CELLULASE PRODUCTION BY *ASPERGILLUS FUMIGATUS* USING DIFFERENT PLANT-BASED AGRICULTURAL BIOMASS FOR PADDY STRAW SACCHARIFICATION

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Six different plant-based agricultural residues, *viz*. paddy straw, soybean pod husk, sugarcane bagasse, groundnut shells, corn stalks and pigeon pea pod husk were used for cellulase production. The studies on cellulase production by *Aspergillus fumigatus* CTS2 with different agricultural residues as substrate revealed that maximum filter paper, carboxymethyl cellulase, cellobiase and xylanase activities of 114.97, 512.95, 203.25 and 4295.51 nm/min/gds, respectively, were observed 72 hours after incubation with soybean pod husk. Scanning electron micrographs of fungi treated husk showed non-flaky, even surface features, probably due to consumption/hydrolysis of the debris (of host sample origin) by the enzyme secretions of the fungi. The maximum filter paper (89.54 nmol/min/gds), carboxymethyl cellulase (405.37 nmol/min/gds), cellobiase (171.26 nmol/min/gds) and xylanase 3765.76 (nmol/min/gds) activities were observed for equal proportions of paddy straw and soybean pod husk. The saccharification of pretreated paddy straw by *A. fumigatus* cellulase yielded maximum reducing sugar content of 486 mg/g 72 hours after incubation. The study revealed that soybean pod husk is a potential lignocellulosic residue that could be used as a supplement with paddy straw for maximizing cellulase production and saccharification of straw.

Keywords: agricultural biomass, cellulase, soybean pod husk, paddy straw, saccharification

INTRODUCTION

There is an increasing interest in the production of biofuels as an alternative solution to the energy shortage and greenhouse gas emissions.[1] Currently, bioethanol is predominately synthesized *via* the first-generation production process, where food-based crops, such as corn, sugar cane and wheat, are used as raw material. However, the conversion of food materials into biofuels has raised serious concerns regarding global food security and also adversely affected the public acceptance of biofuels.[2] Therefore, recent research on biofuels has been focused on the development of advanced generation biofuels. The production of bioethanol from lignocellulosic raw materials, *e.g.* wheat straw, corn stover and bagasse,[3] is a promising option.

Paddy straw is one of the most abundant lignocellulosic wastes of the world, with an annual production of 731 million tons, to which Asia contributes with about 667.6 million tons.[4] Paddy straw contains high cellulose (33-47%) and hemicellulose content (19-27%), which can be hydrolysed to sugars and thus, can be fermented into ethanol. The recalcitrance of straw has been identified as a major hindrance in its depolymerization. The resistance to enzymatic hydrolysis is caused by morphological and physicochemical factors, such as high lignin content, degree of crystallinity, degree of polymerization, hemicellulose sheathing, accessibility of inner microfibrils, porosity, moisture content and particle size of the substrate.[5] Multiple enzymes with different

specificities are required to deconstruct the complex lignocellulosic structure.[6] The synergetic action of lignocellulases – cellulases, hemicellulases, lignases – and lytic polysaccharide mono-oxygenases (LPMO) is required for an effective deconstruction of biomass for providing sugar-rich feedstock and its fermentation to produce ethanol.

Solid state fermentation (SSF) is rapidly gaining interest as a cost-effective technology for production of enzymes.[7] The most important factors to be considered during the development of a SSF are the choice of microorganisms and substrates.[8] The microorganisms that are particularly suitable for SSF are filamentous fungi, since the technique simulates their natural habitat and, under these conditions, they are able to synthesize considerable amounts of enzymes and other metabolites.[9] The most promising residues for SSF include agricultural and forestry residues, which are very abundant and normally underutilized. A fraction of them is utilized to generate electricity, while another large fraction is burnt without energy recovery or remains in the field, posing negative environmental impacts.[10] Agro-residues that can be used as substrates for SSF include sugarcane bagasse, cassava bagasse, cereal bran, such as wheat bran, rice bran, oat bran and soybean bran, coffee pulp and husks, fruit peels and pulps, corn cobs, straws and husks of different origins. These agro-residues not only provide solid support for fungal growth, but they are also a source of carbon and nutrients.[11] These agro-residues differ greatly in composition, chemical nature, mechanical properties, particle size, water retention capacity and surface area. The variation in these residue characteristics can affect the overall process design and product development.[12]

In this paper, we report fungal cellulase production under solid state fermentation using different plant-based agricultural residues. The best selected agricultural residue was used for large-scale enzyme production. The enzyme was extracted and subsequently utilized for hydrolysis of paddy straw into fermentable sugars.

EXPERIMENTAL

Materials

Chemicals

Chemicals, such as beech wood xylan, carboxymethyl cellulose (CMC) and bovine serum albumin (BSA), were purchased from SRL Chemicals. All the other chemicals required for medium preparation were procured from HiMedia Laboratories Ltd. (Mumbai, India). The chemicals and reagents used were of analytical grade. The standard cellulase enzyme was purchased from Central Drug House (P) Ltd., New Delhi.

Biomass material

Six different plant-based agricultural residues, *viz*. paddy straw (var. PR 121), soybean pod husk (var. SL 958), sugarcane bagasse (var. CoJ 88), groundnut shells (var. SG 99), corn stalks (PMH 1) and pigeon pea pod husk (var. PAU 881), were procured from the Department of Plant Breeding and Genetics, Punjab Agricultural University, Ludhiana. The different agricultural residues were thoroughly washed with tap water to remove soil, dust and other unwanted materials prior to sun drying. The residues were then pre-milled and screened, and the 30 mesh size fractions were used for further experiments.

Strain maintenance

A fungal strain identified as *Aspergillus fumigatus* (CTS2) stored in our laboratory was originally isolated from soil collected near Pathak Energy Workshop, PAU, Ludhiana. The fungus was maintained by repetitive sub-culturing on Potato Dextrose Agar (PDA) slants after 10 days (Fig. 1).

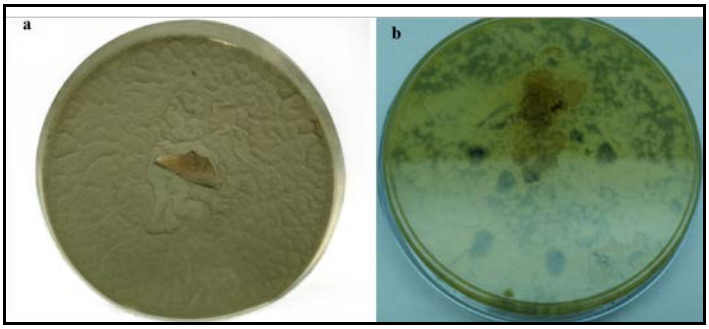

Figure 1: Maintenance of *Aspergillus fumigatus* CTS2 on potato dextrose agar medium; growth of fungus on (a) the front side, and (b) the back side

Methodology

Pretreatment of paddy straw

The sequential acid steam and microwave alkali pretreatment of paddy straw was carried out as per the method of Kaur.[13] For this, 5 g of powdered paddy straw was soaked in a 250 mL reagent bottle containing 87.5 mL of 2% sulfuric acid (w/v) and kept overnight. The samples were then autoclaved at 121 °C, 15 psi for 52 minutes. The treated slurry was filtered through Whatmann No. 1 filter paper and the residue was dried overnight at 60 °C. The thus obtained dried residue was then soaked in 3.75% sodium hydroxide (w/v) overnight and subjected to microwave treatment at 480 W for 10 minutes. The acid filtrate was analyzed for reducing sugars and total soluble sugars by the method of Nelson[14] and Dubois,[15] respectively.

Inoculum preparation

The fungus was cultured on potato dextrose agar plates at 45 °C for 3-4 days. The spore suspension was prepared from actively growing fungal culture by rinsing the agar plates with 10 mL sterile saline water (0.9%) and the spores were collected in a sterile tube. The spores were counted and adjusted to 1×10^8 spores/mL by using a haemocytometer.

Production medium

Mandels and Weber[16] medium was used for cellulase and xylanase production. The chemical composition of the medium (per litre) was as follows: 2 g KH_2PO_4, 1.4 g $(NH_4)_2SO_4$, 1 g peptone, 0.3 g $MgSO_4.7H_2O$, 0.3 g $CaCl_2$, 5 mg $FeSO_4.7H_2O$, 1.56 mg $MnSO_4.H_2O$, 1.4 mg $ZnSO_4.7H_2O$, 2.0 mg $CoCl_2.6H_2O$ and 1 mL Tween 80. The pH of the medium was adjusted to 5.0. The medium was sterilized by autoclaving at 121 °C for 20 min before inoculations.

Cellulase and xylanase production

A. fumigatus was grown on different plant-based agricultural residues under solid state fermentation to test the cellulolytic potential of this fungus on different substrates. For this, 2.5 g of each of the six agricultural residues were placed separately in different flasks. Then, 10 mL of Mandels and Weber[16] medium was added to each flask containing agricultural residues with a moisture content of 80 percent. The residues were then subjected to steam pretreatment by autoclaving at 121 °C for 20 minutes and cooled before inoculation. One millilitre of fungal spore suspension per gram of residue was inoculated into the different flasks, which were then incubated at 45 °C for 168 hours. The extraction of the crude enzyme mixture was carried out by adding 30 mL of 100 mM chilled citrate buffer (pH 4.8) into each flask, followed by shaking at 100 rpm and 27 °C for 10 minutes. The mixture was then centrifuged at 10,000 rpm at 4 °C for 15 minutes. The supernatant thus obtained was dialyzed against the same buffer (diluted 10 times) and assayed for different cellulolytic enzyme activities.

Co-substrating of paddy straw with plant-based agricultural residues

The agricultural residue that supported maximum production of enzyme by *A. fumigatus* was identified and was further used in different proportions, *i.e.* 1:1 and 3:1 ratios with paddy straw for cellulase production. The CMCase, filter paper, cellobiase and xylanase activities were determined.

Bulk production of cellulases

The bulk production of cellulase was carried out by taking 100 g of mixed agricultural residue as per the method described above. The enzyme was extracted and dialysed against sodium citrate buffer of pH 4.8 (diluted 10 times). The enzyme filtrate was then subjected to 80 percent ammonium sulphate precipitation. The precipitates were collected by centrifugation at 10,000 rpm in a cold centrifuge at 4 °C for 10 minutes and were dissolved in a minimal amount of 100 mM citrate buffer (pH 4.8) and then dialyzed against the same buffer (diluted 10 times). The enzyme was lyophilized and further used for hydrolysis of pretreated paddy straw.

Enzymatic saccharification of pretreated paddy straw

Paddy straw was pretreated with sequential acid steam, followed by microwave alkali treatment, as described above. The enzymatic saccharification of pretreated straw was carried out by incubating 1 g of pretreated straw with crude cellulase (5 FPU) in a stoppered 250 mL flask with a total volume of 50 mL made with 100 mM citrate buffer (pH 4.8). The hydrolysis was carried out at 50 °C and 100 rpm on a rotary shaker for 120 h. The samples (5 mL) were withdrawn after every 24 h, and then centrifuged at 10,000 rpm for 10 min. The total reducing sugars in the supernatant were estimated by the Nelson method. The percent saccharification was calculated as per the formula given by Singh.[17]

$$\text{Saccharification (\%)} = \frac{\text{Reducing sugars released (g)} \times 0.9 \times 100}{\text{Amount of cellulose (g)}}$$

Morphological characterization of fungal culture and agricultural residue

Morphological characterization of *A. fumigatus* was carried out by Optical Research Microscopy using a bright field condenser (primary staining). A drop of fungal suspension was spotted on the glass slide, covered with a cover slip and observed under the bright field condenser (20x and 40x). The morphology of untreated and fungi treated agricultural residue was studied using a Scanning Electron Microscope (SEM) (S-3400 N, Hitachi Science Limited, Japan) as per the standard protocol of Bozzola and Russell.[18] The samples were sputter-coated in a gold ion sputter coater prior to imaging under the Scanning Electron

Microscope at 250 and 500x magnification.

Analytical methods

Compositional analysis

The cellulose, hemicellulose and lignin contents of agricultural residues were determined by the method of Goering and Van Soest (1970).[19] The ash content and total nitrogen content of the residues were determined as per the standard method of AOAC.[20,21] The crude protein content was determined by multiplying the percent nitrogen content with the nitrogen-to-protein conversion factor.[22]

Enzyme assays

Crude enzyme was used for the assay of different cellulolytic activities. FPase activity of the enzyme filtrate was assessed by the method of Mandels *et al*. (1976).[23] FPase activity was determined by adding a strip of Whatman filter paper No. 1. (1 cm x 6 cm) to a reaction mixture that contained 0.5 mL of 100 mM citrate buffer (pH 4.8) and 0.5 mL enzyme preparation. CMCase activity was determined as described by the method of Wood and Bhat (1988).[24] CMCase activity was determined in a total reaction volume of 1 mL containing 0.3 mL enzyme preparation and 0.5 mL carboxymethyl cellulose solution (0.5%, w/v) in citrate buffer (50 mM, pH 4.8). The reaction mixture was incubated at 50 °C for 30 min for CMCase and 60 min for FPase assay. The reducing sugars released were measured by the method of Nelson (1944).[14] Cellobiase activity was measured according to the method of Toyama and Ogawa (1977).[25] Cellobiase activity was determined in a total reaction volume of 1 mL containing 0.5 mL enzyme preparation and 0.5 mL 0.05 percent (w/v) cellobiose solution in citrate buffer (100 mM, pH 4.8). This mixture was incubated at 50 °C for 120 min. The amount of reducing sugars was measured by Nelson's method. Xylanase activity was determined by the method of Bailey *et al.* (1992).[26] An amount of 1 g of beechwood xylan was dissolved in 100 mL citrate buffer (50 mM, pH 4.8), followed by heating to boiling under stirring. For determination of xylanase activity, 0.5 mL of this suspension (1%, w/v, xylan) in citrate buffer was incubated at 50 °C with 0.5 mL enzyme preparation for 10 min, and the hydrolysis product (reducing sugars) was measured by the Nelson method.[14] The enzyme activities were expressed as nanomoles of the reducing sugars released per min per g dry substrate (nmol/min/gds).

Statistical analysis

All the values presented in graphs and figures are the means of three replications. The data were statistically analyzed using the analysis of variance (ANOVA) and expressed as mean ± standard error (SE). The least significant difference was considered at $P \leq 0.05$.

RESULTS AND DISCUSSION

Chemical analysis of different plant-based agricultural biomass

The different plant-based agricultural residues were subjected to chemical analysis. The cellulose, hemicellulose, lignin, ash and crude protein contents of different residues have been presented in Table 1.

Table 1
Chemical composition of different plant-based agricultural residues

Agricultural residues	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Ash (%)	Crude protein (%)
Paddy straw	41.34 (39.99)	23.20 (28.78)	4.13 (11.72)	14.13 (22.07)	3.25 (10.38)
Soybean pod husk	39.72 (39.05)	12.50 (20.70)	9.17 (17.62)	8.13 (16.56)	6.86 (15.17)
Sugarcane bagasse	36.68 (37.26)	22.90 (28.58)	21.85 (27.86)	3.30 (10.46)	2.12 (8.36)
Groundnut shells	52.30 (46.31)	7.70 (16.10)	20.03 (26.58)	5.65 (13.74)	5.97 (14.13)
Corn stalks	40.97 (39.78)	17.37 (24.62)	18.25 (25.28)	5.37 (13.39)	3.53 (10.82)
Pigeon pea pod husk	40.14 (39.30)	9.60 (18.04)	8.30 (16.74)	3.54 (10.85)	6.46 (14.72)
CD (p = 0.05)	(0.20)	(0.39)	(0.31)	(0.32)	(0.48)

Values in parentheses are transformed arcsine means

The cellulose content was significantly high in groundnut shells, *i.e.* 52.30 percent, followed by paddy straw with a cellulose content of 41.34 percent. However, significantly high hemicellulose content of 23.20 percent was observed in paddy straw, followed by sugarcane

bagasse (22.90 percent). Significantly high lignin content was observed in sugarcane bagasse (21.85 percent), followed by groundnut shells with the lignin content of 20.03 percent, while the lignin content was reported to be significantly low in paddy straw (4.13 percent). High cellulose and hemicellulose content and low lignin content of paddy straw showed that it is a good lignocellulosic biomass for ethanol production. Significantly high ash content of 14.13 percent was observed in paddy straw, followed by soybean pod husk (8.13 percent). The high ash content in paddy straw could be due to the large amount of silica and other metal ions present in this residue, thus making it a low-quality feedstock for thermal processes.[27] The crude protein content was significantly high in soybean pod husk, *i.e.* 6.86 percent, which was statistically at par with the crude protein content of pigeon pea pod husk (6.46 percent), thus suggesting that these feedstocks are nutritionally rich for growth and multiplication of fungi.

Table 2
Chemical composition of paddy straw after sequential two-step chemical pretreatment

Chemical constituents	Composition (%)	
	Untreated paddy straw	Two-step pretreated paddy straw
Cellulose	41.34	81.93
Hemicelluloses	23.20	4.07
Lignin	4.13	2.90
Total soluble sugars	-	19.20
Reducing sugars	-	15.95
Ash	14.13	1.93
Crude protein	3.25	1.95

Effect of sequential (acid steam followed by microwave alkali) pretreatment on chemical composition of paddy straw

The paddy straw was subjected to sequential acid steam followed by microwave alkali pretreatment. The chemical analysis of straw (Table 2) revealed that the relative proportion of cellulose (81.93%) increased, while that of hemicellulose and lignin was drastically reduced (4.07 and 2.90%, respectively) in pretreated paddy straw, as compared to the relative proportion of cellulose, hemicellulose and lignin in untreated paddy straw (41.34, 23.20 and 4.13%). The total soluble sugar and reducing sugar content of 19.20 and 15.95 percent, respectively, was observed in the acid hydrolysate obtained after the acid steam pretreatment step, thus indicating a major breakdown of hemicellulose during this step. The main effect of acid treatment consisted in the reduction in the hemicellulose content, which is due to the susceptibility of amorphous xylan in hemicellulose to acid hydrolysis,[28] while the effect of alkali pretreatment consisted in lignin removal and biomass swelling by solvation and saponification.[29] The relative ash content was reduced from 14.13 percent in untreated paddy straw to 1.93 percent in pretreated straw, whereas crude protein content was reduced from 3.25 percent in the untreated paddy straw to 1.95 percent in the two-step pretreated straw.

The sequential acid and microwave alkali pretreatment process in our study thus led to the enrichment of cellulose in the solid fraction, containing 81.93 percent cellulose, along with a small amount of hemicellulose and lignin, as compared to the single-step pretreatments employed in previous studies, in which the cellulose content of 40 to 65 percent was obtained from various pretreated biomass using dilute acid, alkaline and ionic liquid treatments.[30-32] The two-step acid steam followed by microwave alkali pretreatment process can be thus used to increase the digestibility of paddy straw under mild conditions, as reported in this work.

Cellulase and xylanase production by *A. fumigatus* with different agricultural biomass as substrate

Cellulolytic (filter paper, CMCase, cellobiase) and xylanase activities of *A. fumigatus* CTS2 were studied with different agricultural biomass as substrate. The filter paper activity *A. fumigatus* CTS2 grown on different agricultural residues at different incubation times has been presented in Figure 2. The filter paper activity of *A. fumigatus* grown on paddy straw, soybean pod husk,

groundnut shell and pigeon pea pod husk was found to be maximum 72 hours after incubation and declined thereafter, while the activity was maximum 96 hours after incubation when the fungus was grown on sugarcane bagasse and cornstalk residues. The interaction between different incubation times and agricultural residues as substrates revealed that there were significant differences in the filter paper activity of *A. fumigatus* CTS2. The filter paper activity was observed to be maximum with soybean pod husk at different times after incubation with significantly higher activity, of 114.97 nmol/min/gds, 72 hours after incubation. The filter paper activity was found to be minimum for pigeon pea pod husk, with the maximum activity of 22.67 nmol/min/gds 72 hours after incubation.

The carboxymethyl cellulase (CMCase) activity of *A. fumigatus* (CTS2) on different agricultural residues as substrate and at different incubation times has been given in Figure 3. The interaction studies showed significantly higher CMCase activity, of 512.95 nmol/min/gds, on soybean pod husk 72 hours after incubation, followed by the CMCase activity of 259.71 nmol/min/gds 96 hours after incubation. The CMCase activity was found to be minimum on pigeon pea pod husk with the maximum activity (88.41 nmol/min/gds) 72 hours after incubation.

The data pertaining to cellobiase production by *A. fumigatus* (CTS2) on different agricultural residues as substrate have been given in Figure 4. The interaction studies revealed significantly higher cellobiase activity, of 203.25 nmol/min/gds, was recorded for soybean pod husk 72 hours after incubation, followed by the activity 96 hours after incubation (178.60 nmol/min/gds). The minimum cellobiase activity was observed when the fungus was grown on pigeon pea pod husk with the maximum activity of 65.05 nmol/min/gds 72 hours after incubation.

The xylanase activity of *A. fumigatus* (CTS2) on different residues and at different incubation times has been presented in Figure 5. The xylanase activity of *A. fumigatus* (CTS2) was higher on soybean pod husk, as compared to other agricultural residues, at different hours after incubation. The interaction studies revealed significantly higher xylanase activity, of 4295.51 nmol/min/gds, on soybean pod husk 72 hours after incubation, followed by the activity of 4173.36 nmol/min/gds 96 hours after incubation. The minimum xylanase activity was reported for pigeon pea pod husk at different periods after incubation, with the maximum activity of 548.17 nmol/min/gds 72 hours after incubation.

Soybean is a leguminous crop and its straw is superior in nitrogen content,[33] as compared to other straws, and it provides sufficient nutrients for the growing fungus with high cellulolytic activity. The husk particles may also remain well aerated under moist conditions during solid state fermentation and facilitate the free exchange of gases for growth and respiration of the fungus.[34] This is the first study that reports that soybean pod husk is a good substrate for large-scale production of both cellulases and xylanases by *A. fumigatus* fungus without supplementation of any nutrients in the solid state fermentation medium.

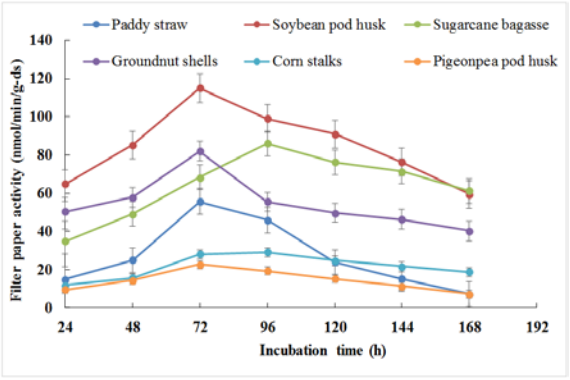

Figure 2: Filter paper activity of *A. fumigatus* CTS2 using different agricultural residues as substrate under solid state fermentation (LSD (p = 0.05): residue (R) – 1.06, incubation time (h) – 1.14, Rxh – 2.80)

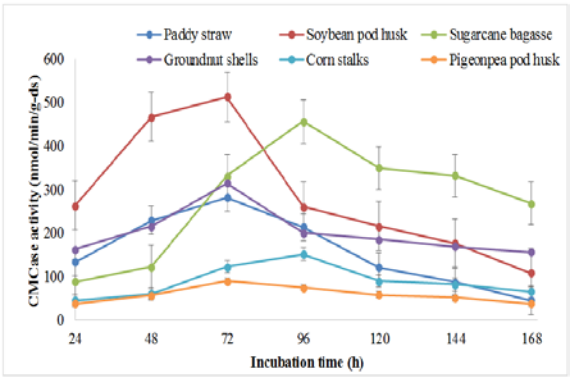

Figure 3: CMCase activity of *A. fumigatus* CTS2 using different agricultural residues as substrate under solid state fermentation (LSD (p = 0.05): residue (R) – 3.54, incubation time (h) – 3.83, Rxh – 9.37

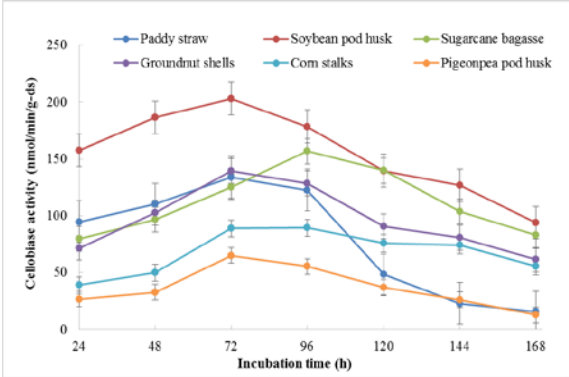

Figure 4: Cellobiase activity of *A. fumigatus* CTS2 using different agricultural residues as substrate under solid state fermentation (LSD (p = 0.05): residue (R) – 0.73, incubation time (h) – 0.79, Rxh – 1.94

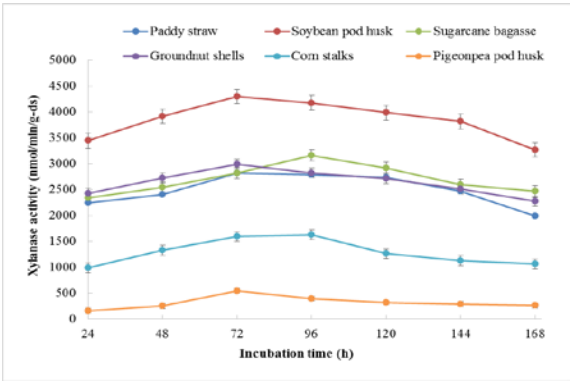

Figure 5: Xylanase activity of *A. fumigatus* CTS2 using different agricultural residues as substrate under solid state fermentation (LSD (p = 0.05): residues (R) – 5.63, incubation time (h) – 6.08, Rxh – 14.89

Morphological characterization of fungal culture and soybean pod husk

The optical microscopy observation of *A. fumigatus* using the bright field condenser showed the occurrence of thin septate hyphae bearing chains of circular exogenous spores on aerial sporangiophores (Fig. 6). The SEM micrograph of the ground soybean pod husk (control) showed the presence of debris on the surface (Fig. 7 (a and c)), which may have been produced by the mechanical grinding of the substrate. At higher magnifications, the pod husk surface did not show the occurrence of microbial cells. The surface topography of the sample appeared to be rough, dry and flaky, probably because of the presence of lignin, silica and other hydrophobic components. The inoculation of ground soybean pod husk with *A. fumigatus* CTS2 resulted in vigorous ramification of the vegetative hyphae of the inoculated fungus on the surface. The images at 250 and 500x magnifications showed the presence of columnar, uniseriate spore heads with asexual spore chains formed by basipetal succession on short conical conidiophores of the test fungus (Fig. 7 (b and d)). There were marked differences in the surface topography of the soybean pod husk after fungal inoculation. The surface of the fungus-inoculated husk appeared non-flaky or was devoid of substantial amounts of debris, contrary to the surface of the uninoculated husk. This may be due to the consumption/hydrolysis of the debris (of host sample origin) by the enzyme secretions of the fungi.

Cellulase production by *A. fumigatus* CTS2 with different proportions of paddy straw and soybean pod husk under solid state fermentation

The data pertaining to cellulase production by *A. fumigatus* CTS2 under solid state fermentation with paddy straw and soybean pod husk as substrates in 1:1 and 3:1 ratios at different incubation times have been presented in Table 3. Significantly higher filter paper, CMCase, cellobiase and xylanase activities, of 89.54, 405.37, 171.26 and 3765.76 nmol/min/gds, were observed 72 hours after incubation with a ratio of 1:1 of paddy straw and soybean pod husk. The carbon/nitrogen ratio (C:N value) of paddy straw is very high, *i.e.* about 80:1, as compared to other agricultural residues.[35] Thus, the addition of soybean pod husk to paddy straw decreased the C:N value of the resulting mixture, thereby improving the culture conditions for fungal growth and cellulase production.[36] The carbon/nitrogen ratio of fungal growth medium has a major role in evaluating the efficiency of the solid state fermentation process for enzyme production.[37]

Bulk production of cellulase by *A. fumigatus* CTS2 with equal proportions of paddy straw and soybean pod husk as substrate

Bulk production of cellulase by *A. fumigatus* on a mixed substrate of paddy straw and soybean pod husk (1:1 ratio) has been presented in Table 4.

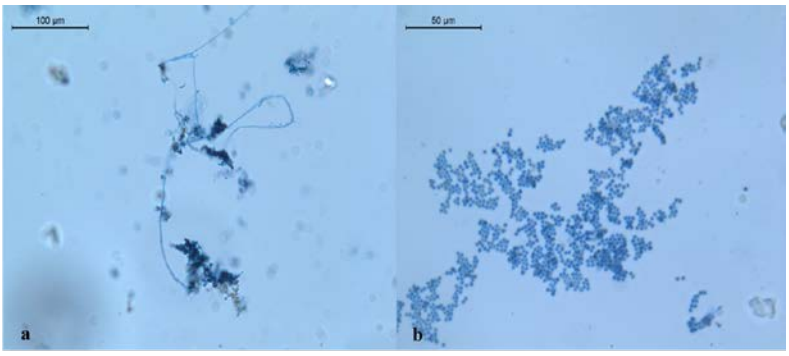

Figure 6: Morphological details of *Aspergillus fumigatus* CTS2 using a bright field condenser at (a) 200x and (b) 400x magnification

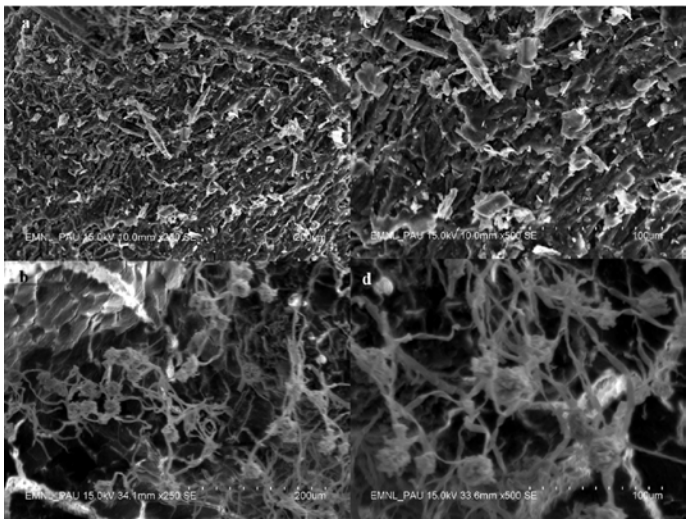

Figure 7: Scanning electron micrographs of ground soybean pod husk (control) (a and c), and fungi treated soybean pod husk (b and d) at 250x and 500x magnification

Table 3
Cellulase activities of *Aspergillus fumigatus* CTS2 using paddy straw and soybean pod husk in different proportions as substrate under solid state fermentation

Incubation time	Filter paper activity (nmol/min/gds)		CMCase activity (nmol/min/gds)		Cellobiase activity (nmol/min/gds)		Xylanase activity (nmol/min/gds)	
	1:1	3:1	1:1	3:1	1:1	3:1	1:1	3:1
24 h	29.88 (5.56)	20.20 (4.60)	127.43 (11.33)	104.98 (10.29)	122.66 (11.12)	102.62 (10.18)	2671.04 (51.69)	2408.23 (40.08)
48 h	60.83 (7.86)	36.65 (6.14)	254.34 (15.98)	235.17 (15.37)	146.92 (12.16)	122.05 (11.09)	3060.20 (55.33)	2729.69 (52.26)
72 h	89.54 (9.51)	71.76 (8.53)	405.37 (20.16)	338.25 (18.42)	171.26 (13.12)	142.07 (11.96)	3765.76 (61.37)	3322.74 (57.65)
96 h	76.54 (8.81)	61.31 (7.89)	327.38 (15.73)	265.41 (16.32)	151.38 (12.34)	130.39 (11.46)	3254.78 (57.06)	2949.05 (54.31)
120 h	60.28 (7.83)	45.76 (6.84)	246.67 (15.73)	169.71 (13.06)	130.75 (11.48)	111.03 (10.58)	2933.09 (54.17)	2853.29 (53.43)
144 h	45.76 (6.84)	27.46 (5.33)	186.48 (13.69)	138.08 (11.79)	116.89 (10.86)	94.05 (9.75)	2647.59 (51.46)	2522.48 (50.23)
168 h	33.02 (5.83)	17.58 (4.31)	133.57 (11.60)	116.16 (10.82)	101.64 (10.13)	73.87 (8.65)	2471.41 (49.72)	2205.98 (46.98)
CD (p = 0.05)	(0.14)	(0.13)	(0.29)	(0.26)	(0.05)	(0.05)	(0.12)	(0.10)

Figures in parentheses are transformed square root means

Table 4
Bulk production of cellulases from *Aspergillus fumigatus* (CTS2)

Purification step	Filter paper activity		CMCase activity		Cellobiase activity		Xylanase activity	
	Total activity (IU)	Purification fold	Total activity (IU)	Purification fold	Total activity (IU)	Purification fold	Total activity (IU)	Purification fold
Crude extract (dialyzed)	13.96 (0.04)	1.00	41.94 (0.12)	1.00	16.08 (0.04)	1.00	397.35 (1.09)	1.00
Ammonium sulphate precipitation (0-80%)	10.36 (0.17)	4.25	23.85 (0.38)	3.17	12.51 (0.20)	5.00	161.82 (2.60)	2.39

Enzyme activity expressed as IU = μmol/min; Value in parentheses is specific activity (IU/mg)

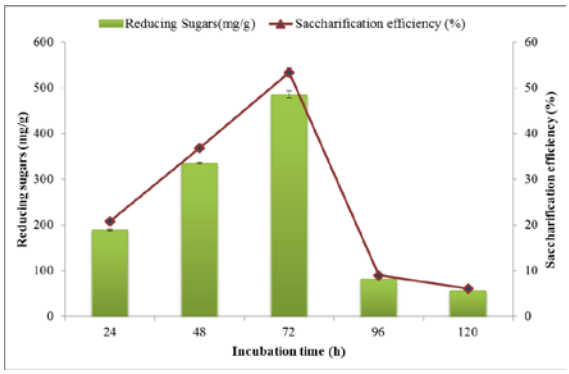

Figure 8: Reducing sugar content (mg/g) of paddy straw hydrolysate and saccharification efficiency (%) of *Aspergillus fumigatus* CTS2 at different incubation times

Total filter paper, CMCase, cellobiase and xylanase activities of 13.96, 41.94, 16.08 and 397.35 IU were observed in the dialysed fraction of crude extract, respectively. The ammonium sulphate precipitated fractions recorded total filter paper, CMCase, cellobiase and xylanase activities of 10.36, 23.85, 12.51 and 161.82 IU and specific activities of 0.17, 0.38, 0.20 and 2.60 IU/mg, respectively. The purities of the filter paper, CMCase, cellobiase and xylanase activities were calculated as approximately 4.25-, 3.17-, 5.00- and 2.39-fold, respectively, by 80% ammonium sulphate precipitation, followed by dialysis, which was greater than that of the crude enzyme. The cellulase produced in bulk was used subsequently for saccharification of pretreated paddy straw. Mehboob *et al.*[38] suggested that microorganisms cultured on lignocellulosic wastes could be used for the production of cellulolytic enzymes on a large scale. Maximum endoglucanase, exogluconase and β-glucosidase production was observed after 72 h at 55 °C, pH 5.5, and 70 percent moisture level. The endoglucanase, exogluconase and β-glucosidase enzymes were purified to 2.63-, 3.30-, 4.36-fold, respectively, by 40 percent ammonium sulphate precipitation, followed by gel filtration chromatography.

Enzymatic saccharification of pretreated paddy straw

The studies on enzymatic hydrolysis of pretreated paddy straw with *A. fumigatus* cellulase revealed an increase in reducing sugar content from 24 to 72 hours after incubation, which declined thereafter. The highest reducing sugar content, of 486 mg/g, was observed 72 hours after incubation with saccharification efficiency of 53.39 percent (Fig. 8). The release of reducing sugars from enzymatically saccharified biomass depends on the specific binding of the celluloytic enzymes with the substrates, as well as on the availability of cellulose in the biomass.[39] The acid steam followed by microwave alkali pretreatment of paddy straw increased the availability of cellulose in the pretreated biomass for enzymatic hydrolysis and the release of reducing sugars. The

decline in reducing sugar content after 72 hours of incubation could be caused by the depletion of the more amorphous substrate, by product inhibition and enzyme inactivation.[40]

CONCLUSION

From the present study, it may be concluded that, among the plant-based agricultural residues considered, soybean pod husk is a superior lignocellulosic residue for cellulase production. The addition of soybean pod husk to paddy straw in equal proportions stimulated high cellulolytic enzyme production by *A. fumigatus*, which was subsequently required for the hydrolysis of paddy straw into reducing sugars, which, in turn, can be fermented into ethanol. This can contribute to reducing the overall cost of bioethanol production from paddy straw.

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