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SACCHARIFICATION OF MAIZE AGROWASTES BY CELLULOLYTIC FUNGI ISOLATED FROM EJURA FARMS IN EJURA, GHANA.

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ABSTRACT
Maize is the most abundant cereal grown in Ghana and is accompanied by enormous amount of maize agrowastes. This waste which is currently underutilized can be used to produce bio-ethanol. Saccharification of lignocellulosic materials into simple sugars is a crucial and costly step towards bio-ethanol production from them. This work isolated cellulolytic fungi from soil sampled from Ejura farms which can efficiently hydrolyse maize agrowastes. Ten of the isolates exhibited cellulase activities when screened on Mandel's agar media. Aspergillus niger had the highest significant filter paper activity, carboxymethyl cellulose activity and protein concentration of 0.37 FPU/ml 0.7025 U/ml and 5.62 mg/ml respectively when the cellulolytic isolates were assayed on corncob based broth media.

Keywords: *cellulolytic fungi, enzyme activity, lignocellulose*

INTRODUCTION

Maize (*Zea mays*) is the most abundant cereal produced in Ghana (Asante, 2004). According to Asante (2004), production between 1995 and 2004 had hovered around one million metric tons per annum. This was accompanied by large quantities of maize agro wastes (husk, cobs and stove).

These agrowastes are underutilized. Currently the corncobs are burnt as fuel in the households of peasant rural farmers. The husks are used for wrapping kenkey and the stoves are usually burnt or left in the field. In large commercial farms where harvesting is mechanized, the agrowastes are left on the field and ploughed into the soil.

Production of bio-ethanol from maize agrowastes with cellulase is feasible but is relatively expensive and enzyme cost contributes about 40% (Howard *et al.*, 2003; Miyamoto, 1997). Search for high cellulase-producing organisms is one way of reducing the higher production cost (Bon and Ferarra, 2007). The objective of this work is to isolate fungi from soil samples from Ejura farms which will efficiently hydrolyze maize agrowastes into simple sugars for bio-ethanol production. Soil was sampled from Ejura farms because the agrowastes are left on the field and ploughed back into the soil making it a good habitat for cellulolytic fungi but has not been explored (Agana, 2007).

MATERIALS AND METHODS

Isolation of fungi

Fungi were isolated from soil samples collected from Ejura farms in Ashanti region. Potato Dextrose Agar (PDA) containing chloramphenicol (500 mg/ml) to suppress bacterial growth was used as isolation medium. The inoculated plates were incubated at 25°C for 4-5 days. The cultures were further purified by subculturing on PDA. The methods of Burnett and Hunter (1972), Malloch, (1997), Guarro *et al*., (1999), www.doctorfungus.org, 2008; www.mycologyonline.adelaide.edu.au, 2008 were employed for identification of the isolates.

Screening of fungal isolates for cellulase activity

Preliminary screening of isolates was carried out on Mandel agar medium (MAM). MAM was prepared as described by Jeffries (1987) and consisted of the following per liter: $(NH_4)_2SO_4$ 1.4 g, KH_2PO_4 2.04 g, $CaCl_2$ 0.3 g, $MgSO_4.7H_2O$ 0.3 g, Urea 2.1g, Citric acid 0.25 g, Tween 80 2 ml, Avicel cellulose 10 g, Peptone1 g, and Trace metal stock solution 1 ml. The trace metal stock solution consisted of the following chemicals per 500 ml: $FeSO_4$ 2.55 g, $MnSO_4.H_2O$ 0.93 g, $ZnSO_4.H_20$ 1.78 g, Co $(NO_3)_2.6H_2O$ 1.25 g, and Conc. HCl 5 ml.

Colony diameter and clearing zone diameter of the isolates were measured and clearing zone diameter to colony diameter ratios were calculated. Based on these ratios the nine highest isolates were selected for further screening.

Further screening was performed in corncobs-based broth (CBB) medium. Preparation of CBB was similar to MAM except that cellulose was substituted with corncob powder. Corncobs were sun dried and pounded. It was then oven dried at 120˚C for 3 days after which it was milled and sieved (Mesh series 70, Endecott filter) to obtain the powder. Hundred milliliters of CBB medium was dispensed into each 250 ml conical flasks. Inoculum of each isolate was prepared by flooding a 4-days old slant of that isolate with 10 ml sterile distilled water. With the aid of flamed inoculating loop, conidia were scratched and the tubes were shaken vigorously to break the clumps of conidia to obtain a homogenous suspension. The spores of each isolate were estimated with hyaemocytometer (Improved Neubauer Haemocytometer). Approximately 1×10^7 spores of each isolate were inoculated into a conical flask containing 100 ml of autoclaved CBB under laminar flow. The inoculated conical flasks were incubated on a shaker (G24 Environmental incubator shaker) at 150 rpm at room temperature (25˚C) for 6 days. Five milliliters aliquots were taken from each inoculated medium 3 and 6 days after inoculation. The aliquots were centrifuged in centrifuge (Centrikon T-42K) at 10000g for 10 min at 4˚C, the supernatants decanted into new tubes and stored frozen for few days for enzyme assay**.** Freezing and thawing has less effect on enzyme activity for short period (Waravdekar *et al., 1964*)

Determination of enzyme activity

Cellulase activities were determined by filter paper assay (FPA) and carboxymethyl cellulose assay (CMCA). The procedures for these assays were adopted from those previously described (Jeffries, 1987; Ghose, 1987 and Adney and Baker, 1996). Whatman No.1 filter paper and carboxymethyl cellulose (degree of substitution = 0.7) were substrates in FPA and CMCA respectively. Reducing sugars released from these assays were quantified by DNS assay. The DNS reagent (1% DNS solution and 40% potassium sodium tartrate or Rochelle salt solution) was prepare as described by Wang (2008). Three milliters of DNS reagent and one ml potassium sodium tartrate solution were added to the content of each assay tube. The contents of the tubes were boiled in a vigorously boiling water bath for 5 minutes and then added to each tube and transferred to cold ice-water bath. One ml of potassium sodium tartrate was then added to the content of each tube and transferred to ice-cold water bath. Intensity of colour formation was determined by measuring absorbance at 540 nm with spectrophotometer (Helios UV Visible Spectrometer). Amount of reducing sugars released was deter-

mined from glucose standard curve (glucose (mg/0.5ml) against absorbance at 540nm). FP and CMC activities were estimated: Filter paper unit, FPU = mg glucose released ×0.185 and CMC Unit = mg of glucose released × 0.37.

Total proteins of the isolate supernatants were determined by Biuret method. Four milliliters of Biuret reagent was added to 1 ml of the supernatant and incubated at 37°C for 20 minutes. The colour intensity was determined at an absorbance of 540 nm and the protein concentration calculated from egg albumin curve.

Experimental Design and Statistical Analysis
All the tests were laid in complete randomized design and each treatment was tested in triplicate. ANOVA analyses were carried out with Assistat 7.5 beta. Graphs were plotted with Microsoft excel.

Table 1: Number of species isolated in each genus

Genera	Number of species
Aspergillus	4
Trichoderma	1
Penicillium	2
Mucor	1
Fusarium	1
Rhodotorula	1
Acremonium	1
Coccidioides	1

RESULTS AND DISCUSSION

Twelve species from 8 different genera were isolated from the soil. These were *Aspergillus terreus, Trichoderma sp. Aspergillus versicolor, Penicillium sp.9, Mucor sp. Aspergillus flavus, Aspergillus niger, Fusarium sp. Penicillium sp. 7, Rhodotorula sp. Acremonium sp.* and *Coccidioides sp.*

The number of species from each genus is illustrated in Table 1. The genus *Aspergillus* was the most diverse followed by *Penicillium* species.

Ten out of the 12 isolated species exhibited cellulolytic activity. The number of cellulolytic fungi isolated is comparable to those cited in literature. Kader and Omar (1998), in a scientific expedition to Sayab-Kinabalu park at Sabah in Malaysia isolated 16 cellulolytic fungi species. Baig *et al.*,(2003), in a similar work isolated 12 cellulolytic fungi from a banana farm at Maharashtra in India. *Coccidioides sp* was not tested for cellulolytic activity because it is highly infectious as human pathogen (www.mycology.adelaide.edu.au/Mycoses/ Dimorphic_systemic, 2008)

Table 2: Mycelial diameter in centimeters of the isolates on MAM

Isolates	Mean diameter at 3 days	Mean diameter for 6 days
A. terreus	3.4^{b}	4.22^{b}
Trichoderma	7.26^{a}	8.31^{a}
A. versicolor	1.50^{d}	2.13^{d}
Penicillium sp.9	2.50^{c}	3.00^{c}
Mucor sp.	1.23^{d}	0.41^{f}*
A. flavus	3.5^{b}	4.36^{b}
A. niger	2.4^{c}	3.93^{b}
Fusarium sp	1.57^{d}	2.49^{cd}
Penicillium sp.7	1.30^{d}	2.220^{d}
Rhodotorula sp.	0.13^{e}	0.21^{f}
Acremonium sp.	1.10^{d}	1.18^{e}

The alphabets represent the order of the means within a column which are significantly different at P = 5% by Duncan's Multiple Range Test (DMRT). Means in a column with different superscripts are significantly different.
** Measurement was not taken after 3 days.*

The isolates were cultured on Mandel agar media (MAM). Mycelial and clearing zone diameters were measured on the 3, 5 and 6 days after inoculation. The average was calculated as mean (Table 2). The 3 days mycelia diameter mean is of importance because *Mucor sp.* exhibited profuse aerial growth which made measurement after third day impracticable. The mean of mycelial diameter is an indicator of the ability of the isolates to hydrolyze Avicel cellulose (pure crystalline cellulose).

There were significant differences ($p<0.05$) between the mean mycelial diameters for both the 3 and 6 days period. *Trichoderma sp.* had the highest mean mycelial diameter for 3 days period and was significantly different from other species, followed by *A. flavus, A. terreus, Penicillium sp.9 and A. niger* whilst *Rhodotorula sp.* recorded the least. For the mean mycelia diameter over the 6 days period, *Trichoderma sp.* recorded the highest followed by *A. flavus, A. terreus* and *A. niger*, in that order.

All the 10 cellulolytic species isolated had been reported to exhibit cellulase activity with *Trichoderma sp.* and *Aspergillus sp.* being prominent (Lynd *et al*., 2002; Miyamoto,1997; Bon and Ferrara, 2007; Rajesh *et al.*, 2008). Of the 10 cellulolytic species, 4 were *Aspergillus* species and 2 were *Penicillium* species. *Aspergillus* and *Penicillium* species were the abundant cellulolytic species isolated from Rawalpindi district in Pakistan by Khalid *et al.,* (2006).

Clearing zone diameter to mycelial diameter ratio indicates the ability of the isolates to exude extracellular enzymes into the media. The ratios for 3 and 6 days periods are shown in Table 3. The ratios for 3 days are preferred to 6 days period because the petri plate size restricted the clearing zone diameter of fast growing *Trichoderma* species. Profuse aerial growth of *Mucor sp.* made measurement after 3 days impracticable. *Mucor sp.* had the highest ratio whilst *Rhodotorula sp.* recorded the least ratio of 1 implies that there was no difference between its clearing zone diameter and mycelial diameter.

The ability of a fungus to hydrolyze pure crystalline cellulose does not necessarily translate into ability to hydrolyze lignocellulosics (Lynd *et al.,* 2002). Nine of the cellulolytic isolates were cultured in CBB at pH 5.0 at 25˚C. The mean enzyme activities and protein concentrations of the isolates were determined. The unit of activity (U) is defined as the amount of enzyme liberating l µmol glucose per minute in a standard assay.

Table 3: Clearing zone to mycelial diameter ratios for the isolates

Isolates	Ratio at 3 days	Ratio at 6 days
A. terreus	1.16^{bc}	1.28^{ab}
Trichoderma	1.18^{bc}	1.08^{cd}
A. versicolor	1.09^{bc}	1.12^{bc}
Penicillium sp.9	1.11^{bc}	1.00^{d}
Mucor sp.	1.97^{a}	1.32^{ab*}
A. flavus	1.34^{bc}	1.33^{ab}
A. niger	1.43^{b}	1.47^{a}
Fusarium sp	1.39^{b}	1.2^{ab}
*Penicillium sp.*7	1.09^{bc}	$1.14b^{c}$
Rhodotorula sp.	1.00^{c}	1.00^{d}
Acremonium sp.	1.09^{bc}	1.10^{cd}

The alphabets represent the order of the means within a column which are significantly different at P = 5% by Duncan's Multiple Range Test (DMRT). Means in a column with different superscripts are significantly different.

** Measurement was not taken after 3 days.*

Filter paper activity (FPA) measures the overall activity of all the cellulase components (exoglucanase, endoglucanase and β−glucosidase). There was significant difference ($p<0.05$) between FPA of the isolates. Fig. 1 illustrates the mean FPA of the isolates. *A. niger* had the highest mean FPA of 0.3648 FP U/ml and was significantly different. This is comparable to similar works cited in literature. Immanuel *et al.,* (2007) reported of isolated *A. niger* strain with maximum FPA of 0.262 U/ml on sawdust-based medium. Baig *et al.,* (2003) also reported of *Trichoderma lignorum* isolated from banana field with maximum FPA of 0.45 U/ml on banana-based medium.

A. niger was followed by *Trichoderma sp.* and *A. flavus* with 0.3412 U/ml and 0.3208 U/ml respectively. *Fusarium sp.* and *Penicillium sp.7* recorded the least.

Carboxymethyl Cellulose (CMC) activity measures endoglucanase activity. There was significant difference ($p<0.05$) between the CMC activity of the isolates and the mean CMC activities are illustrated on Fig. 2. *A. niger* had the highest mean CMC activity of 0.7025 U/ml and was significantly different. It was followed by *A. flavus* and *Trichoderma sp.* with 0.5938 U/ml and 0.5878 U/ml respectively. *A. terreus, Penicillium sp. 9, Fusarium sp.* and *Penicillium sp. 7* were the least significant.

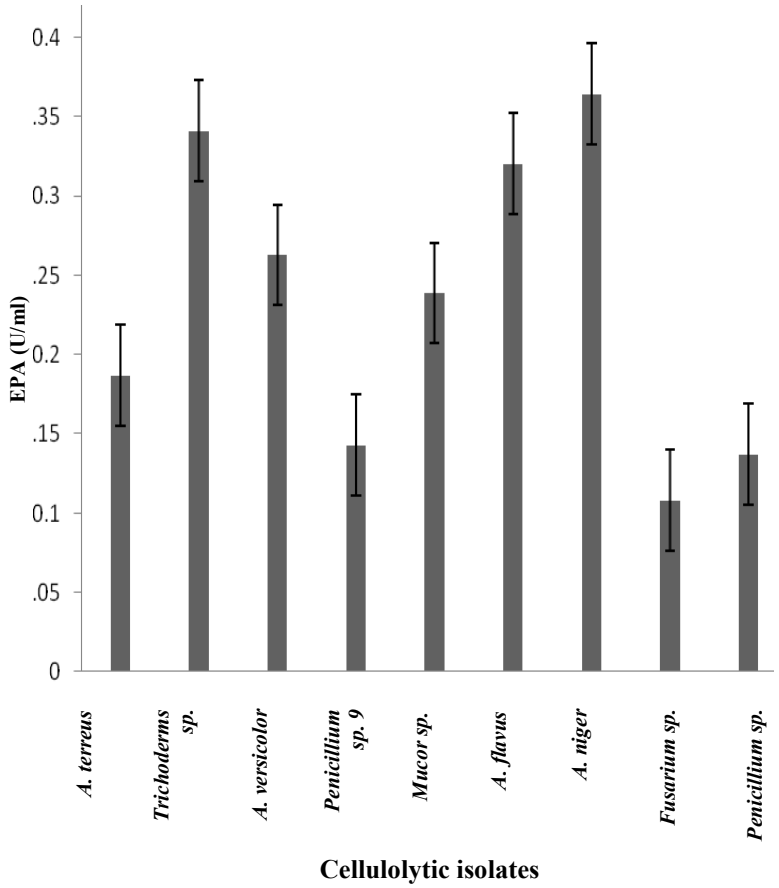

Fig. 1: Mean FPA (U/ml) of the cellulolytic isolates in CBB over 6 days period

Most enzymes are proteins so protein concentration can indicates enzyme concentration. There was significant difference ($p<0.05$) in the mean protein concentration of the isolates. The mean protein concentration of the isolates is illustrated in Fig 3. *A. niger* had the highest protein concentration of 5.6155 mg/ml followed by *A. flavus* with 4.5493 mg/ml though the difference between them is not significant. *Fusarium sp.* was the least significant.

A. niger had the highest enzyme activities (FPA and CMCA) and protein concentration. The higher FPA may be due to superior composition of its cellulase component (Olofsson *et al.,* 2008) or possession of other complementary enzymes such as lignase and hemicellulase (Baig *et al.,* 2004). The effective hydrolysis of lignocellulosics is determined by the constitution of the three components of cellulase.

Aspergillus genus is notable for its higher endoglucanase activity measured as CMC activity (Miyamoto, 1997). Since there is a direct link between protein concentration and enzyme concentration, it might account for the higher protein concentration of *A. niger*.

Although *Trichoderma sp.* had the highest significant mean mycelial diameter on Mandel agar medium: an indicator of cellulase activity (Maheshwari *et al.*, 2000), its enzyme activities (FPA and CMCA) on CBB came next to *A. niger*. This may be due to the impediments posed by the lignocellulosic apart from crystallinity. The optimal composition of cellulase determines lignocellulosic hydrolysis (Olofsson *et al.,* 2008). The cellulase constituents in this case were predetermined by the source of the enzyme (isolate).

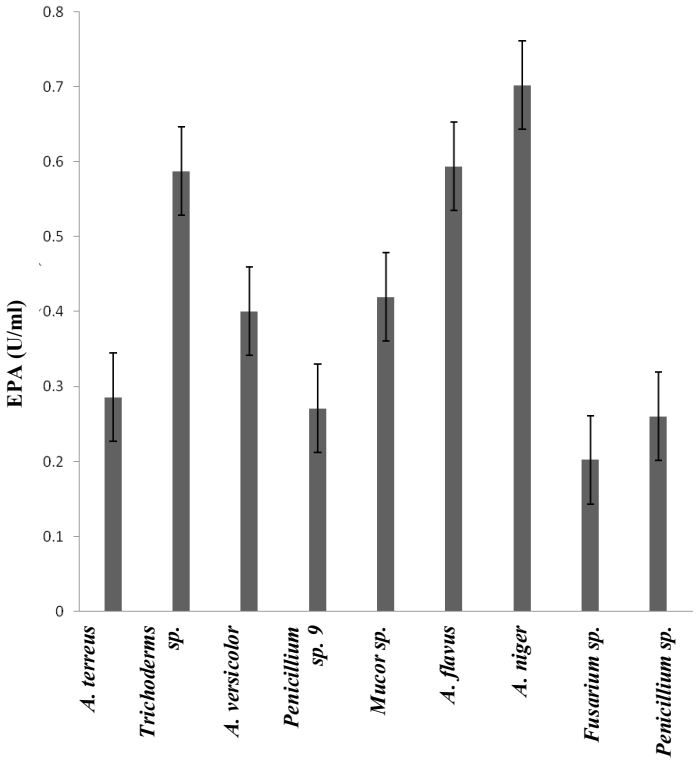

Fig. 2: Mean CMC activity (U/ml) of cellulolytic isolates in CBB over 6 days period

CONCLUSION

Soil from Ejura farms soil harbours cellulolytic fungi out of which *Aspergillus niger* was isolated. *Aspergillus niger* produced cellulase used to efficiently hydrolyze maize agro-waste (corncobs) into reducing sugars. The mean FP activity of 0.3648 U/ml on corncob based medium is appreciable.

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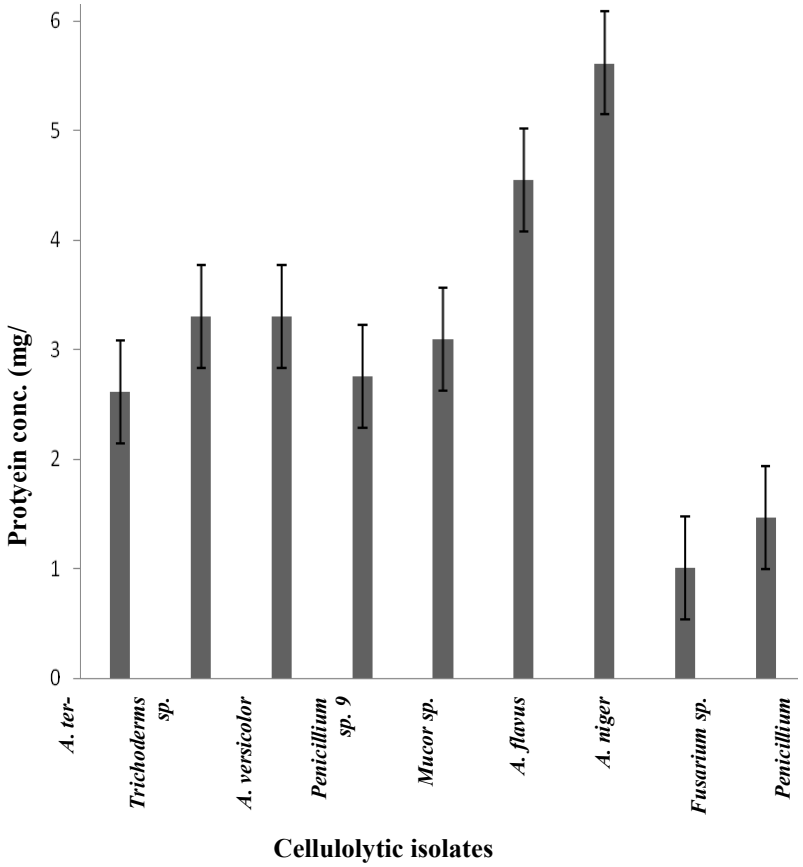

Fig. 3: Mean protein conc. (mg/ml) of the cellulolytic isolates in CBB over 6 days period

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