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ORIGINAL ARTICLE

Influence of Carbon Source and Inoculum Size on Production of Cellulolytic Enzymes by a Local Isolate of *Aspergillus niger* on Wheat bran

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ABSTRACT
Wheat bran is an abundant, potential substrate for fermentation. Czapek Dox liquid broth amended with cellulose (0.5%) was used to moisten solid matrix of wheat bran for Aspergillus niger cultivation. The protein secretion and filter paperase (FPase), carboxymethyl cellulase (CMCase) and β-glucosidase production by Aspergillus niger using different carbon sources and inoculum size were investigated. Glucose, sucrose and cellulose were used as carbon sources. The inoculum size varied from 2×10^4, 2×10^5, 2×10^6 and 2×10^7 were used in the present investigation. Among the different carbon sources, glucose (2.5%, w/v) and inoculum size, 2×10^6 were found to be the best for maximum secretion of protein and cellulase production on locally available cheap substrate wheat bran. Growth of A. niger on 2.5% glucose at optimal inoculum size of 2×10^6 generated higher production of protein (27.84 mg/g), FPase (27.78 FPU/g), CMCase (47.90 U/g) and β-glucosidase activity (0.075 U/g) under optimal conditions.
Keywords: *Cellulose, Aspergillus niger, wheat bran, solid state fermentation, filter paperase, carboxymethyl cellulase, β-glucosidase.*

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INTRODUCTION

Lignocellulose is the most abundant renewable biological resource (1, 2) and the production of biofuels and biobased products from less costly lignocellulosic materials is a promising approach to reducing greenhouse gas emissions and lessening reliance on finite fossil fuels (1-6).

Development of technologies for effectively converting agricultural and forestry lignocellulosic residues as well as energy crops to fermentable sugars offers outstanding potential to benefit the national interest through: (1) improved strategic security, (2) decreased trade deficits, (3) healthier rural economies, (4) improved environmental quality, (5) technology exports, and (6) sustainable energy resource supplies (3-5). Furthermore, tremendous amounts of cellulose are available as municipal and industrial wastes, which today contribute global environmental pollution problems. Thus, there is a great interest in the utilization of cellulosic biomass as a renewable source of energy via breakdown to sugars that can be converted to liquid fuel. The chemical components of the lignocelluloses make them enormous substrate for its biotechnological value (7).

Agricultural wastes and in fact all lignocellulosics can be converted into products that are commercial interest such as ethanol, glucose, single cell protein etc., (8). Cellulose is the world's most abundant renewable carbon source, natural biopolymer and a potentially important source for the production of industrially useful materials (9, 10). Cellulase enzyme has been reported for the bioconversion of lignocellulosics to useful products (8, 11). The great majority of cellulose hydrolysis to date has focused on the genetics, structure, function and interaction of components of cellulase enzyme system (3). Different fungi and bacteria have been explored for production of cellulases using different substrates (3)

but cost of production of cellulase is still prohibitive for commercial application. Enzymatic hydrolysis is an economic process in the conversion of cellulose to easily fermentable low cost sugars (12). Enzymatic saccharification is not cost-effective at present, large quantities of active and cheap cellulase preparations are required (3, 13). To overcome this short-coming, efforts are being directed continuously to reduce the cost of production of cellulase by searching high-yielding cellulase producing strains, mutants that are resistant to catabolite repression, genetic engineering methods and optimizing processes of fermentation methods (14). To date, the production of cellulase has been intensively studied in submerged fermentation (SmF) with different microorganisms in comparison to solid state fermentation (SSF) (3). SSF is advantageous over SmF to obtain concentrated metabolites and subsequent purification procedures are economical (15, 16). SSF is an attractive process to produce cellulase economically due to its lower capital investment and its lower operating conditions (17, 18). In the present study, cellulase production by *Aspergillus niger* on effect of nutrients such as different carbon and nitrogen sources in solid state fermentation were compared.

MATERIALS AND METHODS

Organism

A local isolate of *Aspergillus niger* isolated from the soils contaminated with the effluents of cotton ginning mills, Nandyal, Andhra Pradesh (19) was used in the present study. This fungal culture was maintained on Czapek Dox medium.

Substrate

Air-dried and milled wheat bran was utilized as substrate for solid state fermentation and was purchased from local market in Punganur of Chittoor district.

Culture media

Czapek Dox medium was amended with carbon sources in the right proportion for enhanced production of cellulolytic enzymes. The medium contained (g/L): 2.0 $NaNO_3$; 1.0 K_2HPO_4; 0.5 $MgSO_4.7H_2O$; 0.5 KCl; 0.01 $FeSO_4.7H_2O$; 30 sucrose; 5.0 cellulose; pH=5.0 whereas inoculum size modified Czapek Dox media was used (13). The above medium was used to moisten the solid substrate-wheat bran in experiments (20).

Growth conditions

The fermentation was carried out in 250 mL Erlenmeyer flasks containing 10 g of wheat bran. The solid substrate was initially moistened to 50% (w/v) level with normal Czapek Dox liquid medium containing 0.5 % (w/v) cellulose for carbon sources experiment, whereas inoculum size experiment where we used optimized Czapek Dox medium to moisten the substrate at 40% moisture level (13, 20), cotton-plugged and autoclaved at 121^{0}C for 30 minutes. Sterile solid culture medium in the flasks were inoculated with the spores of *A. niger* at density of $2x10^6$ spores/flask except inoculum size and incubated at ambient temperature (30±2^{o}C). One milliliter of spore suspension containing about $2x10^6$ spores/mL was used to inoculate the wheat bran. During the course of incubation, water loss by evaporation from the flask was aseptically replaced with addition of sterile distilled water to maintain 50% and 40% moisture content (*13,20*). At the regular intervals the flasks were withdrawn for further processing. Three different carbon sources (sucrose or glucose or cellulose) were additionally supplemented to Czapek Dox medium, at 2.5% level. Supplemented Czapek Dox medium was used to moisten wheat bran and *A. niger* was grown in the same manner as described above. Different inoculum sizes were supplemented to the previously had been moistened wheat bran with modified Czapek Dox medium.

Enzyme extraction

During the course of solid state fermentation in carbon source experiments, the flasks were withdrawn for processing at desired intervals and entire fermented bran in the flask was mixed with 0.2 M acetate buffer, pH=5.0 (1:2 w/v), the slurry was filtered (immediately after soaking) through nylon cloth and the filtrate was centrifuged at 10,000 rpm for 20 min at 4 ^{0}C. The clear filtrate/leachate obtained was used for further analysis. Meanwhile, the best solvent, water for extracting cellulolytic enzymes from fermented bran was identified on the basis of results of our study (13). In case of inoculum size experiment the fermented bran in the flask was mixed with the best solvent-distilled water (1:12, w/v) in 250 mL beakers and shaken on an orbital shaker at 150 rpm at 30 ^{0}C temperature for 30 min incubation.

Enzyme assays

Each sample was monitored for protein content, filter paperase (FPase), carboxymethyl cellulase (CMCase) and β-glucosidase activity. Filter paper assay method (21) 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 1 μmole of reducing sugar per minute. Activity of endoglucanase in the culture filtrate was

quantified by Carboxymethyl cellulase method (22). One unit of endoglucanase activity was defined as the amount of enzyme releasing 1 µmole of reducing sugar per minute. β-Glucosidase activity in the culture filtrate of *Aspergillus niger* was determined according to the method of Herr (23).

Protein determination

Aliquots of *Aspergillus niger* culture filtrates with appropriate dilution were used for estimation of soluble protein content according to the method of Lowry et al., (24).

Statistical Analysis

Results presented are the averages of replicates. Duncan's Multiple Range (DMR) test for whole data was performed by Megharaj [24].

RESULTS AND DISCUSSION

The chemical composition of wheat bran

Chemical analysis of agro-residues used in the present study (Table 1) revealed that dry matter of sawdust contained highest carbon content but lowest nitrogen content and it was poor in terms of phosphorus and other minerals (potassium, calcium and magnesium). Wheat bran and rice bran are the best solid substrates on basis of overall composition of carbon, nitrogen and other mineral nutrients followed by groundnut fodder. Further analysis of carbon content indicates extent of chemical components such as soluble fractions, lignin, hemicellulose and cellulose present in agro-residues (Table 2). Wheat bran and groundnut fodder relatively contained higher portion of soluble fraction to the tune of 39% of dry matter as against 23% in sawdust. Content of soluble fractions in other agro-residues (rice bran and sugarcane bagasse) were intermediate between these two agro-residues. As far as other components, in particular, cellulose was concerned, the reverse trend was observed in agro-residues. The presence of easily soluble sugars and nitrogen content in high proportion will support better growth of organism on wheat bran thereby facilitating higher enzyme production. Thus, higher production of cellulolytic enzymes on wheat bran could be attributed to availability of nutrients in larger proportion. Similarly, wheat bran as the best solid substrate for production of cellulolytic enzymes was demonstrated (20, 26). It might be due to that wheat bran provided adequate amount of nutrients (proteins 1.32%, carbohydrates 69.0%, fats 1.9%, fiber 2.6%, ash 1.8%, Ca 0.05%, mg 0.17%, P 0.35%, K 0.45%, S 0.12% and various amino acids) to the microorganism (26). Chandra et al., proved that the maximum β endoglucanase (50) and β exoglucanase (51) was recovered when they cultivated the local strain *A. niger* on wheat bran as a source of substrate in SSF. In another study Yadav P. Suresh et al. concluded that considerably the good amount of enzyme was produced (26.06 U/gram of rice husk) by local isolated strain *A. protuberus* in solid state fermentation (52).

Table 1. The chemical composition of wheat bran

Component	Concentration (% w/w)
Carbon	4.67
Nitrogen	2.52
Phosphorus	0.13
Potassium	1.10
Calcium	0.06
Magnesium	0.86

Table 2. Chemical components such as soluble fractions, lignin, hemicellulose and cellulose present in native wheat bran

Fraction	Wheat bran (%)
Lignin	10.64
Hemicellulose	13.60
Cellulose	38.06

Carbon sources

Maximum secretion of protein (9.48 mg/g of wheat bran) was recorded on 5th day of incubation upon supplementation of glucose at 2.5% to wheat bran at the beginning of cultivation of *A. niger* in SSF followed by cellulose with 7.80 mg/g of wheat bran on 1st day of incubation and 6.96 mg/g of wheat bran in the sucrose amended medium on 3rd day of incubation (Table 3). Rise in protein content in control took place from 6.08 mg/g of wheat bran on the first day of incubation to 6.16 mg/g of wheat bran on the 5th day of incubation. Among the carbon sources used in the present study, glucose supplementation caused maximum secretion of protein on wheat bran.

Glucose exerted maximum effect on yields of FPase with 7.04 FPU/g of wheat bran followed by cellulose with 6.86 FPU/g of wheat bran on 3rd day of incubation and sucrose with 2.85 FPU/g of wheat bran on 1st and 2nd day of incubation (Table 4). Yields of FPase to the extent of 3.13 FPU/g of wheat bran only was obtained from fermented wheat bran without supplementation on the 2nd day of incubation.

Table 3. Effect of supplementation of carbon sources on secretion of protein in wheat bran by *A. niger* in SSF

Carbon source	Protein content (mg/g of wheat bran)				
	Incubation time (days)				
	I	II	III	IV	V
Control	6.08a	6.01a	6.16a	6.60a	6.16a
Sucrose	6.52a	5.12a	6.96b	6.44a	6.64ab
Glucose	7.88c	6.92b	7.96cb	8.44c	9.48bc
Cellulose	7.80d	7.01b	7.49d	7.56d	7.34c
Means, in each column, followed by the same letter are not significantly different ($P \leq 0.05$) from each other according to DMR test.					

Table 4. Effect of supplementation of carbon source on production of FPase in wheat bran by *A. niger* in SSF

Carbon source	FPase* (FPU/g of wheat bran)				
	Incubation time (days)				
	I	II	III	IV	V
Control	2.63a	3.13c	2.13ab	1.83b	1.94a
Sucrose	2.85a	2.85b	1.88a	1.32a	2.37b
Glucose	5.96b	5.81c	7.04c	6.33b	3.93c
Cellulose	5.83b	6.14d	6.86d	6.45b	6.50d
Means, in each column, followed by the same letter are not significantly different ($P \leq 0.05$) from each other according to DMR test.					
*Filter paperase (FPase) is expressed in terms of filter paper units. One unit is the amount of enzyme in the culture filtrate releasing 1 µmole of reducing sugar from filter paper per min.					

A glance of profile of yields of CMCase on wheat bran with/without carbon sources by *A. niger* in SSF indicated a clear cut trend of peak production on the first day of incubation followed by downward trend at subsequent intervals (Table 5). Glucose yielded higher titers of CMCase with 3.11 U/g of wheat bran than cellulose with 1.52 U/g of wheat bran and sucrose 1.54 U/g of wheat bran on the 1st day of incubation only.

Table 5. Effect of supplementation of carbon source on production of CMCase in wheat bran by *A. niger* in SSF

Carbon source	CMCase* (U/g of wheat bran)				
	Incubation time (days)				
	I	II	III	IV	V
Control	1.56a	0.67a	0.63a	0.22a	Nda
Sucrose	1.54a	1.22b	0.61a	0.28a	Nda
Glucose	3.11b	1.56b	0.72b	0.04b	0.52a
Cellulose	1.52a	1.28b	0.83b	0.81c	0.82b
Means, in each column, followed by the same letter are not significantly different ($P \leq 0.05$) from each other according to DMR test.					
*Carboxymethyl cellulase (CMCase) is expressed in terms of units. One unit is the amount of enzyme releasing 1 µmole of reducing sugar from carboxymethyl cellulose per min.					
Nd = Not detected					

Higher yields of β-glucosidase from fermentation of wheat bran with the carbon sources used in the present study on 2nd and 3rd day of incubation only (Table 6). Glucose yielded higher β-glucosidase with 0.0144 U/g of wheat bran on 2nd day of incubation than cellulose with 0.0136 U/g of wheat bran on 3rd day of incubation and sucrose with 0.0129 U/g of wheat bran on 2nd day of incubation.

Table 6. Effect of supplementation of carbon source on production of β-glucosidase in wheat bran by *A. niger* in SSF

Carbon source	β-glucosidase* (U/g of wheat bran)				
	Incubation time (days)				
	I	II	III	IV	V
Control	0.0032b	0.0125a	0.0121a	0.0123a	0.0123b
Sucrose	0.0033b	0.0129a	0.0118a	0.0127a	0.0120b
Glucose	0.0023c	0.0144b	0.0138b	0.0129a	0.0139b
Cellulose	0.0042c	0.0130b	0.0136b	0.0124a	0.0120b
Means, in each column, followed by the same letter are not significantly different ($P \leq 0.05$) from each other according to DMR test.					
*One unit of β-glucosidase activity is defined as the amount of enzyme liberating 1µmole of *p*-nitrophenol per min.					

Many investigators used different soluble sugars for inducing cellulases and carbon sources affected the type of enzyme produced (27, 29). Fadel (30) showed that glucose, maltose and sucrose induced FPase production, whereas, glucose and maltose induced CMCase. High levels of β-glucosidase activity were produced by addition of 1% sucrose than other levels and glucose, cellobiose, xylose and maltose. Addition of glucose at levels 5 and 10% of straw weight stimulated lignin degradation and cellulose hydrolysis when *Polyporus* sp. A-333 was cultivated on oat straw under SSF conditions (31). Low level of glucose (1%) in the growth medium did not repress CMCase synthesis but higher concentrations (10%) prevented further utilization of CMC due to inhibition of synthesis of CMCase (32). Glucose is the best inducer and lactose is found to be the best carbon source for the production of FPase and CMCase whereas β-glucosidase and protein content CMC and fructose was found to be the best inducers and carbon sources, respectively (53). Shruthi et al. (54) reported that the effect of NaoH treatment on the production of cellulose enzyme and found that 1% alkali treatment to ground nut fodder produced maximum filter paperase activity with 5.45 FPU/g of substrate, carboxymethyl cellulase activity of 4.75 U/g of substrate, and β-glucosidase activity of 19.0 U/g of substrate at 1 ml of crude cellulase preparation. Groundnut fodder supported significant production of FPase (5.9 FPU/g of substrate), CMCase (1.1 U/g of substrate) and *β*-glucosidase activity (6.5 U/g of substrate) in SSF among the solid supports screened in the study (55).

Different concentrations of glucose

Supplementation of at all concentrations used in this study was stimulatory to secretion of protein in solid state fermentation by *A. niger* on wheat bran throughout incubation period (Table 7). Additional supply of glucose at 0.5, 1.0, 2.5 and 5.0% levels caused increase in secretion of protein within a range of 14–37, 2.5–41, 15–54 and 18–41% respectively over control without supplementation on all incubation intervals used in this study. However, maximum (9.49 mg/g bran) secretion of protein into bran in solid state fermentation occurred on 5th day of incubation when *A. niger* was grown on wheat bran with 2.5% glucose supplementation.

Table 7. Effect of supplementation of glucose on secretion of protein in wheat bran by *A. niger* in SSF

Concentration of glucose (%)	Protein content (mg/g of wheat bran)				
	Incubation time (days)				
	I	II	III	IV	V
Control	6.08 a	6.01a	6.16 a	6.60 a	6.16 a
0.5	6.96b	7.00 b	8.44 c	8.40 c	8.08 b
1.0	7.68 c	6.16 b	6.72 a	8.48 c	8.68 b
2.5	7.88 c	6.92 b	7.96 cb	8.44 c	9.48 bc
5.0	7.68 c	5.12 a	7.28ab	7.92 b	8.68b
Means, in each column, followed by the same letter are not significantly different ($P \leq 0.05$) from each other according to DMR test.					

FPase production in solid state fermentation on wheat bran with glucose supplementation followed the trend similar to that of protein secretion (Table 8). Amendment of glucose improved FPase yields in solid state fermentation on wheat bran by *A. niger* by 44–245% increase over control. FPase yields reached maximum on 2nd day of incubation in control as against 3rd day of incubation in case of wheat bran with glucose supplementation. Solid state fermentation of wheat bran with 2.5% glucose supplementation by *A. niger* gave the maximum titres of 7.04 FPU/g bran on 3rd day of incubation.

CMCase in solid state fermentation on wheat bran with/without supplementation of glucose occurred higher yields on the first day of incubation and then onwards exhibited downward trend in production (Table 9). Supplementation of glucose at 5% level caused 3 fold increases in CMCase yields over control on the first day of incubation.

There was marginal increase in β-glucosidase production on wheat bran in solid state fermentation by *A. niger* with supplementation of glucose up to 1% level (Table 10). Addition of glucose beyond 1% level did not result in improvement of yields of β-glucosidase, in particular, on early incubation time (1st day) and caused decrease in activity of β-glucosidase.

Table 8. Effect of supplementation of glucose on production of FPase in wheat bran by *A. niger* in SSF

Concentration of glucose (%)	FPase* (FPU/g of wheat bran)				
	Incubation time (days)				
	I	II	III	IV	V
Control	2.63 a	3.13 a	2.13 a	1.83 a	1.94 a
0.5	5.26 b	5.93 c	6.52 b	6.15 c	2.81 b
1.0	5.70 b	6.22 c	6.37 b	6.22 c	4.30 c
2.5	5.96 b	5.81c	7.04 bc	6.33 c	3.93 c
5.0	6.04 bc	4.78 b	6.11 b	4.74 b	5.04 cd
Means, in each column, followed by the same letter are not significantly different ($P \leq 0.05$) from each other according to DMR test.					
*Filter paperase (FPase) is expressed in terms of filter paper units. One unit is the amount of enzyme in the culture filtrate releasing 1 µmole of reducing sugar from filter paper per min.					

Table 9. Effect of supplementation of glucose on production of CMCase in wheat bran by *A. niger* in SSF

Concentration of glucose (%)	CMCase* (U/g of wheat bran)				
	Incubation time (days)				
	I	II	III	IV	V
Control	1.56 a	0.67a	0.63 b	0.22 b	Nd a
0.5	2.89 b	1.19 a	0.67 b	0.37 c	0.178 c
1.0	3.85 bc	1.26 a	0.12 a	0.59 d	0.59 e
2.5	3.11 b	1.56 ab	0.72 b	0.04 a	0.52 d
5.0	4.70 d	1.78 ab	0.99 bc	1.22 e	0.04b
Means, in each column, followed by the same letter are not significantly different ($P \leq 0.05$) from each other according to DMR test.					
*Carboxymethyl cellulase (CMCase) is expressed in terms of units. One unit is the amount of enzyme releasing 1 µmole of reducing sugar from carboxymethyl cellulose per min.					
Nd = Not detected					

Table 10. Effect of supplementation of glucose on production of β-glucosidase in wheat bran by *A. niger* in SSF

Concentration of glucose (%)	β-glucosidase* (U/g of wheat bran)				
	Incubation time (days)				
	I	II	III	IV	V
Control	0.0032 c	0.0125 a	0.0121 a	0.0123 a	0.0123a
0.5	0.0035 d	0.0143c	0.0142b	0.0136ab	0.0132 b
1.0	0.0041 e	0.0139 b	0.0141 b	0.0123a	0.0132 b
2.5	0.0023 b	0.0144 c	0.0138 b	0.0129 a	0.0139 c
5.0	0.0002 a	0.0144 c	0.0139 b	0.0126 a	0.0140 c
Means, in each column, followed by the same letter are not significantly different ($P \leq 0.05$) from each other according to DMR test.					
*One unit of β-glucosidase activity is defined as the amount of enzyme liberating 1µmole of *p*-nitrophenol per min.					

The presence of glucose in the fermentation medium was found to be the most effective for production of glucanase, as well as for production of cellulolytic enzymes by *T. viride* (33). On the other hand sucrose induced cellobiase better than glucose in the same organism. But, glucose induced FPase better than sucrose in other organism *P. chrysogenum* on lignocellulosics in SSF condition. Among carbon sources tested in the present study, glucose (2.5%) served the best source followed by cellulose and sucrose for FPase, β-glucosidase and secretion of extracellular protein. However, CMCase production was maximum on 5.0 % level of glucose. Zhang et al. (34) reported that even in the absence of cellulose, cells of *Trichoderma reesei* C30 in such systems were found to produce some cellulase, with a maximum activity of about 0.12 FPU/mL. Monitoring of synthesis of the three groups of cellulase components, i.e., exoglucanase, endoglucanase and β-glucosidase, in fermentation using 5 g glucose/L plus 5 g cellulose/L as the C-substrates indicated that production profiles of the three components were similar except that the β-glucosidase activity was lower. This observation of synthesis of β-glucosidase to smaller extent in comparison to exoglucanase and endoglucanase in *T. reesei* is consistent with the other studies (35, 36).

Next to sucrose, starch and galactose were effective on production of cellulolytic enzymes. The production of cellulase and the maximum growth of *T. reesei* C5 were obtained with lactose as carbon source. Fermentative production of cellulase was made on substrates cellulose, xylose and lactose using different strains of *T. reesei* in submerged fermentation (37, 38). In the same study, cellulase activity was found to be less when glucose was used as carbon source because of inhibition. Cellulase production appeared to require residual inducer in the culture medium for a period of approximately 30–50 h while cellulose, lactose or glucose was the growth substrate (39). Olsson et al. (40) reported that more cellulose in the media resulted in higher levels of endoglucanase.

Souza and Peralta (41) reported that the enrichment of wheat bran cultures with glucose at 5 or 10% did not cause any catabolite repression. In submerged fermentation, the supplementation of glucose in medium at higher concentration in the media suppressed the utilization of carboxymethyl cellulase in *Aspergillus japonicus* (32) and effected pronounced repression synthesis of cellulase in *A. niger* (42). Glucose represses cellulase synthesis by a catabolite repression mechanism at the transnational level (43, 44). In case of solid state fermentation the reverse trend was observed in the present study, the supplementation of glucose in medium at higher concentration in the media enhanced the production of cellulase and secretion of protein. Maximal protein and high cellulase production i.e., 88.1 mg/g of protein, 31.5 IU/g of FPase, 46.6 IU/g of CMCase and 215.2 IU/g of β-glucosidase activity was achieved with inoculum size at 10% (v/w) in solid state fermentation of radicle by *A. niger* (30).

Spore density (Inoculum size)

Cultivation of *A. niger* was carried out on wheat bran moistened with optimized medium. The influence of spore density on production of cellulase on wheat bran by *A. niger* was assessed. Cultivation of *A. niger* on wheat bran with $2x10^5$ spores/g of bran secreted higher protein content to the extent of 33.60 mg/g of wheat bran followed by $2x10^4$ spores/g of bran with 33.12 mg/g of wheat bran on 2nd day of incubation (Table 11).

Table 11. Effect of spore density on secretion of protein into fermented bran by *A. niger* in SSF

Spore density (spores/g of wheat bran)	Protein content (mg/g of wheat bran)				
	Incubation time (days)				
	I	II	III	IV	V
2×10^4	18.24 a	33.12 c	24.0 a	29.28 bc	24.0 b
2×10^5	19.20 a	33.60 c	25.44 a	27.84 b	23.52 b
2×10^6	22.32 b	27.84 b	26.40 ab	26.88 b	20.64 a
2×10^7	27.84 c	22.08 a	23.52 a	21.60 a	21.60 b
Means, in each column, followed by the same letter are not significantly different ($P \leq 0.05$) from each other according to DMR test.					

Peak production of FPase in fermented bran on 2nd day followed by drop irrespective of spores in inoculum used was in tune with the results of earlier experiments (Table 12). Growth of *A. niger* on wheat bran with spore density with $2x10^6$ spores/g of bran produced maximum FPase activity with 27.78 FPU/g of wheat bran on 2nd day of incubation. Next to this, high FPase activity occurred in the fermented bran with spore density of $2x10^5$ on 2nd day of incubation. Even the peak production of FPase (17.78 FPU/g of wheat bran) in the fermented bran with $2x10^7$ spore density on 1st day of incubation was lowest in comparison to peak yields obtained with other spore densities.

As observed in previous experiments, peak production of CMCase on the first day of incubation followed by drop in its production occurred irrespective of size of inoculum used (Table 13). Fermented bran with

spore density of $2x10^6$ spores/g of bran yielded higher CMCase activity with 47.89 U/g of wheat bran followed by 46.67 U/g of wheat bran in the fermented bran which contained $2x10^4$ spores/g of bran on 1st day of incubation. Use of higher spore density $2x10^7$ spores/g of wheat bran produced less CMCase yields to the tune 43.33 U/g of wheat bran on 1st day of incubation.

Table 12. Effect of spore density on production of FPase on wheat bran by *A. niger* in SSF

Spore density (spores/g of wheat bran)	FPase* (FPU/g of wheat bran)				
	Incubation time (days)				
	I	II	III	IV	V
2×10^4	3.33 a	21.67 b	19.44 b	19.99 c	14.44 c
2×10^5	8.89 b	25.56 c	22.78 b	20.11 c	16.67 d
2×10^6	16.67 c	27.78 c	21.67 b	10.56 b	11.67 b
2×10^7	17.78 c	16.11 a	11.11 a	3.89 a	7.50 a
Means, in each column, followed by the same letter are not significantly different ($P \leq 0.05$) from each other according to DMR test.					
*Filter paperase (FPase) is expressed in terms of filter paper units. One unit is the amount of enzyme in the culture filtrate releasing 1 µmole of reducing sugar from filter paper per min.					

Table 13. Effect of spore density on production of CMCase on wheat bran by *A. niger* in SSF

Spore density (spores/g of wheat bran)	CMCase* (U/g of wheat bran)				
	Incubation time (days)				
	I	II	III	IV	V
2×10^4	46.67 b	46.11 ab	43.89 a	27.22 b	34.44 d
2×10^5	46.11 b	43.89 a	43.33 a	37.78 c	31.67 c
2×10^6	47.89 bc	46.11 ab	37.78 a	29.44 b	21.11 a
2×10^7	43.33 a	42.22 a	39.44 a	16.11 a	28.89 b
Means, in each column, followed by the same letter are not significantly different ($P \leq 0.05$) from each other according to DMR test.					
*Carboxymethyl cellulase (CMCase) is expressed in terms of units. One unit is the amount of enzyme releasing 1 µmole of reducing sugar from carboxymethyl cellulose per min.					

Cultivation of *A. niger* on wheat bran with highest spore density of $2x10^7$ spores/g of bran favored the β-glucosidase activity with 0.081 U/g of wheat bran on 4th day of incubation as against 0.0763 U/g of wheat bran by less spore density with $2x10^4$ spores/g of bran on 4th day of incubation (Table 14).

Table 14. Effect of spore density on production of β-glucosidase on wheat bran by *A. niger* in SSF

Spore density (spores/g of wheat bran)	β-glucosidase* (U/g of wheat bran)				
	Incubation time (days)				
	I	II	III	IV	V
2×10^4	0.00071 a	0.06623 a	0.07539 b	0.07627 b	0.07544 a
2×10^5	0.0014 a	0.07488 b	0.07488 b	0.07488 a	0.07544 a
2×10^6	0.01239 b	0.07544 b	0.07488 b	0.07488 a	0.07479 a
2×10^7	0.0173 c	0.07484 b	0.06447 a	0.08088 c	0.07484 a
Means, in each column, followed by the same letter are not significantly different ($P \leq 0.05$) from each other according to DMR test.					
*One unit of β-glucosidase activity is defined as the amount of enzyme liberating 1µmole of *p*-nitrophenol per min.					

The optimal size of inoculum was 10,000 viable spores for dry solids in solid surface fermentation by *Chaetomium globosum, Phanerochaete chrysosporium* and *T. reesei* on soya hull (45). In the present study, inoculum of $2x10^6$ spores/g of bran was optimal for higher production of cellulases by *A. niger*. Harikrishna et al. (46) reported that enzyme activities were found to be higher with the mycelia inoculum compared to the spore inoculum. Muniswaran and Charyulu (47) reported that the inoculum volume did not affect enzyme production very much on coconut coir pith for cellulase production by *Trichoderma viride* NCIM 1051. Larger inoculum size was detrimental to growth and production apart from adding to the fermentation cost (47). The marginal decrease seen at larger inoculum volumes may be due to the free excess unadsorbed liquid, which imposed an additional barrier for diffusion. Similarly, low yields of protein and all components of cellulase except β-glucosidase occurred in fermented bran cultivated with highest spore density in the present study. Spore density of $2x10^6$ spores/g bran was optimal for production of cellulolytic enzymes on wheat bran by *A. niger* on overall basis of four parameters. Optimal production of bacterial cellulases occurred with 15% inoculum size (v/w, based on dry weight of banana

fruit stalk), but further increase resulted in reduced enzyme yield (48). Fermentation was carried out by inoculating the substrate using $1.25x10^7$ spores/g substrate and incubating at 32^0C for 6 days using *R. arrhizus*, whereas *P. chrysosporium* $1.29x10^7$ spores/g substrate and incubating at 35^0C and *Aspergillus* sp. the inoculum size was $1.75x10^7$ spores/ g substrate (49).

CONCLUSIONS

The tremendous commercial potential of cellulases in a variety of applications remains the driving force for research in this bioconversion of cellulose (agro-residues) to soluble sugars such as glucose. Under optimal conditions, the protein, CMCase, FPase and β-glucosidase were increased up to 20%, 44%, 22% and 0.06%, respectively.

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