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# Growth response and comparative cellulase induction in soil fungi grown on different cellulose media.

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**ABSTRACT**

*The capacity to liberate cellulases by diverse fungi isolated from one of the under studied areas in Nigeria was investigated as well as their growth response to the assay media. Modified Mandel's broth having 1% carboxymethylcellulose (CMC-sodium salt), ball-milled Whatman N$^{o}$ 1 filter paper, or cellulose that was purified by the authors from palm frond via the Kraft method served as the assay media. The enzymes assayed for were β-1, 4-endoglucanase ($C_x$) and total cellulase ($C_x$ + $C_1$). Twenty-two cellulolytic fungal isolates representing 5 identified and 3 unidentified genera were obtained from only 7 of the 10 locations. The identified genera are Aspergillus, Penicillium, Fusarium, Trichoderma and Stachybotrys. The mycelia dry weight (MDW) measurement for the growth responses revealed that A. melleus (UCF1), Penicillum (UMC1) and an unidentified fungus (UHG2) had the highest mycelia growth in cellulose medium, CMC medium and FP medium, respectively. A. melleus (UCF1) recorded the highest $C_x$ activity (0.13 U ml$^{-1}$ min$^{-1}$) while T. reesei (USQ5) and F. semitectum (UMQ1) recorded the highest synergistic activity of the $C_x$ + $C_1$ component (0.09 U ml$^{-1}$ min$^{-1}$) on CMC medium,. On cellulose medium, T. reesei (USQ5) recorded the highest $C_x$ activity (0.11 U ml$^{-1}$ min$^{-1}$) while Penicillium (UMG2) recorded the highest synergistic activity of the $C_x$ + $C_1$ component (0.08 U ml$^{-1}$ min$^{-1}$). T. reesei (USQ5) recorded the highest $C_x$ activity (0.14 U ml$^{-1}$ min$^{-1}$) and synergistic activity of the $C_x$ + $C_1$ component (0.12 U ml$^{-1}$ min$^{-1}$) on filter paper medium. On the average, CMC medium was a more favorable substrate for the induction of both enzyme components and support for more fungal mycelia extension than the other media. This is the first report of the induction of cellulase enzyme production in P. shaze, a novel soil fungus first described in 2006.*

**Keywords:** *Cellulolytic fungi, cellulose, cellulase, carboxymethylcellulase, FPase.*

## INTRODUCTION

Soil fungi are usually described as fungi detected or isolated from soil, soil-borne animals, seeds and roots of plants (Watanabe, 2002). Those isolated directly from soil are mostly saprotrophic (Van Elsas *et al.*, 2007) as just a few are involved in the decomposition of plant litter which is a key ecosystem process that serves to release inorganic nutrients from plant detritus, as well as transform this material into soil organic matter (Swift *et al.*, 1979; Moorhead & Sinsabuagh, 2006). In particular, fungi are important in breaking down and recycling plant cell walls, which are mainly composed of cellulose and hemicelluloses; they (Soft-rot, Brown-rot and White-rot fungi) also degrade plant-derived woody substrates containing lignocelluloses, which is cellulose complexed with lignin (Van Elsas *et al.*, 2007). Typical examples of soil fungal genera are *T.*

*viride* (well known cellulolytic fungi), *Aspergillus* and *Penicillum.* Others include *Cladosporium, Alternaria, Pythium, Fusarium and Chaetomium* (Qian *et al*., 1998; Kuhn & Ghannoum, 2003; Khalid *et al*., 2006; Van Elsas *et al.*, 2007; Ezekiel *et al*., 2009).

Cellulose and lignin compose 60 to 75% of fresh plant litter (Swift *et al*., 1979; Schmidt, 2006). Thus among soil fungi, cellulolytic species which specialize in and produce enzymes that are capable of degrading cellulose are of great importance. Cellulases, a group of hydrolytic enzymes capable of hydrolyzing cellulose into its monomeric glucose units, are liberated by cellulolytic bacteria, fungi, actinomycetes and protozoa with fungi being the main responsible class (Siu, 1951; Cowling, 1958; Gascoigne & Gascoigne, 1958; Enari, 1983).

The cellulases involved in the breakdown of cellulose in plant litter by fungal decay/action in soil (Khalid *et al*., 2006) are of three major types; endoglucanases, cellobiohydrolases and β-glucosidase (Klyosov, 1990; Van Elsas *et al.*, 2007), and they act in a coordinated synergistic manner to hydrolyse cellulose into small oligosaccharides and ultimately glucose (Lynd *et al*., 2002). In microorganisms, these enzymes are either cell-bound or extracellular (Kader & Omar, 1998) as the capacity to produce the extracellular enzyme forms in fungi have been extensively studied (Mandels & Reese, 1957; Enari, 1983; Wood, 1985; Sazci *et al.*, 1986; Kamal & Mathur, 2005; Khalid *et al*., 2006; Peciulyte, 2007). Despite this, little is known about the diversity of cellulolytic microbial communities in soil since novel fungi are being discovered. Also a complementary tool to examine the "cellulolytic guild" is still lacking despite an increasing knowledge of the cellulase enzyme (Covert *et al*., 1992; Chow *et al*., 1994; Gielkens *et al*., 1999; Poças-Fonseca *et al*., 2000) as we have an incomplete knowledge of how the composition and function of fungal communities change during the process of litter decay (Edward *et al*., 2008).

The data from this study will benefit agriculturists, ecologist and industrialists, among many others, as cellulases are used to perform a multitude of functions including the removal of cell walls or crude fibre, release of valuable plant cell components (flavours, enzymes, polysaccharides and other proteins), improvement of nutritional value in animal feeds or the preparation of plant protoplasts for genetic research (Mandels, 1985; Peciulyte, 2007). Therefore this research was designed to investigate the diversity of cellulolytic soil fungi from one of the under studied areas in Nigeria, induce their capacity to liberate cellulases when grown on different media under stated environmental conditions as well as study their growth response to the assay media.

## MATERIALS AND METHODS

### Isolation of cellulolytic fungi

Cellulolytic soil fungi were isolated from the 10 randomly selected locations described by Ezekiel *et al.* (2009). The spore sedimentation method was employed at this stage and the method involved placing carefully a complete 12.5 cm-in-diameter piece of Whatman $N^o$ 1 filter paper in a sterile Petri plate. The filter paper was moistened by adding 1 ml tap water and the plate covered. Petri plate containing filter paper was sterilized by autoclaving at 121$^o$C under 15 lbs pressure for 15 mins and upon cooling, the upper lid was carefully removed at the soil location and the lid portion harboring the filter paper was buried in the soil at a depth of 0-15 cm for 7 d. This was repeated twice.At the end of the 7 d, the plates were excavated from the soil locations and the soil in each plate was poured off gradually to leave the dampened soiled filter paper firmly on the plate. The filter paper was then scrapped off the Petri plate and serially diluted till the $10^{-7}$ dilution. One milliliter aliquot of the $10^{-4}$ dilution was then inoculated unto freshly prepared modified cellulose-enriched basal medium (Mandel's agar medium) (Mandels & Reese, 1957) intended to support the growth of only the cellulolytic fungi that adhered to the serially diluted filter paper. The basal medium contained completely shredded Whatman $N^o$ 1 filter paper (10g), $(NH4)_2SO_4$ (1.4g), Urea (0.3g), $KH_2PO_4$ (2.0g), $MgSO_4.7H_2O$ (0.3g), $CaCl_2$ (0.3g), trace metal stock (1 ml) and agar (15g), all dissolved in a liter of distilled water. The trace metal stock consisted of $CoCl_2.6H_2O$ (1.83g), $FeSO_4.7H_2O$ (2.6g), $ZnCl_2$ (0.83g), Conc. HCl (5 ml) and distilled water (495 ml). Plates were incubated in the dark at 30$^o$C for 7 d. Pure cultures of isolates were made and preserved on PDA slants, respectively. Identification of fungal species was by macroscopic and microscopic observations and comparison with descriptions, illustrations and pictures in mycological literature (Gilman, 1971; Bulmer, 1978; Domsch *et al.*, 1980; Klick, 2002; Khalid *et al*., 2006; Paul & Yu, 2008).

### Cellulase Assay

The isolated cellulolytic fungi were grown in shake flask cultures containing the earlier described modified mineral medium (Mandel's broth) of Mandels & Reese (1957) which was intended to induce cellulase enzyme production. The supplemented carbon sources were varied as 1%

carboxymethylcellulose (CMC-sodium salt, ultra low viscosity, Aldrich, USA), ball-milled Whatman N$^o$ 1 filter paper, or cellulose that was purified by us from palm frond via the Kraft method. The media were homogenized properly on a vortex mixer for 15 min before autoclaving. One hundred milliliter of autoclaved assay medium was dispensed in 250 ml flask and inoculated with about 5-6 × $10^7$ spores/ml which was obtained by flooding a 5 d old culture of the specific fungus with sterile water. Flasks were then incubated at 30$^o$C in the dark for 6 d: first, for 3 d without shaking followed by another 3 d with shaking at 230 rpm (ZHP-100 Intelligent Thermostatic Shake Cultivation Cabinet, Serial N$^o$: 15179, China).At the end of the incubation period, the medium in each flask was filtered through a piece of Whatman N$^o$ 1 filter paper placed in a glass funnel. The mycelia mat was oven dried at 200$^o$C for 2 h to determine the constant mycelia dry weight. The filtrate was centrifuged at 13,000 *g* for 20 min in a high speed refrigerated centrifuge (4$^o$C) (Model: GL-16G-II, LENG DONG LI XIN JI, 2002) to obtain the extracellular enzymes in the clear supernatant. Protein content of the medium was determined following the Folin protein method of Lowry *et al.* (1951) with casein as standard. The enzymes assayed for were CMCase (β-1, 4-endoglucanase) and FPase (total cellulase) according to the methods of Mandels *et al.* (1976) using DNS. Enzyme activity expressed in international units (IU) is defined as micromoles (µmol) of glucose released per min per ml of culture filtrate.

**RESULTS AND DISCUSSION**

A total of 22 cellulolytic fungal isolates were obtained from only 7 of the 10 locations (Main Gate, BUMG; Medical Center, BUMC; Female Resident Halls, BUFH; MSQ, BUMQ; Staff Quarters, BUSQ; Horticultural Garden, BUHG and University Cafeteria, BUCF) and they belong to 5 identified and 3 unidentified genera. The identified genera include; *Aspergillus*, *Penicillium*, *Fusarium*, *Trichoderma* and *Stachybotrys* (Table 1). The soils within the Staff Quarters (BUSQ) emerged as the community with the highest cellulolytic fungal species richness (8 species) while soils around the University Cafeteria (BUCF) and Medical Centre (BUMC) had the least (1 species). The other 3 locations; Male Resident Halls (BUMH), Water Industry (BUWI) and Maintenance (BUMT), showed no promise for cellulolytic fungi after repeated isolation attempts from the soils.The locations with very low or no occurrence of cellulolytic species have histories of low or reduced agricultural practices and little exposure to cellulose-rich materials over the years and this corresponds with the reports of Nesci *et al.* (2006) and Ezekiel *et al*. (2009) who suggested that based on their research data, different agricultural practices impact soil fungal inocula; good and high practices enhance species richness and diversity while low or poor practices reduce drastically species richness. This also explains in part the reason for the high richness and diversity of fungi in the other well cultivated soils which on the contrary are exposed to a good feed of degradable plant litter, paper, disposed worn out clothing or other plant-based food waste. The lying cellulose-rich materials may have attracted and supported the growth of many species hence enriching the species richness of these sites as opposed to the sites with very low or even no cellulolytic species.The growth responses of the cellulolytic fungi to the various assay media used for cellulase studies were determined following the mycelia dry weight (MDW) measurement. The MDW measurements (g/100ml assay medium) of the 10 selected isolates from the total cellulolytic fungi obtained are shown in Fig. 1. The results revealed that *A. melleus* (UCF1), *Penicillum* (UMC1) and an unidentified fungus (UHG2) had the highest mycelia growth in cellulose medium, CMC medium and FP medium, respectively. The lowest mycelia growth was recorded in cellulose medium by *T. reesei* (USQ5) and *A. niger* (USQ1); in CMC medium by *F. semitectum* (UMQ1); in filter paper (FP) medium by *T. reesei* (USQ5), *A. tamarii* (USQ3) and *Penicillium* spp. (UMG2). On the average, CMC medium supported more fungal mycelia extension than the other media. The ability of CMC to support more fungal mycelia growth may be attributed to its nature bearing in mind that it is a fully purified and modified cellulose in salt form while the cellulose and filter paper used in the enrichment of the other media still have the "complex" nature which could have made cellulose hydrolysis and glucose utilization more difficult (Mandels & Reese, 1957; Gruno *et al.*, 2004).

The enzyme activity was recorded as specific cellulase activity (U $ml^{-1}$ $min^{-1}$) based on the enzyme component assayed for which included the $C_x$ and $C_x$ + $C_1$ components also known as the CMCase (β-1, 4-endoglucanase) and total cellulase (FPase), respectively. The results revealed that CMC medium was a more favorable medium, of the 3 tested assay media, for the induction of both enzyme components in most of the isolates. On CMC medium (Table 2), *A. melleus* (UCF1) recorded the highest $C_x$ activity (0.13 U $ml^{-1}$ $min^{-1}$) while *T. reesei* (USQ5) and *F. semitectum* (UMQ1) recorded the highest synergistic

activity of the $C_x + C_1$ component (0.09 U $ml^{-1}$ $min^{-1}$). On cellulose medium (Table 3), *T. reesei* (USQ5) recorded the highest $C_x$ activity (0.11 U $ml^{-1}$ $min^{-1}$) while *Penicillium* (UMG2) recorded the highest synergistic activity of the $C_x + C_1$ component (0.08 U $ml^{-1}$ $min^{-1}$). The $C_x$ component was not detectable in assays involving *F. semitectum* (UMQ1), *Penicillum* (UMC1) and the unidentified isolate (UMG3). *T. reesei* (USQ5) recorded the highest $C_x$ activity (0.14 U $ml^{-1}$ $min^{-1}$) and synergistic activity of the $C_x + C_1$ component (0.12 U $ml^{-1}$ $min^{-1}$) on filter paper medium (Table 4). The $C_x$ component was not detectable in assays involving *A. tamarii* (USQ3), *A. niger* (USQ1), *F. semitectum* (UMQ1), *P. shaze* (USQ2) and the unidentified isolate (UMG3).Peciulyte (2007) stated that the filter paper activity termed FPase is usually an expressed summation of a simultaneous synergistic action of endoglucanases, cellobiohydrolases and β-glucosidase in a cellulase preparation. The FPase activity results on the 3 tested assay media therefore show that all the isolates exhibited the capacity to liberate total cellulase components acting in a synergistic manner in other to accomplish complete hydrolysis of cellulose. The isolates that did not show any CMCase activity in our own purified cellulose and filter paper media could have a cellulase system that is deficient in cellobiose due to poor induction by both media thereby causing the accumulation of the disaccharides cellobiose which produces repression and end-product inhibition of the enzymes (Gruno *et al.*, 2004).Our investigation therefore, shows that there are other promising cellulolytic soil fungi that can act naturally on cellulose for the benefit of agriculturists and others in need of this enzyme or even better if exploited using biotechnological approaches. This is the first report of the induction of cellulase enzyme production in *P. shaze*, a novel soil fungus first described by Khalid *et al*. (2006). Further study will be targeted at investigating other poorly studied areas in Nigeria for cellulolytic soil fungi such that contribution can be made to the global database of fungal species taxonomy.

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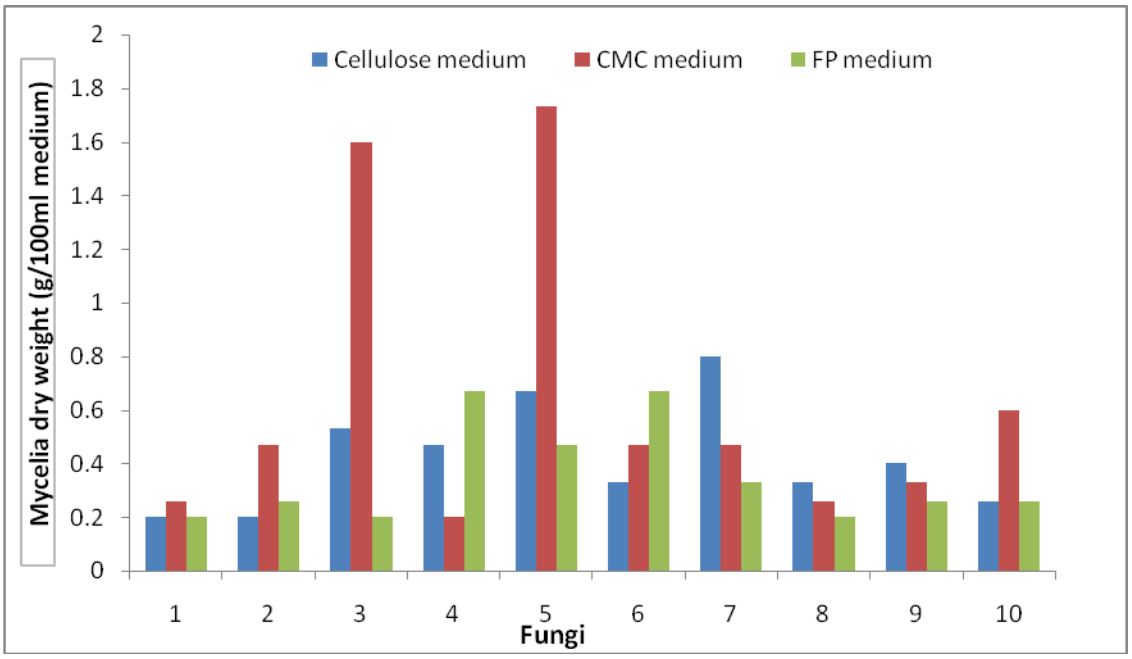
**Table 1: Occurrence of cellulolytic fungal species in the different soil types (communities) by location**

| Community | Code | Species | Number | Frequency (%) |
|---|---|---|---|---|
| BUMG | UMG1 | *A. niger* | 2 | 50.0 |
| | UMG2 | *Penicillium* spp. | 1 | 25.0 |
| | UMG3 | Unidentified | 1 | 25.0 |
| | **Σ** | **3 species** | **4** | **100.0** |
| BUMC | UMC1 | *Penicillium* spp. | 1 | 100.0 |
| | **Σ** | **1 species** | **1** | **100.0** |
| BUFH | UFH1 | *A. tamari* | 2 | 50.0 |
| | UFH2 | *T. reesei* | 1 | 25.0 |
| | UFH3 | Unidentified | 1 | 25.0 |
| | **Σ** | **3 species** | **4** | **100.0** |
| BUMQ | UMQ1 | *F. semitectum* | 1 | 100.0 |
| | **Σ** | **1 species** | **1** | **100.0** |
| BUSQ | USQ1 | *A. niger* | 3 | 37.5 |
| | USQ2 | *P. shaze* | 1 | 12.5 |
| | USQ3 | *A. tamarii* | 2 | 25.0 |
| | USQ4 | *S. chartarum* | 1 | 12.5 |
| | USQ5 | *T. reesei* | 1 | 12.5 |
| | **Σ** | **5 species** | **8** | **100.0** |
| BUHG | UHG1 | *A. niger* | 2 | 66.7 |
| | UHG2 | Unidentified | 1 | 33.3 |
| | **Σ** | **2 species** | **3** | **100.0** |
| BUCF | UCF1 | *A. melleus* | 1 | 100.0 |
| | **Σ** | **1 species** | **1** | **100.0** |

BUMG= Main Gate, BUMC= Medical Center, BUFH= Female Resident Halls, BUMQ= MSQ, BUSQ= Staff Quarters, BUHG= Horticultural Garden, BUCF= University Cafeteria.




1: *T. reesei* (USQ5); 2: *A. niger* (USQ1); 3: *A. tamarii* (USQ3); 4: *F. semitectum* (UMQ1); 5: *Penicillium* (UMC1); 6: unidentified (UHG2); 7: *A. melleus* (UCF1); 8: *Penicillium* (UMG2); 9: *P. shaze* (USQ2); 10: unidentified (UMG3).

**Fig. 1: Mycelia dry weight (g/100ml medium) of selected cellulolytic fungi.**

**Table 2: Specific cellulase activity (U ml$^{-1}$ min$^{-1}$) of isolates on CMC medium (100ml)**

| Code | Isolate | Specific Enzyme Activity (U ml$^{-1}$ min$^{-1}$) | |
|---|---|---|---|
| | | CMCase ($C_x$) (β-1,4-endoglucanase) | FPase ($C_x + C_1$) (Total cellulase) |
| USQ5 | *T. reesei* | 0.07 ± 0.002 | 0.09 ± 0.001 |
| USQ1 | *A. niger* | 0.09 ± 0.001 | 0.05 ± 0.001 |
| USQ3 | *A. tamari* | 0.07 ± 0.005 | 0.04 ± 0.001 |
| UMQ1 | *F. semitectum* | 0.11 ± 0.003 | 0.09 ± 0.002 |
| UMC1 | *Penicillium* | 0.06 ± 0.004 | 0.04 ± 0.002 |
| UHG2 | Unidentified | 0.08 ± 0.001 | 0.06 ± 0.000 |
| UCF1 | *A. melleus* | 0.13 ± 0.002 | 0.08 ± 0.001 |
| UMG2 | *Penicillium* | 0.08 ± 0.003 | 0.06 ± 0.002 |
| USQ2 | *P. shaze* | 0.04 ± 0.001 | 0.06 ± 0.001 |
| UMG3 | Unidentified | 0.04 ± 0.000 | 0.04 ± 0.002 |

Values are means of triplicate assays with standard deviations.

**Table 3: Specific cellulase activity (U ml$^{-1}$ min$^{-1}$) of isolates on Cellulose medium (100ml)**

| Code | Isolate | Specific Enzyme Activity (U ml$^{-1}$ min$^{-1}$) | |
|---|---|---|---|
| | | CMCase ($C_x$) (β-1,4-endoglucanase) | FPase ($C_x + C_1$) (Total cellulase) |
| USQ5 | *T. reesei* | 0.11 ± 0.010 | 0.07 ± 0.015 |
| USQ1 | *A. niger* | 0.02 ± 0.000 | 0.07 ± 0.002 |
| USQ3 | *A. tamari* | 0.03 ± 0.004 | 0.04 ± 0.005 |
| UMQ1 | *F. semitectum* | *nd | 0.04 ± 0.006 |
| UMC1 | *Penicillium* | *nd | 0.02 ± 0.002 |
| UHG2 | Unidentified | 0.09 ± 0.040 | 0.06 ± 0.002 |
| UCF1 | *A. melleus* | 0.01 ± 0.000 | 0.02 ± 0.005 |
| UMG2 | *Penicillium* | 0.05 ± 0.004 | 0.08 ± 0.009 |
| USQ2 | *P. shaze* | 0.08 ± 0.012 | 0.06 ± 0.006 |
| UMG3 | Unidentified | *nd | 0.04 ± 0.001 |

Values are means of triplicate assays with standard deviations.
*not detectable

**Table 4: Specific cellulase activity (U ml$^{-1}$ min$^{-1}$) of isolates on FP medium (100ml)**

| Code | Isolate | Specific Enzyme Activity (U ml$^{-1}$ min$^{-1}$) | |
|---|---|---|---|
| | | CMCase ($C_x$) (β-1,4-endoglucanase) | FPase ($C_x + C_1$) (Total cellulase) |
| USQ5 | *T. reesei* | 0.14 ± 0.001 | 0.12 ± 0.003 |
| USQ1 | *A. niger* | *nd | 0.05 ± 0.004 |
| USQ3 | *A. tamari* | *nd | 0.05 ± 0.028 |
| UMQ1 | *F. semitectum* | *nd | 0.04 ± 0.002 |
| UMC1 | *Penicillium* | 0.04 ± 0.002 | 0.03 ± 0.003 |
| UHG2 | Unidentified | 0.05 ± 0.006 | 0.05 ± 0.003 |
| UCF1 | *A. melleus* | 0.01 ± 0.003 | 0.06 ± 0.001 |
| UMG2 | *Penicillium* | 0.03 ± 0.004 | 0.04 ± 0.001 |
| USQ2 | *P. shaze* | *nd | 0.04 ± 0.003 |
| UMG3 | Unidentified | *nd | 0.05 ± 0.002 |

Values are means of triplicate assays with standard deviations.
*not detectable

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