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ORIGINAL ARTICLE

Cellulolytic enzymes on lignocellulosic substrates in solid state fermentation by *Aspergillus niger*

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Abstract The production of cellulolytic enzymes by *Aspergillus niger* on lignocellulosic substrates groundnut fodder, wheat bran, rice bran and sawdust in solid state fermentation in a laboratory scale was compared. Czapek Dox liquid broth amended with cellulose (0.5%) was used to moisten lignocellulosic solid supports for cultivation of *Aspergillus niger*. The production of filter paperase, carboxymethyl cellulase and -glucosidase were monitored at daily intervals for 5 days. The peak production of the enzymes occurred within 3 days of incubation. Among solid supports used in the study, wheat bran was the best solid matrix followed by groundnut fodder in production of cellulolytic enzymes in solid state fermentation. Groundnut fodder supported significant production of FPase (2.09 FPU/g), CMCase (1.36 U/g) and -glucosidase activity (0.0117 U/g) in solid state fermentation. Considerable secretion of protein (5.10 mg/g) on groundnut fodder at peak time interval 1st day of incubation was recorded.

Keywords Cellulolytic enzymes · Lignocellulosic substrates · Wheat bran; Groundnut fodder · Rice bran · Sawdust · Solid state fermentation · *Aspergillus niger*

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Introduction

Lignocellulose is the most abundant renewable biological resource[1,2] continually replenished by photosynthetic reduction of carbon dioxide by sunlight energy[3] Lignocelluloses constitute a major portion of agricultural wastes and forest wastes[4]. Thus they are the most promising feedstock for the production of energy, food and chemicals[4,5] and their utilization could allow self-sustainable processes and products. The bioconversion of cellulosic materials at economic rate will lead to the development of large-scale processes beneficial to mankind[6]. Such processes as suggested by[3,6] would help alleviate shortages of food and animal feeds, solve modern waste disposal problem and diminish man's dependence on fossil fuels by providing a convenient and renewable source of energy in the form of glucose. Formation of soluble sugars from cellulose in agricultural residues relies on the sequential/coordinated action of individual components such as -exoglucanase, -endoglucanase and -glucosidase in cellulase enzymes[7]. Cellulase production by different organisms in submerged state fermentation has received more attention and is found to be cost-prohibitive because of high cost of process engineering. Solid state fermentation (SSF) is successfully used for large scale production of fungal metabolites and bioconversion of plant, animal wastes into useful products[8,9]. SSF, a highly attractive and alternative process needs to be exploited for generation of cellulolytic enzymes with use of cheaply available lignocellulosic residues and low input of process engineering[10]. The lignocellulose materials are water-insoluble so that its utilization can be fruitfully improved by solid-state bioconversions (SSF). In the present study the production of cellulolytic enzymes by a local isolate of *A. niger* on lignocelluloses in solid state fermentation is reported.

Materials and methods

Lignocellulosic substrates: Lignocellulosic substrates such as groundnut fodder, wheat bran, rice bran and sawdust were chosen as solid matrices for use in solid state fermentation in this study because of their abundance in the local area at cheaper rates. Coverage of the huge extensive area of cultivable land with groundnut crop in a single district of Anantapur generates high volumes of groundnut vegetative biomass. Groundnut fodder was collected from local farmers whereas rice bran and sawdust were obtained from rice mill and saw mill in Anantapur respectively. The substrates were individually sieved through a 2 mm screen, for uniform particle size.

Culture medium: Ten gram samples of different lignocelluloses were dispensed into 250 ml Erlenmeyer flasks. One liter of Czapek Dox liquid medium contained $NaNO_3$ - 2.0 g, K_2HPO_4 - 1.0 g, $MgSO_4$. $7H_2O$ - 0.5 g, KCl - 0.5 g, $FeSO_4$. $7H_2O$ - 0.01 g, Sucrose - 30.0 g and Cellulose - 5.0 g. The different lignocellulosic matrices require different volumes of water within a range of 10-15 ml for 50% moisturization of 10-gram samples. Ten milliliters of the above Czapek Dox medium was only once added to all matrices in the flasks at the beginning and the remaining balance for getting 50% moisture level was provided to the respective matrix in the form of distilled water. These flasks were cotton-plugged and autoclaved at 121°C for 30 minutes.

Pure culture and preparation of inoculum: A local isolate of *Aspergillus niger* isolated from the soils contaminated with the effluents of Cotton ginning mills, Nandyal, Andhra Pradesh[11] was used in the present study. This fungal culture was maintained on Czapek Dox medium. Spore suspension of *Aspergillus niger* was prepared by adding 2 ml of sterile distilled water to 7-day grown slants.

Fermentation method: Sterile solid culture medium in the flasks were inoculated with the spores of *A. niger* at density of 2×10^6 spores/flask and incubated at ambient temperature (30 ± 2°C). The weight of flasks along with matrices was daily measured from 0-day (immediately after inoculation) over a period of 5 days. Difference in the weight of flasks at daily interval and 0-day time indicates water loss and was aseptically replaced with addition of sterile water for maintenance of 50% moisture content. At the regular intervals the flasks were withdrawn for processing. Entire fermented bran in the flask was mixed with acetate buffer (0.2 M; pH 5.0), the slurry was filtered through nylon cloth and the filtrate was centrifuged at 10,000 rpm for 20 min at 4°C. The clear filtrate obtained was used for enzyme assays up to 5 days of incubation.

Enzyme assays: Each sample filtrate was monitored for pH, filter paperase (FPase), carboxymethyl cellulase (CMCase) and -glucosidase activity . Filter paper assay method[12] was employed to measure total cellulase activity of *Aspergillus niger* grown on solid state fermentation. Activity of cellulase was expressed in filter paper units. One unit of filter paperase activity was defined as the amount of enzyme releasing 1µmole of reducing sugar per minute. Activity of endoglucanase in the culture filtrate was quantified by Carboxymethyl cellulase method[13]. One unit of endoglucanase activity was defined as the amount of enzyme releasing 1µmole of reducing sugar per minute. -Glucosidase activity in the culture filtrate of *Aspergillus niger* was determined according to the method[14]. Activities of FPase, CMCase and -glucosidase were measured on substrate – filter paper, carboxymethyl cellulose and *p*-nitrophenyl -glucoside, respectively with appropriate enzyme control and substrate control.

Protein determination: Aliquots of *Aspergillus niger* culture filtrates with appropriate dilution were used for estimation of soluble protein content according to the method[15].

Statistical analysis: Data presented are the averages of three replicates. Duncan's Multiple Range (DMR) test for all data was carried out[16].

Results and discussion

A. niger was cultivated in solid state fermentation on a variety of locally available and cheap lignocelluloses. The production of filter paperase activities on the lignocellulosic substrates was monitored for a period of 5 days (Fig. 1). Maximum FPase activity was recorded on 3rd day of incubation on all the lignocellulosic materials except sawdust used. Growth of *Aspergillus niger* on wheat bran gave the highest FPase activity of 2.9 FPU/g of solid support, while groundnut fodder and rice bran gave 2.09 and 1.62 FPU/g of solid support, respectively. Sawdust supported poorly FPase activity as reflected by recovery of 0.28 FPU/g of solid support. The decrease in FPase activity on wheat bran, groundnut fodder and rice bran occurred from 4th day onwards. Thus, wheat bran is the most suitable for filter paperase production followed by groundnut fodder.

Unlike FPase activity, maximum titres of CMCase on all lignocelluloses except sawdust due to the growth of *A. niger* was registered on the first day of incubation (Fig. 2). Wheat bran yielded highest titres of 3.24 U/g of CMCase in solid state fermentation as against 1.36 U/g by groundnut fodder and 1.09 U/g by rice bran. CMCase activity on all substrates declined on 2nd day onwards. Wheat bran is

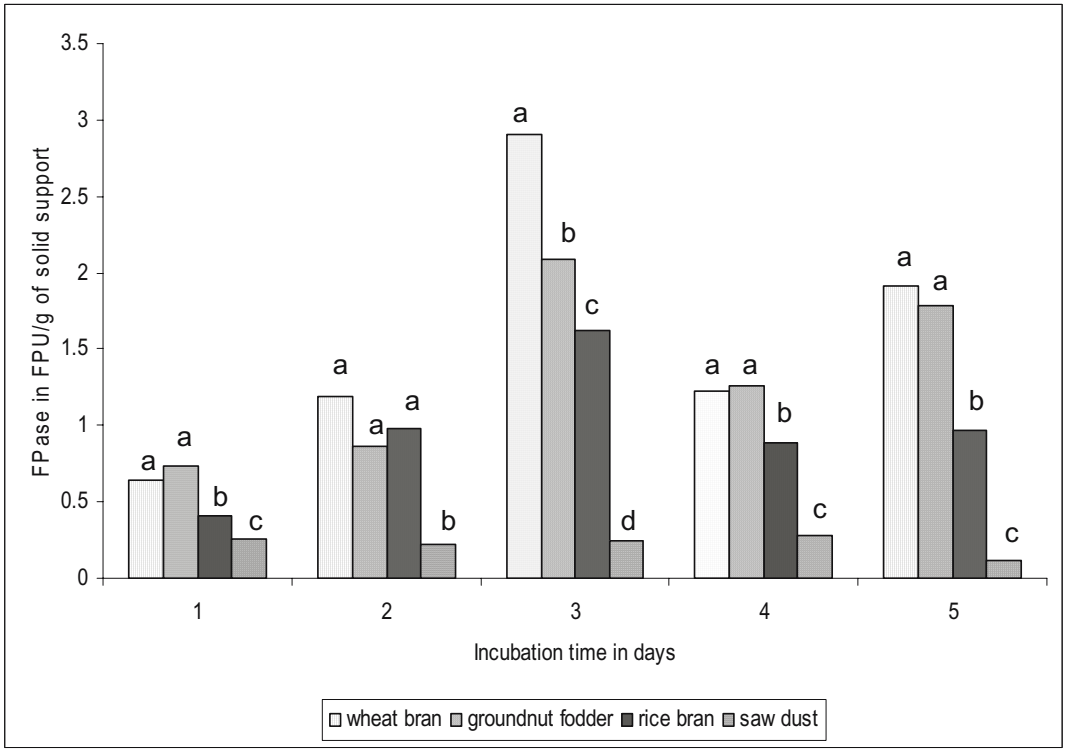

Fig. 1 Production of filter paperase by *A. niger*.
Means, in each bar, followed by the same letter are not significantly different
(P 0.05) from each other according to Duncan's Multiple Range (DMR) test.

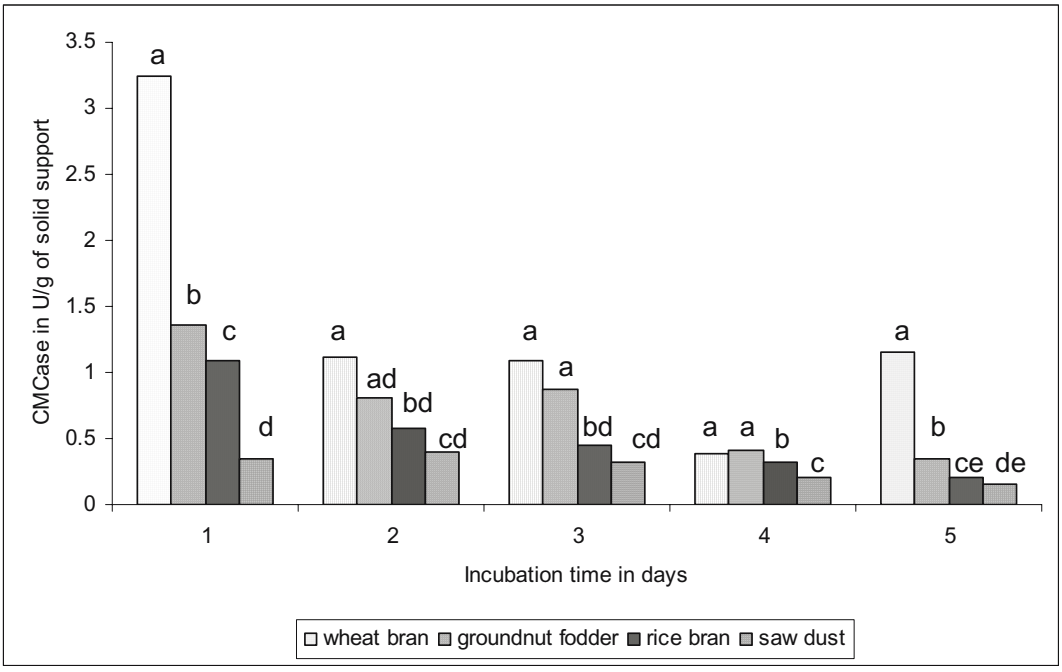

Fig. 2 Production of carboxymethyl cellulase by *A. niger*.
Means, in each bar, followed by the same letter are not significantly different
(P 0.05) from each other according to Duncan's Multiple Range (DMR) test.

Fig. 3 Production of ß-glucosidase by *A. niger.*
Means, in each bar, followed by the same letter are not significantly different
(*P* 0.05) from each other according to Duncan's Multiple Range (DMR) test.

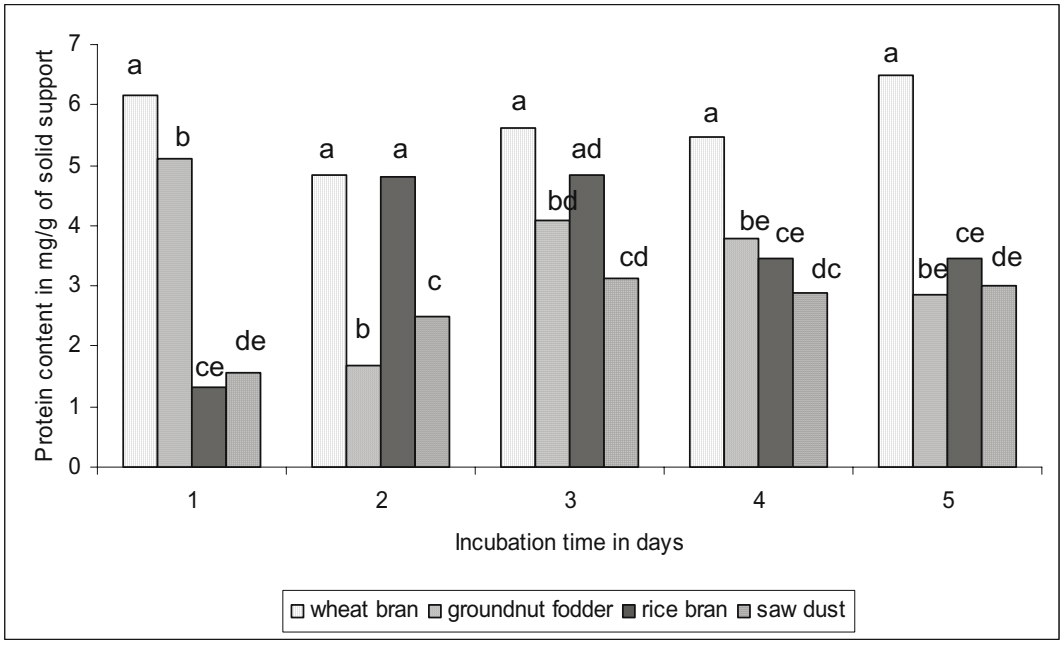

Fig. 4 Production of extracellular protein content by *A. niger* .
Means, in each bar, followed by the same letter are not significantly different
(*P* 0.05) from each other according to Duncan's Multiple Range (DMR) test.

the most suitable for carboxymethyl cellulase production among all lignocellulosic solids tested in this study.

The pattern of secretion of -glucosidase followed the reverse trend in comparison to those of CMCase and FPase (Fig. 3). Yields of -glucosidase were initially low or undetectable on the first day of incubation and improved by 2nd day of incubation on all substrates and maintained at the same level during the rest of period of incubation. Maxi-

mum titres (0.0169 U/g of solid support) of -glucosidase were recovered on 2nd day of incubation from sawdust as against maximum titre of 0.0117 U/g of solid support of groundnut fodder on 5th day of incubation. Production of -glucosidase on sawdust was highest when compared to other solid substrates at their respective time intervals of peak production. Secretion of extracellular protein including cellulolytic enzymes by *A. niger* on wheat bran at all time intervals was high in comparison to that on other solid matrices (Fig. 4). Maximum secretion of protein (6.48 mg/g of solid support) on wheat bran at peak time interval-5th day of incubation was recorded whereas the protein content was low (3.12 mg/g of solid support) on saw dust at peak time interval-3rd day of incubation. Groundnut fodder and rice bran yielded protein content of 5.1 and 4.84 mg/g of solid support on 1st and 3rd day of incubations respectively. pH changes in acetate buffer extract derived from brans with different ages were recorded (Table 1). pH decreased up to 2nd day or 4th day of incubation in all brans except sawdust. There was recovery in pH towards the end of incubation period.

Advances in industrial biotechnology offer potential opportunities for economic utilization of lignocellulosic substrates[17]. Solid state fermentation is a microbial process in which the microorganisms grow under conditions closer to their natural habitat and produce larger amounts of extracellular enzymes and other enzymes than do in submerged fermentation[18]. Fungi, *Trichoderma* spp., *A. niger, A. flavus* and *Penicillium* sp. have been reported to be main sources of cellulase, hemicellulase, pectinase and xylanase[19] on the non-starch polysaccharides (NSPs) including cellulose. Provision of right mix of sugars (0.03 g of sucrose and 0.05 g of cellulose per g of lignocellulose) on to lignocellulosic substrates in the form of limited volume of Czapek Dox medium only at the beginning will allow proliferation and build up of biomass of *A. niger* at the cost of easily utilizable sugar accompanied by rapid secretion of cellulolytic enzymes in the presence of cellulose. Release of cellulolytic enzymes will lead to initiation of attack on cellulosic components of lignocelluloses. Titres of cellulolytic enzymes at peak production time interval in solid state fermentation were higher on wheat bran than on other solid matrices in the present study. Increased production of cellulase in wheat bran is known for presence of ample nutrients, existence of loose texture in moist conditions, and a large surface area[20]. Individual components of cellulolytic enzymes reached peak production at different time intervals in the present study. High activity of endoglucanase in one-day old fermented bran was probably due to secretion of endoglucanase in larger amounts to cleave maximum number of accessible sites in cellulose component of lignocelluloses in the initial stages. Peak time production of -glucosidase was delayed in comparison to -endoglucanase in this study. This is probably due to onset of -glucosidase after the accumulation of the products like cellobiose formed by the action of -endoglucanase and other enzymes. Yields of FPase and CMCase on sawdust were low and steady in comparison to titres of same enzymes on other matrices. On the other hand, sawdust supported -glucosidase at the same levels as noticed on other matrices from second day onwards. Consistent production of -glucosidase on sawdust in solid state fermentation could be attributed to continuous demand of hydrolyzing products generated by steady action of -endoglucanase and other enzymes from highest cellulose content and low soluble fractions (data not shown) in sawdust. It is clear from these results that groundnut fodder ranks the second next to wheat bran for higher production of cellulase enzymes but it can be considered because of it's more abundance in our local area due to cultivation of groundnut in larger area. Groundnut fodder is used as cattle feed. Its nutritive value can be improved due to increase in protein content by the growth of *A. niger* on groundnut fodder. The yields of cellulase production in the present study were higher when compared to the results in the study of Muniswaran and Charyulu[21]. According to this study, the yields of FPase, CMCase by *T. viride* NCIM 1051 on coconut pith were 0.514, 1.077 IU/g of substrate and no activity of -glucosidase. The yields of cellulase production obtained in the present study were low in comparison to titres of CMCase (61.0 IU/g substrate) and FPase (20.7 IU/g substrate dry matter (SDM) by *Trichoderma harzianum Rifai*, strain CCMF-470 on wheat bran[22]. Further higher titres were obtained in a most recent study with *A. niger* on different lignocelluloses[23]. Differences in titres of enzyme yields in different studies can be attributed to use of different materials as solid matrix, different cultural practices and different organisms.

The association of cellulose with lignin and hemicelluloses in the lignocellulosic materials is an important

Table 1 pH changes in buffer leachates of fermented brans.

Incubation period in days	pH of buffer leachates derived from fermented brans			
	Rice bran	Wheat bran	Groundnut fodder	Sawdust
I	5.37	5.30	4.96	5.31
II	4.91	5.16	4.90	5.89
III	4.89	5.38	5.27	5.56
IV	4.85	6.05	5.11	5.60
V	5.22	6.19	5.42	5.30

factor limiting the hydrolysability. Removal/degradation of hemicelluloses and lignin by pre-treatments such as NaOH or H_2SO_4 or H_2O_2 etc., open up the cell wall structure, thus increasing the accessibility of cellulases to cellulose[24]. Pretreatment process may improve substrate utilization by the microbes and enhance enzyme yields[25,26]. In the present study, only native lignocellulosic substrates without pretreatment were used. Use of pretreated lignocelluloses may further increase yields of cellulolytic enzymes by microorganisms in solid state fermentation and needs to be further explored.

Conclusion

It can be concluded from the present study, groundnut fodder serves as the best solid substrate next to wheat bran for production of cellulolytic enzymes in solid state fermentation by *Aspergillus niger*. Increase in protein content in groundnut fodder due to cultivation of *A. niger* is an interesting observation and it has implication for improvement of nutritive value of groundnut fodder for cattle.

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