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Full Length Research Paper

Efficiency of rice straw lignocelluloses degradability by *Aspergillus terreus* ATCC 74135 in solid state fermentation

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The ability of *Aspergillus terreus* for the production of cellulolytic enzymes and reduction of lignocellulose contents of rice straw in solid state fermentation was investigated in this study. Results suggested that, 8 days fermentation was appropriate, with enzymes activities as follows: FPase = 410.76 U/gDM, CMCase = 351.96U/gDM, β-glucosidase = 16.37 U/gDM, xylanase = 6166.01 U/gDM and amyloglucosidase = 425.04 U/gDM (with maximum 993.71 U/gDM on day 6). In addition, the solid state fermentation significantly (P < 0.01) reduced the concentrations of NDF, ADF, cellulose and hemicellulose in the rice straw by 19.96, 13.8, 16.32 and 32.87%, respectively. The high degradation of the hemicellulose was reflected by the high activity of xylanase enzyme, which hydrolyses xylan in hemicellulose to xylose. Higher reducing sugar and microbial cell mass productions were also obtained after 8 days fermentation. Present data showed that, *A. terreus* is capable of producing high quantity of cellulolytic enzymes for the reduction of lignocellulose contents of biomass in a shorter incubation time when compared with the previously reported for biological treatment of agricultural by-products using white rot fungi.

Key words: *Aspergillus terreus*, biomass, biological treatment, enzyme activity, solid state fermentation.

INTRODUCTION

Huge quantities of agro-industrial biomass are produced worldwide annually, that is including about 900 million tons of rice straw (RS) which more than 90% are produced in Asia. Although, these materials are potential feed resources for ruminant livestock, their use is limited because of the high indigestible fiber components. The burning of these materials in the field is of pollution concerns. Biodegradation of cellulose and hemicelluloses content of agro-biomass and convert into microbial mass and bioactive materials is alternative method for enhancement the quality of these materials for used as animal feed. Solid state fermentation (SSF) is the growth of microorganisms on moist solid materials in the absence or near absence of free water (Lonsane et al., 1985) and has been used for production of enzymes (Cen and Xia, 1999), bioethanol (Amin, 1992), antibiotics (Adinarayana et al., 2003), biologically active secondary metabolites (Balakrishnan and Pandey, 1996) and for enhancement of nutritive value of agricultural biomass as animal feed (Iluyemi et al., 2006). Rice straw can be used as a potential substrate for biomaterial production in SSF. Cellulose and hemicelluloses are the main components in this agricultural biomass and during the solid state fermentation process, these materials break down into compounds of smaller molecules and high quality compo-

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nents (such as glucose and microbial protein) and their digestibility were improved for used as animal feed.

Microorganisms that are selected for solid state fermentation should have the capability to produce sufficient quantity, the appropriate enzymes to degrade the specific cellulose and hemicellulose in the substrate. Cellulose enzymes, which can hydrolyze cellulose to produce glucose, galactose and cellobiose, can be divided into three enzymes: endoglucanase (endo-1,4-b-D-glucanase, EC 3.2.1.4); cellobiohydrolase (exo-1,4-b-D-glucanase, EC 3.2.1.91) and β-glucosidase (1,4-b-D-glucosidase, EC 3.2.1.21) (Li et al., 2004). Xylanase (EC 3.2.1.8) is the potential enzyme for hydrolysis of hemicellulose to yield xylose, while starch can be hydrolyzed by amyloglucosidase. These enzymes are the most important catalyzers in feed industry and consumption of these enzymes can increase the digradability and digestability of agro-biomass for ruminants.

Studies have shown that, *Aspergillus terreus* can produce cellulase and hemicellulase to degrade lignocellulose in biomass (Workman and Day, 1982; Emtiazi et al., 2001; Gao et al., 2008). Gao et al. (2008) demonstrated the ability of *A. terreus* M11 for endoglucanase activity (581 U/gDM), filter paper activity (243 U/gDM) and β -glucosidase activity (128 U/gDM) using corn stover and yeast extract as carbon and nitrogen sources. However, there is a lack of inforrmation on the use of *A. terreus* for reduction of lignocellulose content in agro-biomass, such as rice straw for use as animal feed. The objective of this study was to identify the types of cellulolytic enzymes produced by *A. terreus* ATCC 74135 in SSF and the extent of reduction of lignocellulose content in rice straw at different incubation times to assess the potential use of this method to improve the nutritive value of RS as animal feed.

MATERIALS AND METHODS

Substrate

Rice straw was collected from the local rice fields from the state of Selangor, Malaysia. The material was dried and ground to uniform size (No. 6 meshes) and stored in plastic bags at 4°C for later use as substrate in this study.

Microorganism and preparation of spore suspension

A. terreus ATCC 74135 used in this study was obtained from American type culture collection (ATCC). It was maintained on potato dextrose agar (PDA) slants at 25°C for 7 days, stored at 4°C and sub-cultured every two weeks.

For the preparation of spore suspension, 10 ml of sterilized 0.1% Tween-80 solution was added to the 7-day old culture slants of the fungus. At the end of incubation, the surface of the culture was scratched with sterilized loop and agitated thoroughly using a shaker to suspend the spores. The number of the spore was measured using a haemocytometer and adjusted to approximately 10^7 spores/ml and was used as inoculums throughout the study.

Solid state fermentation

Solid state fermentation of rice straw was carried out in 500 ml Erlenmeyer flasks. 30 g of RS was put in individual flasks and 30 ml distilled water (contain 1% urea as nitrogen source) were added to give moisture content of approximately 50%. The flasks were plugged with cotton-wool and autoclaved at 121°C for 15 min. Each flask was inoculated with 10% (v/w) inoculums containing 10^7 spores per ml for the fermentation. The flasks were incubated at 25°C and after 2 days intervals, 3 flasks were removed from the incubator, dried at 60°C for 24 h and stored in a refrigerator (4°C) for future analysis.

Chemical analysis

Dry matter (DM) loss was determined by the difference between dry weight of sample before and after fermentation and described as percentage of initial weight of the sample. Neutral detergent fibre (NDF) and acid detergent fibre (ADF) were determined by the detergent system (Van Soest et al., 1991) and acid detergent lignin (ADL) by method described by AOAC (1990). Hemicellulose contents were estimated as the difference between NDF and ADF, while cellulose content was the difference between ADF and ADL.

Enzyme extraction

At periodic intervals, 30 ml of citrate buffer (50 mM, pH 5.0) were added into 1 g fermented substrate and the mixtures were shaken (200 rpm) for 60 min at 4°C and centrifuged at 5520 x g for 5 min. The supernatants were filtered (Whatman no. 1 filter paper) to remove suspended spores and later stored at 4°C for future assays of enzymes activity.

Enzyme assay

Filter paper activity (FPase), endoglucanase (carboxymethyl cellulase) and beta-glucosidase were determined according to the method of Grajek (1987). Filter paper activity was assayed by incubating 1 ml of extracted enzyme, Whatman filter paper No.1 (50 mg, 1×6 cm) and 1 ml citrate buffer at 60°C for 30 min in an incubating shaker. The glucose concentration was determined by the dinitrosalicylic acid (DNS) method (Miller, 1959).

Endoglucanase (carboxymethylcellulase, endo-1,4-b-D-glucanase; EC 3.2.1.4) activities were determined by estimation of the reducing sugar liberated by the action of this enzyme on carboxymethyl cellulose (CMC). One percent of carboxymethyl cellulose in citrate buffer (50 mM, pH 5) was used as substrate and 1 ml of substrate was aded into 1 ml of the extracted enzyme and incubated for 30 min in 60°C in an incubating shaker. The reaction was stoped by adding 1 ml of potassium sodium tartrate (Rochelle salt). Concentration of free carboxymethyl glucose units which reacted with dinitrosalicylic acid reagent was estimated using the DNS method.

β-Glucosidase (β-D-glucoside glucohydrolase; EC 3.2.1.21) activities were estimated using *p*-nitrophenyl-b-Dglucopyranoside (*p*NPG) as substrate. The total assay mixture (2 ml) containing 1 ml of *p*NPG (1 mM) and 1 ml of extracted enzyme was incubated at 60°C for 60 min. After incubation, 4 ml of 0.01 M sodium hydroxyde was added to stop the reaction. *p*-nitrophenol liberated was measured spectrophotometrically at 420 nm.

Xylanase activity was estimated by method of Bailey et al. (1992). 1% Xylan from oat (Sigma) was used as substrate in the reaction with the extracted enzyme and incubated at 50°C for 30 min. At the end of the incubation, the concentration of xylose was

determined by the DNS method and xylose (sigma) was used as standard.

Amyloglucosidase activity was determined by incubating 1 ml extracted enzyme and 1 ml of substrate solution contain 1% starch from barley in 50 mM citrate buffer (pH 5) at 55°C for 20 min. The reducing sugars were measured with the DNS method using glucose as standard (Bernfield, 1955). Results of enzyme activities are expressed in terms of units per gram dry mater (U/gDM), where one unit of enzyme is the amount of enzyme that can produce 1 µM glucose from the substrate in one minute.

Fungal mass estimation

Fungal mass estimation was done by N-acetyl glucosamine released after acid hydrolysis of chitin present in fungal cell wall (Ramachandran et al., 2005). For conversion of chitin into glucosamine, 0.1 g of fermented sample was mixed with 0.5 ml concentrated sulphuric acid and incubated at 30°C for 24 h. This mixture was diluted with distilled water to make 1 N solution and autoclaved in 15 psi for 15 min. Sodium hydroxide (2 N) was used to neutralize the solution and later made up to 30 ml with distilled water. Extracted glucosamine was mixed with 1 ml of acetyl acetone reagent (1 ml of acetyl acetone in 50 ml of 0.5 N sodium carbonate) and incubated in a boiling water bath for 20 min. After cooling, 6 ml of ethanol was added, followed by the addition of 1 ml of Ehrlich reagent (2.67 g *p*-dimethylaminbenzaldehyde in 1:1 mixture of ethanol and HCl and later made up to 100 ml) and incubated at 65°C for 10 min. At the end of the incubation period, the solution was cooled and its optical density was read at 550 nm. Glucosamine hydrochloride from sigma was used as standard with different concentrations between 0 to 50 µg/ml. Cellmass is expressed in terms of mg of N-acetyl glucosamine released per g DM (mg/gDM).

Scanning electron microscope

Microscopy analysis was conducted using a scanning electron microscope to determine the morphological growth of *A. terreus* on the surface of rice straw. The samples were dried, cut into 1 mm and affixed to a metal SEM stub and sputter coated in gold by using SEM coating unit (BAL-TEC SCD 005 Spotter coater). The coated specimens were viewed using PHILLIPS XL30 environmental scanning electron microscope at accelerating voltage of 15 to 25 KV.

Statistical analysis

Data were analysed using the general linear model (GLM) procedure of SAS 6.12 (1988). All multiple comparisons among means were performed using Duncan's new multiple range test.

RESULTS AND DISCUSSION

Enzymatic activities

Enzyme activities of *A. terreus* in solid state fermentation of rice straw are shown in Table 1. Total Cellulase activity of *A. terreus* was indicated by the activity of filter paper activity, carboxymethylcellulase (CMCase) and β-glucosidase. Filter paper activity that represent total cellulase activity (Badhan et al., 2007), was at its highest at 8 and 10 days of fermentation (410.76 and 480.48 U/gDM,respectively) and thereafter, the activity declined. This amount is higher than the activity that was reported by others (Table 2). The activities of carboxymethylcellulase were highest in samples harvested from 6, 8 and 10 days fermentations, while those for β-Glucosidase were from 6 and 8 days fermentations (16.37 and 15.97 U/g DM, respectively). Kang et al. (2004) have reported the 129 units of CMCase activity using *Aspergillus niger KK2* and RS as substrate in solid state fermentation. Kalogeris et al. (2003b) have reported the 1709 units of CMCase activity using *Thermoascus aurantiacus* and wheat straw as substrate. The highest activity recorded in this study was xylanase, which catalyzes xylan to xylose and many reports have shown the high ability of different fungi for production of xylanase (Table 3). In this study, maximum activity of this enzyme was from 8 days of fermentation (6166.01 U/gDM). The maximum activity of amyloglucosidase was obtained from 6 days fermentation (993.71 U/gDM). Different studies have reported the ability of *A. terreus* for production of FPase, CMCase and β-glucosidase (Gao et al., 2008), xylanase (Suvarna Lakshmi et al., 2009) and amyloglucosidase (Ali et al., 1990). These enzymes are important in the process of cell wall degradation of biomass. Abilities of different fungi for production of cellulases and xylanase from the literature are shown in Tables 2 and 3, respectively. The data clearly indicated high variation for production of enzyme using solid state fermentation. Carbon source and microorganism are most important factors for production of these enzymes in SSF and the presence results show that, rice straw is a suitable carbon source for production of cellulolytic enzyme by *A. terreus* in solid state fermentation.

Lignocelluloses content

The effects of *A. terreus* on lignocellulose contents of rice straw are shown in Table 4. Fermentation has significant effect on acid detergent fibre, neutral detergent fibre, cellulose and hemicellulose contents of RS ($P < 0.01$) but not on acid detergent lignin. The results also suggested that, 8 days fermentation is most appropriate to achieve significant reduction of lignocellulose by *A. terreus*. Duration of fermentation is of practical importance in the biological treatment of biomass, as the growth rate of the microorganism used for this purpose should be high and thus, achieved the target of improving the quality of the biomass in a short time. White rot fungi are widely studied in biological treatment, however, their growth rates are slow and need a long incubation period, often more than 21 days and even up to 2 to 3 months to be effective (Kirk and Moore, 1972). Long incubation period is of disadvantage under farm-scale conditions because space and storage is limited in the farm and it is not economically acceptable for farmers to keep the feed for a long time. On the other hand, long time incubation will increase the dry mater loss of fermented samples to

Table 1. Enzyme activity of *A. terreus* in solid state fermentation of RS.

Incubation time (day)	Enzyme activity (U/gDM)				
	FPase	CMCase	B-Glucosidase	Xylanase	Amyloglucosidase
2	193.20 ± 12.68^{c}	126.84 ± 18.23^{c}	5.24 ± 0.46^{d}	4035.17 ± 105.53^{d}	410.76 ± 62.39^{d}
4	237.72 ± 25.37^{c}	269.64 ± 46.67^{b}	14.67 ± 0.42^{b}	5848.88 ± 201.69^{ab}	659.40 ± 72.92^{b}
6	397.32 ± 81.90^{b}	363.72 ± 5.25^{a}	16.37 ± 1.03^{a}	5884.81 ± 136.02^{ab}	993.71 ± 41.97^{a}
8	410.76 ± 29.06^{ab}	351.96 ± 54.88^{a}	15.97 ± 0.09^{a}	6166.01 ± 92.59^{a}	425.04 ± 46.49^{d}
10	480.48 ± 32.11^{a}	348.60 ± 32.11^{a}	14.69 ± 42.99^{b}	5758.28 ± 304.32^{b}	519.12 ± 42.99^{c}
12	204.12 ± 24.04^{c}	237.72 ± 22.02^{b}	12.62 ± 0.54^{c}	5273.99 ± 208.56^{c}	283.92 ± 7.70^{e}
Significance	**	**	**	**	**

**: Significantly different at 1% level ($P < 0.01$); a, b, c and d: indicating means within row differed significantly

Table 2. Cellulases enzyme production by different fungi in solid state fermentation.

Microorganism	Carbon source	Enzyme activity (U/g DM)			Reference
		CMCase	Fpase	β-Glucosidase	
Fusarium oxysporum	Corn stover	304		0.14	Panagiotou et al. (2003)
Trichoderma reesei MGG77	Rice bran		2.314		Latifian et al. (2007)
Funalia trogi IBB 146	Wheat straw	356	26		Kachlishvili et al. (2006)
Sporotrichum thermophile	Beet pulp, cellulose	109		12.1	Grajek (1986)
Trichoderma reesei ZU-02	Corncob		158		Xia and Cen (1999)
Trichoderma koningi F244	Wheat bran	287.3	94	184	Li et al. (2004)
T. aurantiacus	Wheat straw	1709	5.5	79	Kalogeris et al. (2003b)
Aspergillus niger KK2	Rice straw	129	19.5	100	Kang et al. (2004)
T. aurantiacus	Wheat straw	1572		101.6	Kalogeris et al. (2003a)
A. terreus M11	Corn stover	581	243	128	Gao et al. (2008)
A. terreus ATCC74135	Rice Straw	363.72	480.48	16.37	Present research

Table 3. Xylanase activity (U/gDM) by different fungi in solid state fermentation.

Microorganism	Carbon source	Xylanase (U/gDM)	Reference
A. terreus (MTCC 8661)	Palm oil fibre	115,269	Suvarna Lakshmi et al. (2009)
Thermomyces lanuginosus	Sorghum straw	48,000	Bakri et al. (2003)
Trichoderma longibrachiatum	Wheat bran and wheat straw	592.7	Azin et al. (2007)
A. terreus ATCC:74135	Rice straw	6,166.01	Present research

more than 50%. Results of this study show that, *A. terreus* has faster growth rate than other fungi, including white rot fungi in biological treatment for reduction of lignocellulose contents in biomass.

Reduction rates of different components of the ligno-celluloses are reflected by the activities of the specific enzymes. Based on the data of 8 days fermentation, 32.86% of the hemicelluloses were degraded, while only 16.32% of the cellulose was degraded within the same duration (Table 4). The 2 folds higher degradation rate of the hemicelluloses than the cellulose is reflected by the significantly higher activity of xylanase (6,166 U/gDM), purportedly to hydrolyze xylan in hemicelluloses to xylose, when compared with the total activity of the cellulose enzymes (FPase, CMCase and B-glucosidase, 778 U/gDM) (Table 1). High xylanase activity of fungi was also reported by other researchers (Table 3).

Dry matter loss, pH and reducing sugars

The effect of *A. terreus* on DM loss, pH and reducing sugar are shown in Table 5. As discussed previously, based on the enzyme activity and lignocellulose degrada-bility data, 8 days fermentation was sufficient for the biological treatment of RS using *A. terreus*. At this incubation period, the DM loss was 12.27%, which is lower than that reported using white rot fungi (Jung et al.,

Table 4. Effect of incubation time on lignocellulose composition of RS (% of dry matter).

Incubation time (days)	NDF	ADF	ADL	H-cellulose	Cellulose
0	79.26 ± 0.97^{a}	53.64 ± 0.27^{a}	5.47 ± 0.18	25.62 ± 1.22^{a}	48.17 ± 0.14^{a}
2	73.92 ± 1.42^{b}	52.47 ± 1.30^{a}	5.54 ± 0.35	21.46 ± 0.15^{b}	46.92 ± 1.42^{a}
4	67.68 ± 1.64^{c}	47.69 ± 1.04^{b}	5.99 ± 0.97	19.99 ± 1.10^{bc}	41.70 ± 1.86^{b}
6	64.95 ± 0.50^{d}	46.93 ± 1.20^{b}	5.53 ± 0.60	18.02 ± 1.16^{cd}	41.40 ± 1.61^{b}
8	63.44^{d} ± 1.19^{e}	46.24 ± 0.72^{b}	5.93 ± 0.94	17.20 ± 1.62^{de}	40.31 ± 1.49^{bc}
10	64.18 ± 1.41^{de}	46.86 ± 0.79^{b}	5.25 ± 0.65	17.32 ± 2.02^{de}	41.61 ± 0.67^{b}
12	62.61 ± 0.53^{e}	47.66 ± 2.02^{b}	5.97 ± 1.17	14.94 ± 1.51^{e}	38.36 ± 1.12^{c}
Significance	**	**	NS	**	**

NS: Not significantly different (P > 0.05) **: significantly different at 1% level (P < 0.01); a, b, c and d: indicating means within row differed significantly.

Table 5. Effect of incubation time on pH, dry matter (DM) loss and sugar content of RS.

Incubation time (day)	Reducing sugar (mg/gDM)	pH	DM loss (%)
0	6.47 ± 0.07^{d}	7.39 ± 0.16^{a}	-
2	8.5 ± 0.40^{d}	7 ± 0.16^{b}	3.74 ± 0.09^{f}
4	15.16 ± 0.78^{bc}	6.66 ± 0.06^{c}	7.77 ± 0.68^{e}
6	16.04 ± 0.21^{abc}	6.47 ± 0.08^{d}	10.33 ± 0.14^{d}
8	17.63 ± 1.73^{a}	6.39 ± 0.01^{d}	12.27 ± 0.31^{c}
10	17.11±1.12^{ab}	6.39 ± 0.02^{d}	13.54 ± 0.36^{b}
12	14.7±2.17^{c}	6.48 ± 0.03^{d}	18.63 ± 0.16^{a}
Significant	**	**	**

**: Significantly different at 1% level (P < 0.01); a, b, c, d, e and f: indicating means within row differed significantly.

1992; Jalc et al., 1998). One of the problems of biological treatment using white rot fungi is high DM loss as the results of long incubation period. Jung et al. (1992) studied the effect of five white rot fungi (*basidiomycetes*) for the improvement of the quality of oat straw. Although,30 days of fermentation using *Phanerochaete chrysosporium* had shown enhancement in *in vitro* DM digestibility, but 42.3% of DM were lost due to the long incubation time required. Jalc et al. (1998) reported 43% DM loss after biological treatment of wheat straw using *Daedalea guercina* (white rot fungi).

Solid state fermentation using *A. terreus* had significant (P < 0.01) effect on reducing sugar content of rice straw (Table 5). Enhancement of reducing sugar over incubation times was correlated with enzyme activity and reduction of lignocelluloses described earlier. Zadrazil (1984) reported that, cellulose and hemicelluloses are converted to soluble (partly sugars) materials during the incubation. Reducing sugars, include glucose, xylose and mannose, have higher digestibility for the animals than their original macromolecules (cellulose and hemicelluloses), therefore, enhancing the concentration of these sugars is an additional advantage besides the reduction of lignocelluloses in the improvement the quality of biomass through biological treatment

Reduction of pH over days of fermentation is due to the. production of acids (such as itaconic acid) by *A. terreus* (Petruccioli et al., 1999). Rumen microorganisms are sensitive in acidic condition and ruminant feed ingredient with low pH significantly reduces the activity of these microorganisms (pH <6). Although, biological treatment reduces the pH of the fermented samples, they are still within the acceptable range of rumen microorganisms.

Fungal cell-mass

Determination of fungal cell-mass in solid state fermentation is more difficult than in liquid state fermentation. This is because it is difficult to separate the fungal mycelia from the solid substrate in SSF. Chitin (a component of fungal cell-mass) was used as indicator for the determination of fungal cell-mass in SSF (Fang et al., 2010). In the process of hydrolysis of the fermented samples, chitin is converted into glucosamine which can be determined using spectrophotometer. The glucosamine (index for cell-mass) contents of the fermented rice straw during the fermentation are shown in Figure 1, with maximum microbial cell-mass production at 8 days fermentation (60 mg/gDM). One of the benefits of biological treatment is the production of microbial cell-mass; because of its high content of true protein, fatty

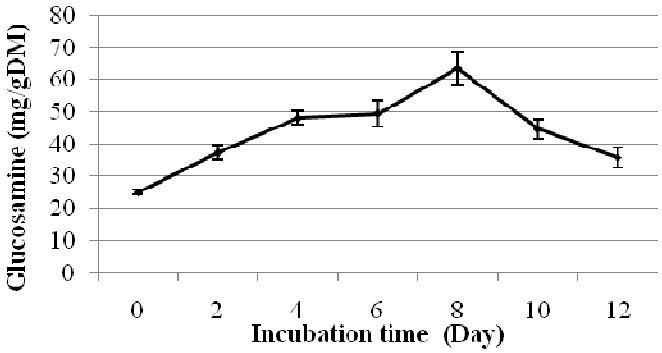

Figure 1. Effect of incubation time on glucosamine formation.

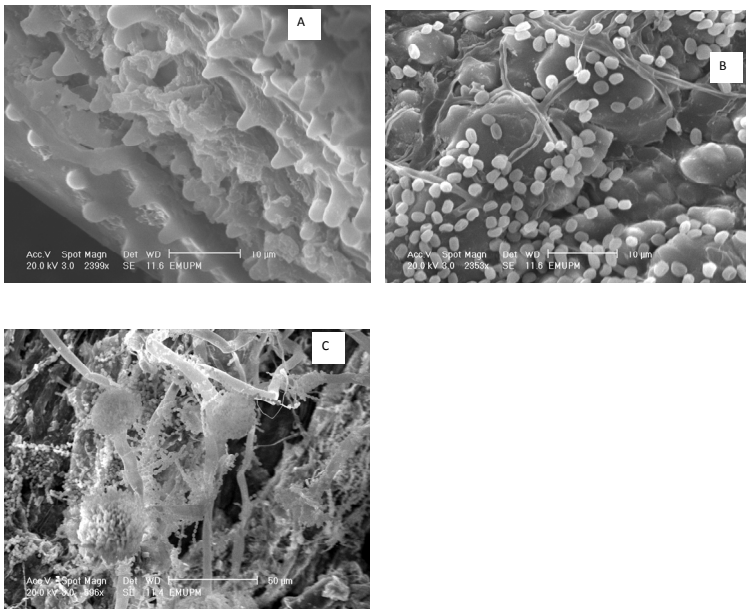

Figure 2. Scanning electron micrographs. (A) Non fermented rice straw; (B) fungal hyphae and spores of *A. terreus* on the surface of rice straw; (C) sporangium of *A.*

acids and soluble carbohydrates (Shojaosadati et al., 1999; Iluyemi et al., 2006) as reflected by an enhancement in the digestability of protein for the fermented materials (Iconomou et al., 1998).

Extensive attachment of mycelium, spores (Figure 2b) and sporangium (Figure 2c) of *A. terreus* on the surface of treated rice straw when compared with the untreated sample (Figure 2a) suggesting the efficacy of *A. terreus* for production of lignocellulolytic enzymes and degrada-tion of lignocellulose in rice straw.

One of the applications of solid state fermentation is the use of this technique for enhancement of biomass quality as animal feed. The microorganisms to be selected for SSF should be able to produce the appropriate lignocellulolytic enzymes at sufficient quantity to effectively degrade the fibre contents. The enzymes that had been determined in this study (cellulase, xylanase and amyloglucosidase) are among the widely used enzymes in animal feed additive for the degradation of lignocelluloses in feed. Huge quantity of agricultural biomass are produced worldwide annually and are potential sources of pollution if not appropriately managed. Although, these biomass can be used as animal feed, their high cell wall content is the inhibiting factor for their practical usage; because cellulose, hemicellulose and lignin contents of these materials have negative correlation with their degradability and digestibility. Reduction of these components as shown in the present study, is a useful indicator for improvement of the quality of lignocellulotic materials using biological treatment. In addition, the cellulolytic enzymes present in the treated biomass has an additional potential benefit to continue to be active in the rumen ecosystem to futher degrade the lignocellulosic contents of feed in the rumen.

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