gave the best yields of extracellular protein (Fig-7) and cellulolytic enzymes (Fig-8).

Majority of studies on microbial production of cellulolytic enzymes were conducted with one fermentation method – either submerged fermentation or solid state fermentation

method but not both. Among four lignocelluloses – cassava bagasse, sugar cane bagasse, rice straw and wheat bran tested in solid state fermentation by *Trichoderma reesei* NRRL 11460, sugar cane bagasse yielded maximum titers of FPase after 96 hours of growth (Singhania *et al*., 2006). A comparative study (Sukumaran *et al*., 2009) with two fungal cultures on wheat bran in solid state fermentation indicated that *T. reesei Rut C-30* relatively produced higher yields of FPase and endoglucanase whereas *A. niger* gave relatively higher titers of β – glucosidase. Cultivation of *Trichoderma reesei ZU02* in deep trough fermentor for 5days in solid state fermentation generated 128 U/g of FPase (Xia and Cen, 1999). Among different lignocelluoses tested in SSF for production of cellulases, wheat bran was the best substrate followed by groundnut fodder (Subhosh Chandra *et al.,* 2007). Protein content in these koji materials reached to about 5mg/gDS submerged fermentation of rice straw by *Trichoderma harzianum*gave yields of 0.13, 0.15 and 1.65 U/mL in respect of exoglucanase, endoglucanase and cellobiase under optimal conditions (Kocher *et al*., 2007). *Fusarium oxysporum* produced FPase, CMCase and –glucosidase to the extent of 1.34, 1.92 and 1.78 U/ mL respectively (Ramanathan *et al*., 2010). (Victor *et al.,* 2003). Obtained 0.0743, 0.0573 and 0.0502 IU/mL of cellulase within 12 h by *Aspergillus flavus* on substrates- sawdust, bagasse and corncob, respectively. Yields of FPase and CMCase obtained with *Trichoderma viridae* in submerged fermentation were 1.5 and 1.0 U/mL (Nathan *et al*., 2014). Cultivation of *A. niger* on saw dust in liquid culture yielded 2.42 U/mL of FPase (Narasimha *et al*., 2006).

Figure.1 Secretion of extracellular protein lignocelluloses by Aspergillus niger in SmF

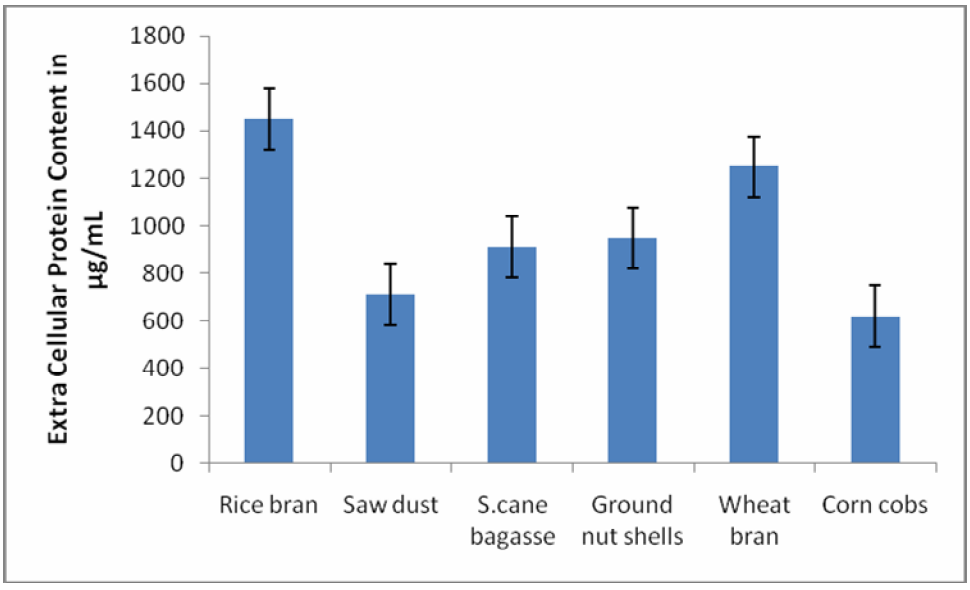


Figure.2 Cellulase productions on natural lignocelluloses by *Aspergillus niger* in SmF

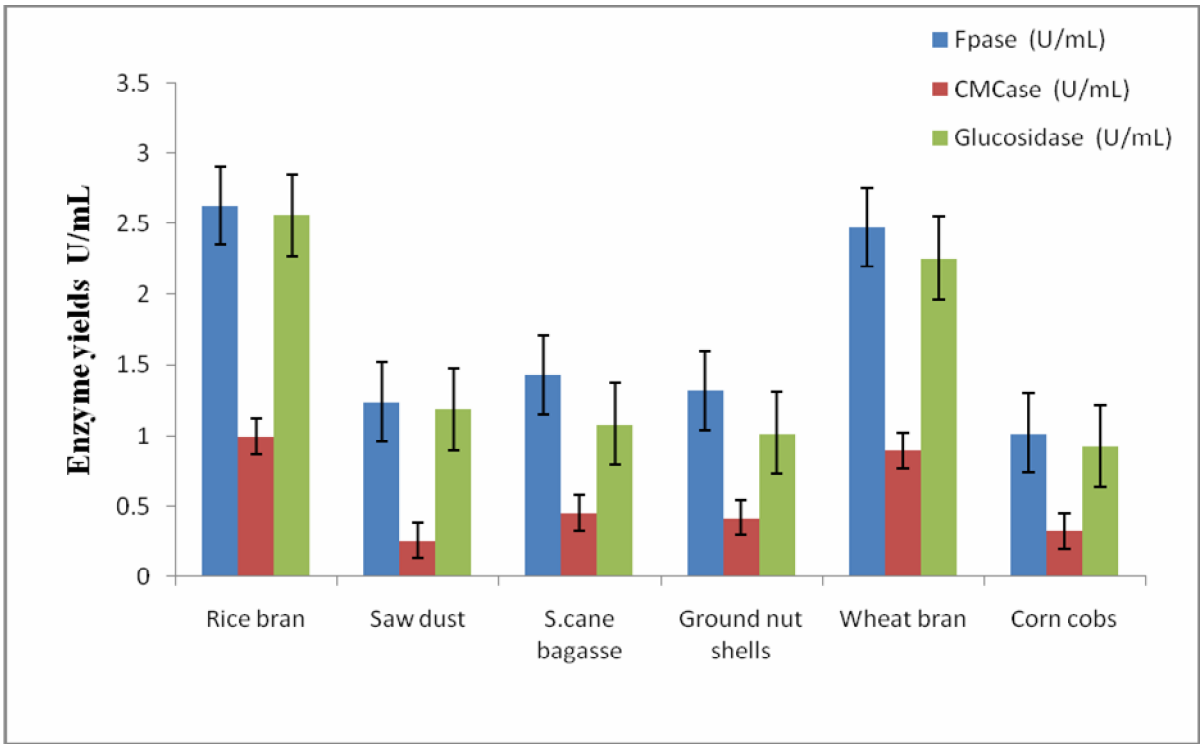


Figure.3 Secretion of extracellular protein on combination of natural lignocelluloses by *Aspergillus niger* in SmF

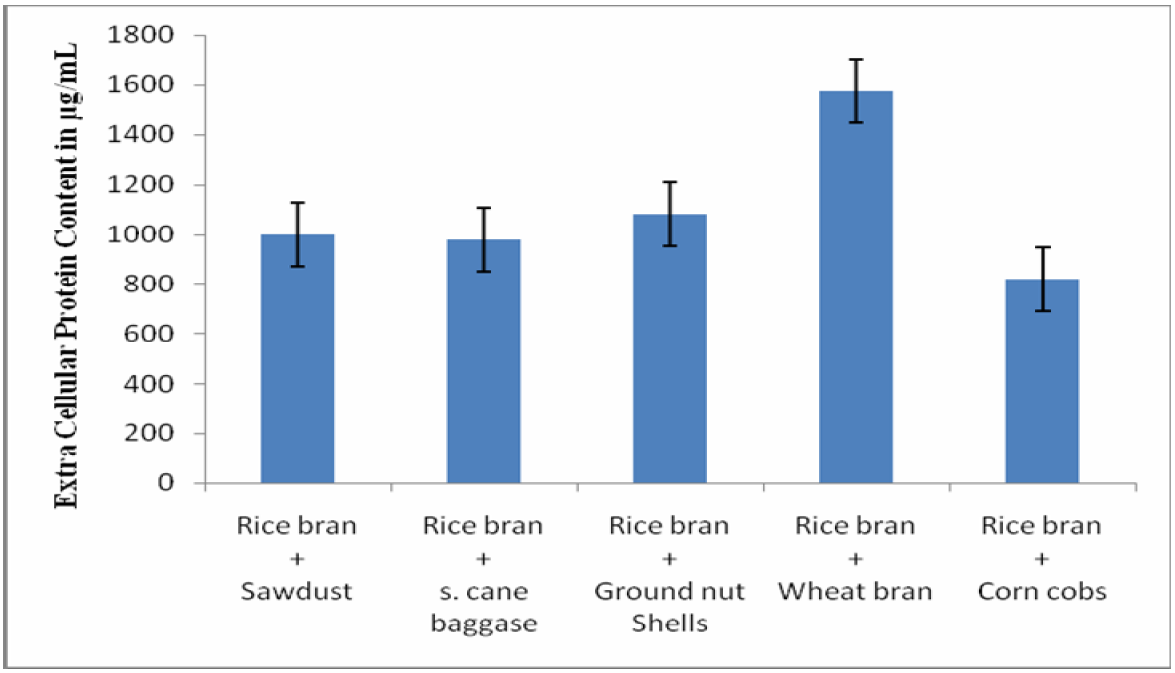


Figure.4 Cellulase production on combination of natural lignocelluloses by *Aspergillus niger* in SmF

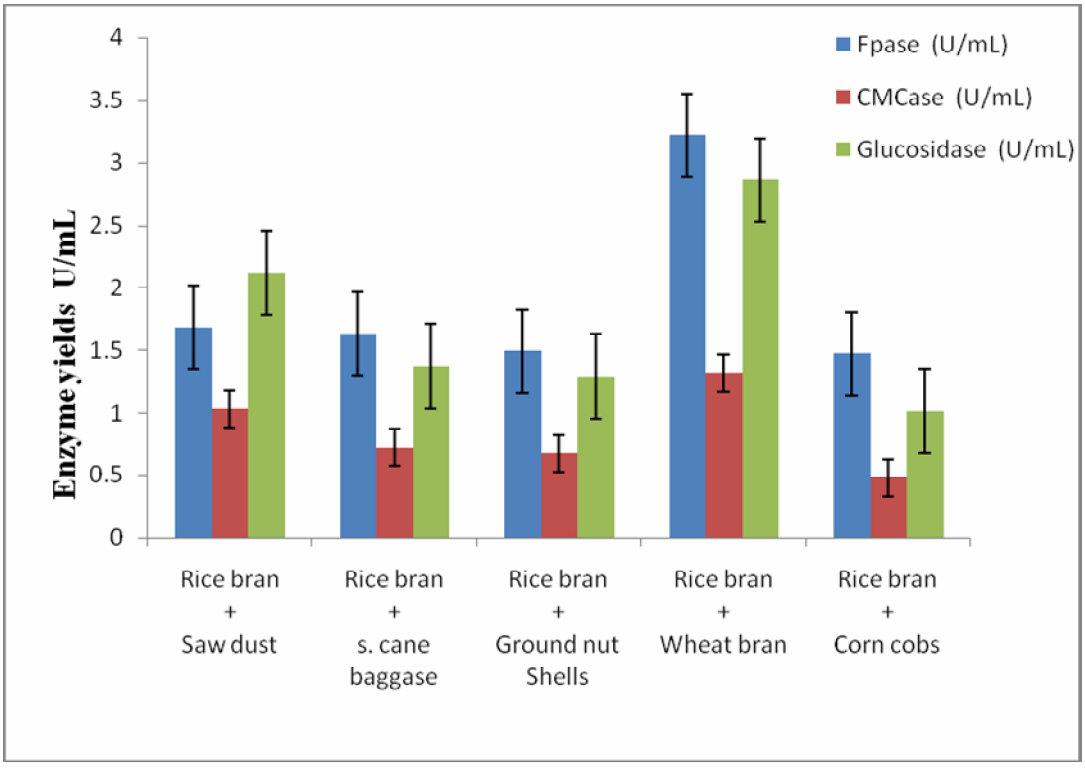


Figure.5 Secretion of extracellular protein on natural lignocelluloses by Aspergillus niger in SSF

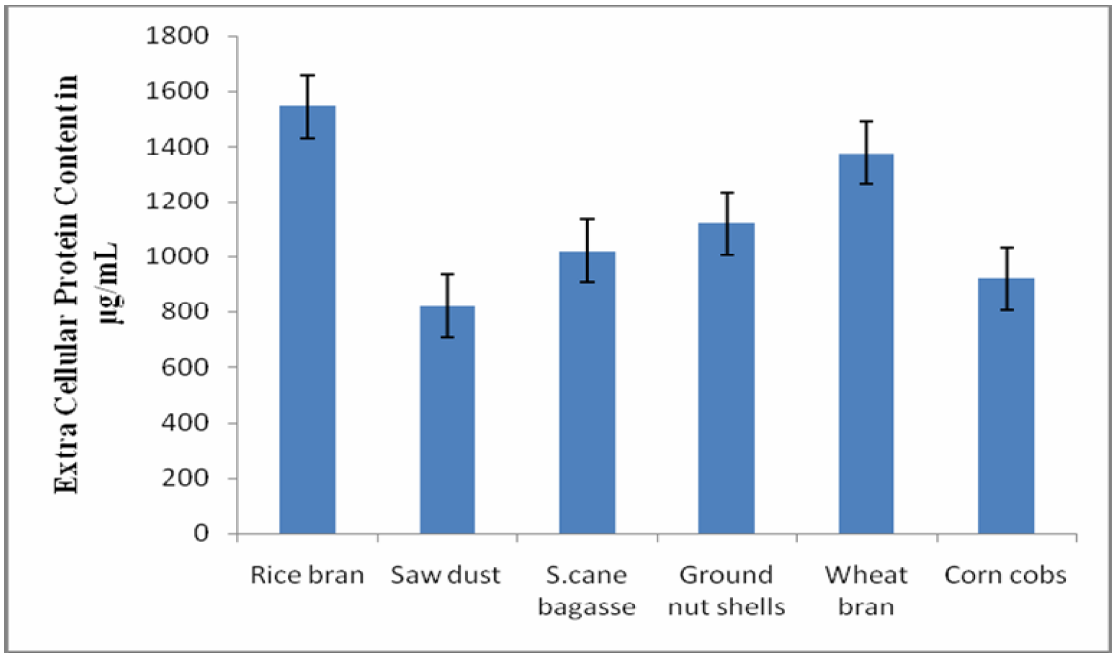


Figure.6 Cellulase production on natural lignocelluloses by *Aspergillus niger* in SSF

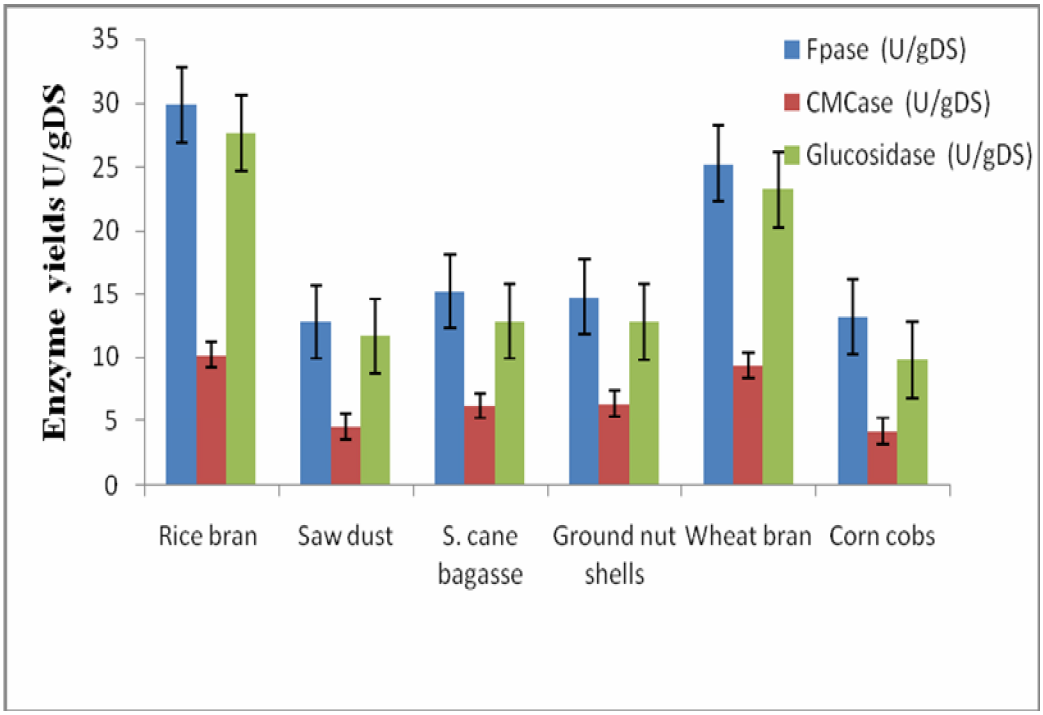


Figure.7 Secretion of extracellular protein on combination of natural lignocelluloses by *Aspergillus niger* in SSF

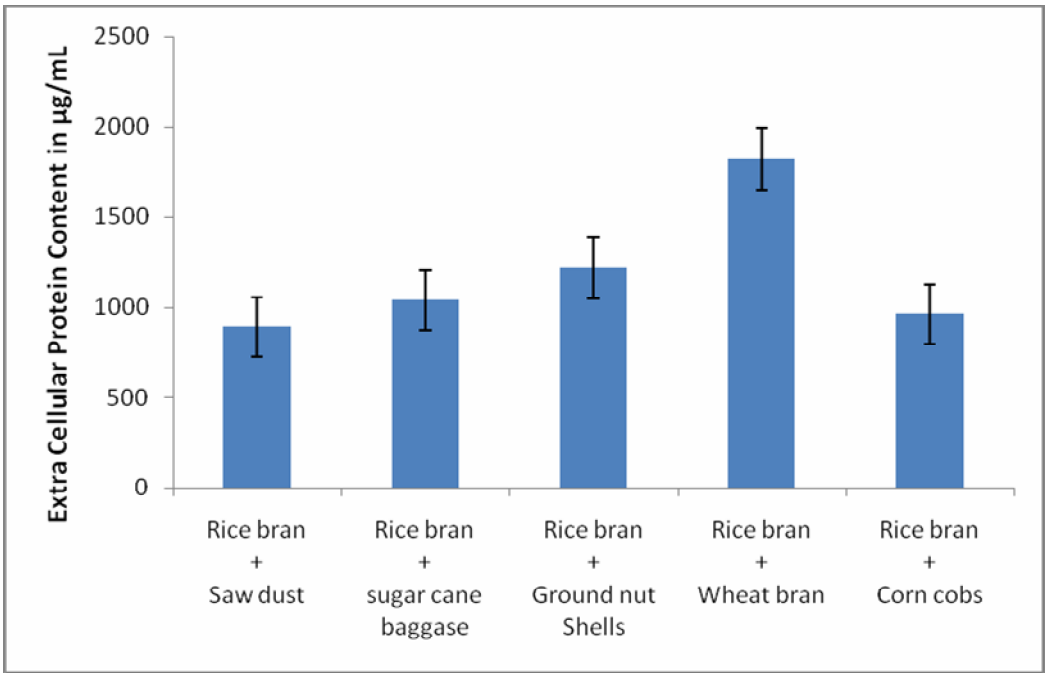
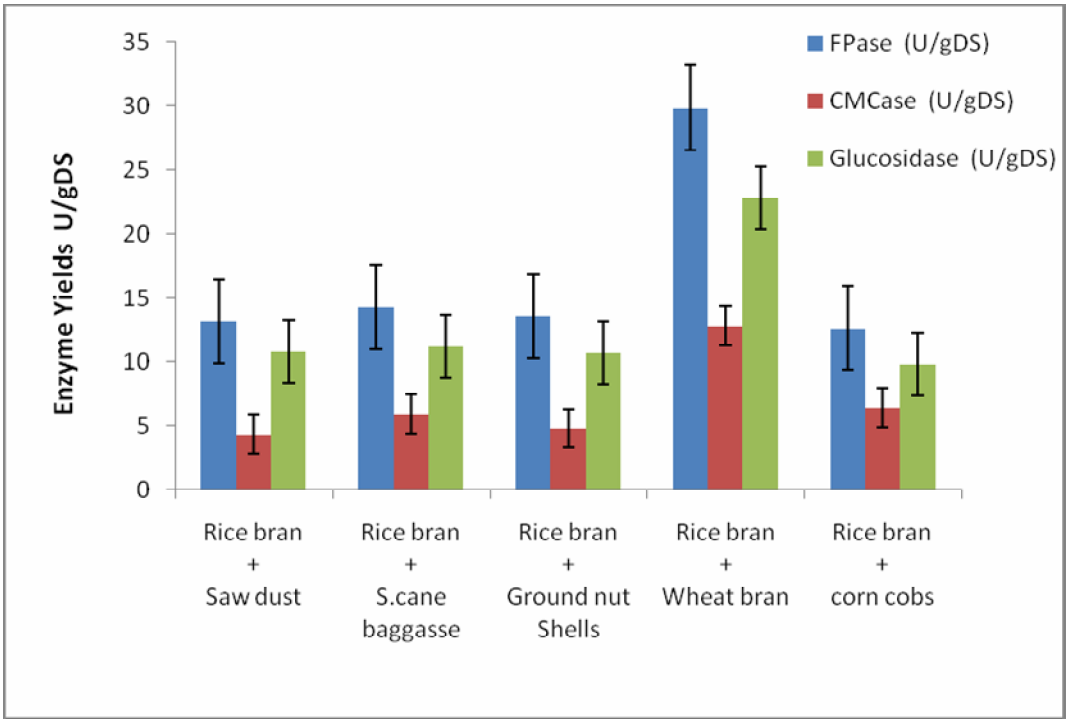


Figure.8 Cellulase production on combination of natural lignocelluloses by *Aspergillus niger* in SSF



Different yields obtained in different studies could be attributed to inherent capacities s of organism used, different cultural practices and different lignocelluloses used.

The local isolate of *A. niger* in the present study was also able to efficiently utilize rice bran and wheat bran for production of cellulolytic enzymes in both fermentation methods. Our isolate produced 2.632, 2.478 and 2.984U/mL of FPase on rice bran, wheat bran and rice bran + wheat bran respectively in SmF whereas the corresponding figures of FPase on rice bran, wheat bran and rice bran + wheat bran were 29.81, 25.2 and 32.18 U/gDS respectively in SSF. Comparison on production of cellulolytic enzymes by *A. niger* in SmF and SSF were made under non-identical conditions earlier (Subhosh Chandra *et al.,* 2008). However, based on results obtained and extent of nutrients (mineral salts in SSF and Czpek Dox medium) along with cost used in cultural practices adapted in the present study, SSF on rice bran and wheat bran and its combinations appeared to be a better choice for cellulase production by *A. niger.*

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