

Assessment of Growth and Cellulase Production of Wild-Type Microfungi Isolated from Ota, Nigeria

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Abstract: The aim of the study was to isolate and identify filamentous microfungi involved in wood-waste decomposition in Canaanland, Ota, South-West Nigeria and to evaluate their potentials for cellulose saccharification. Microbiological techniques were used to isolate and identify the fungi. Four filamentous microfungi, identified as *Aspergillus niger*, *Aspergillus flavus*, *Penicillium chrysogenum* and *Trichoderma* sp., were isolated. All the isolates, particularly *Trichoderma* sp., grew rapidly on Sabouraud's agar and Czapek-Dox agar. Two of the isolates, *Aspergillus niger* and *Trichoderma* sp., was cultivated for 168 h by submerged fermentation in modified Czapek-Dox liquid medium containing cellulose as sole carbon source and harvested at 24 h intervals. The mycelia weight of the harvested cultures, and the protein content and cellulase activity of the filtrates were determined. The peak mycelia weight of 4.6 and 3.0 mg mL⁻¹ was, respectively obtained for *Trichoderma* sp. and *A. niger* at 48 h. The protein and cellulase activity of *Trichoderma* sp. peaked at 72 h whereas for *A. niger*, the peak protein content and peak cellulase activity was obtained at 96 and 72 h, respectively. The peak protein and cellulase activity values of *A. niger* were 0.175 and 0.077 unit mL⁻¹, respectively. *Trichoderma* sp. yielded a protein peak of 0.180 mg mL⁻¹ and peak cellulase activity of 0.108 unit mL⁻¹. There is a correlation between the protein content and cellulase activity of the culture filtrates. The strains of *A. niger* and *Trichoderma* sp. obtained from this study are potential tools for the saccharification and bioconversion of cellulosic materials.

Key words: Microfungi, mycelia weight, extracellular proteins, cellulase activity, cellulose hydrolysis

INTRODUCTION

Cellulose, a major component of all vegetation, is one of the world's most abundant resources (Lynd *et al.*, 2002). It is constantly replenished by photosynthesis and accounts for nearly one-half of the 18 to 20 metric tons of organic carbon annually fixed by photosynthesis (Romanelli *et al.*, 1975). Cellulose is also a major component of solid waste daily produced in large quantities from such activities as food processing, lumbering operations, paper making, cereal grain harvesting, sugar cane processing, domestic and office activities (Abu *et al.*, 2000). Attempts at utilizing cellulose as a commercial fermentation substrate have been unsatisfactory mainly due to the low rate of hydrolysis by mesophilic microorganisms (Romanelli *et al.*, 1975).

Cellulase is a generic name for the group of enzymes which catalyze the hydrolysis of cellulose and cellooligosaccharide derivatives (Chinedu *et al.*, 2008).

The enzymes play important role in natural biodegradation processes in which plant lignocellulosic materials are degraded by a wide variety of fungi, bacteria, actinomycetes and protozoa (Peciulyte, 2007). They are widely used for the extraction of valuable compounds from plant cells, improving nutritional values of animal feed and in preparing plant protoplasts for genetic research (Abdul *et al.*, 1999). They are also applied in the chemical industry for the production of fermentable sugars, ethanol, organic acids, detergents and other chemicals (Howard *et al.*, 2003). Cellulases also find important application in the pulp and paper industry, textile industry, animal feed and food industry and also in processing cellophane and rayon (Peciulyte, 2007). Cellulase is potentially useful for industrial saccharification of cellulosic biomass (Chinedu *et al.*, 2008). The growing interest in the conversion of lignocelluloses into bulk chemicals and biofuels as a means of alleviating energy shortages has heightened the

search for highly active, thermostable and specific cellulases (Howard *et al.*, 2003; Chinedu *et al.*, 2008).

Fungi produce extracellular cellulase enzymes that break down cellulose into two or three glucose units which are readily degraded and assimilated as glucose monomers (Willey *et al.*, 2008). Fungal cellulases are extracellular and inducible (Abdul *et al.*, 1999) and consist of three components namely exoglucanase, endoglucanase and β -glucosidase (Khan, 1980). The ability of filamentous fungi to secrete cellulases and other cell wall hydrolyzing enzymes into their culture media has led to the harvesting of these useful enzymes (Berry and Patterson, 1990; Chinedu *et al.*, 2008). In this study, the growth, protein and cellulase production of some microfungi isolated from wood-waste dump in Covenant University, Canaanland, Ota, Nigeria were evaluated. The potentials of two of the isolates for cellulose saccharification are indicated.

MATERIALS AND METHODS

Sample collection and preparation: Sample of decomposing wood-waste was collected in a sterile bag from a construction site at Covenant University, Ota, Ogun State in March 2, 2009 and stored at room temperature. One gram of the sample was aseptically introduced into 9.0 mL of sterile distilled water in a sterile test-tube and shaken vigorously. The stock culture was used for fungal isolation.

Media preparation: Sabouraud's Agar (Merck, Germany) was prepared according to manufacturer's instruction.

Czapek-dox agar: One liter of Czapek-Dox agar consists of: 30.0 g Sucrose, 2.0 g NaNO_3 , 1.0 g KH_2PO_4 , 0.5 g KCl, 0.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.01 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and 20.0 g Agar (Oxoid, England) and 1.0 mL of trace solution (containing 1.0 g ZnSO_4 and 0.5 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O L}^{-1}$).

Modified czapek-dox agar had the same composition as Czapek-Dox Agar except that sucrose was replaced with equal quantity of crystalline cellulose.

Modified Czapek-Dox liquid medium had the same composition as Modified Czapek-Dox Agar except that it did not contain agar. Each medium was supplemented with 0.05% (w/v) Ampicillin to prevent bacterial growth. The pH was adjusted to 5.4 before sterilization by autoclaving at 121°C for 15 min.

Isolation of the fungal isolates: One milliliter of the stock culture was inoculated on freshly prepared modified Czapek-Dox agar and incubated at 25°C for 120-168 h. Pure fungal isolates were obtained through careful isolation and successive sub-culturing of the growing fungal colony on fresh Sabouraud's agar.

Identification of the fungal isolates: Each isolate was identified based on its colonial and cultural properties, and the microscopic features of its sporulating structures (Harrigan and McCance, 1966). The characteristics of the vegetative mycelia (hyphae) and color of the colonies were observed over a period of 24 to 168 h. The morphology of the isolates, stained with lactophenol-cotton blue, was studied using a light microscope and compared according to the description given by Thom and Raper (1945), Alexopoulos (1952), Talbot (1972) and Deacon (1980).

Growth studies: Colony diameter: A flamed, sterile cork borer (Pore size; 5.0 mm diameter), was used to bore a hole enclosing a disc of the pure culture of the respective pure fungus on Czapek-Dox agar plate. One disc of each fungus was aseptically transferred and placed at the center of sterile, freshly prepared Sabouraud's and Czapek-Dox agar plates. The average diameter of the growing colony was measured at intervals of 24 for 120 h using a calibrated transparent ruler.

Mycelia weight: Ten milliliter of 1.0% Tween 80 was placed on top of 3 day Czapek-Dox agar plate containing the pure isolate of fungus. Conidia were harvested using sterile cotton swab and transferred into a sterile test tube and diluted with equal volume of sterile distilled water. Two milliliter of the spore suspension was inoculated into 100 mL of sterile modified Czapek-Dox liquid medium in 250 mL Erlenmeyer flask. The flask was covered with sterile cotton wool and incubated in the dark at 25°C with continuous agitation at 100 rpm using the Griffin flask shaker. Cultures were harvested at 24 h intervals by filtration over a 168 h period. The mycelium was washed and dried in the oven at 80°C for 2 h, and carefully weighed. The cell-free filtrates were used for protein and enzyme analyses.

Protein assay: Protein content of the culture filtrates was determined by the folin ciocalteau method described by Lowry *et al.* (1951) using Bovine Serum Albumin (BSA) as standard.

Enzyme assay: A modified reducing sugar method of Khan (1980) as described by Chinedu *et al.* (2008) was used to assay for cellulase (EC 3. 2. 1. 4) activity. Carboxymethyl-cellulose (CMC) was used as enzyme substrate. The released reducing sugar was measured using 3, 5-dinitrosalicylic acid and read at 540 nm using a spectrophotometer (Miller, 1959). A unit of activity was defined as amount of enzyme required to liberate 1 μmol of Glucose per minute under the assay conditions.

RESULTS

Isolation and identification: Four different filamentous microfungi were isolated from the wood-waste and identified as *Aspergillus niger*, *Aspergillus flavus*, *Penicillium chrysogenum* and *Trichoderma* species based on the colonial and cultural characteristics and microscopic features of the sporulating structures of the fungal isolates (Table 1). Colony of the fungal isolate growing on Sabouraud's agar

plate and the microscopic features of the sporulating structure are shown in Fig. 1a,b-4a,b.

Growth studies

Growth on sabouraud's and czapek-dox agar: Figure 5 shows the growth of the fungal isolates on Sabouraud's agar. The growth on Czapek-Dox Agar is shown in Fig. 6. All the organisms grew rapidly on both media, filling the entire plate (8.5 cm diameter)

Table 1: Colonial and microscopic characteristics of the fungal isolates

Isolate No.	Hyphae	Pigmentation	Sporulating features	Identification (Organism)
1.	Aseptate	Dark brown to black colony	Non-septate conidiospores; mop-like conidial head	<i>Aspergillus niger</i>
2.	Aseptate	Yellowish-green colony	Non-septate conidiospores; Columnar conidial head	<i>Aspergillus flavus</i>
3.	Aseptate	Bluish - Ash colony	Branched conidiospores with a brush-like conidial head	<i>Penicillium chrysogenum</i>
4.	Septate	White to grey colony forming dark green concentric rings	Irregularly branched conidiospores; clusters of grape-like conidial head	<i>Trichoderma</i> sp.

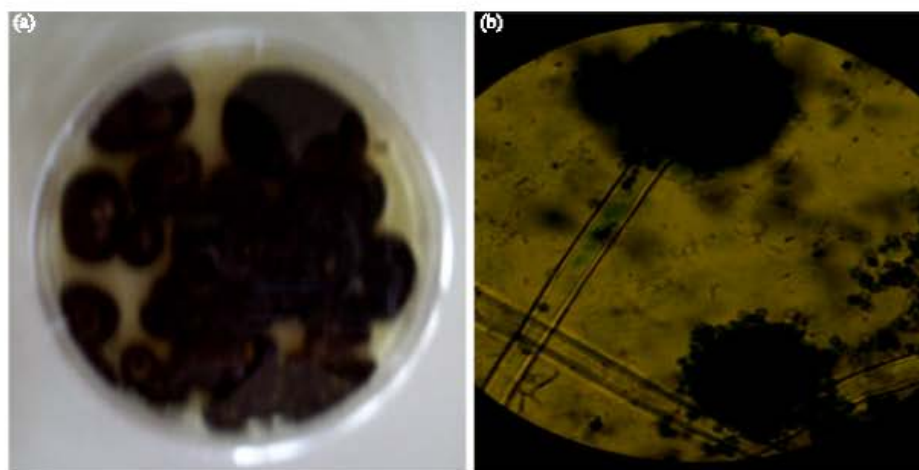


Fig. 1: *Aspergillus niger*: (a) 5-day culture on Sabouraud's agar plate and (b) Microscopic picture (x100) of the sporulating structure

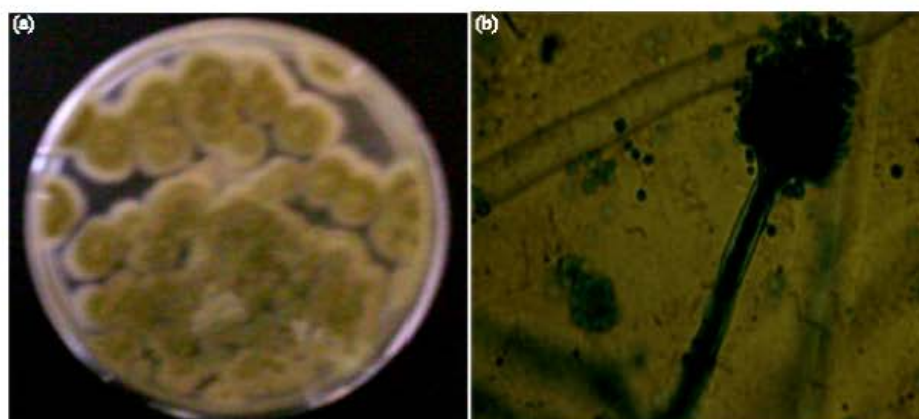


Fig. 2: *Aspergillus flavus*: (a) 5-day culture on Sabouraud's agar plate and (b) Microscopic picture (x100) of the sporulating structure

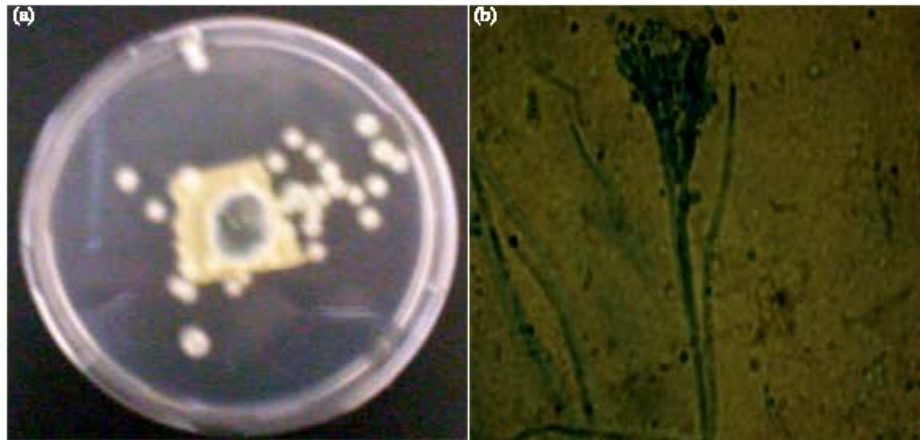


Fig. 3: *Penicillium chrysogenum*: (a) 3-day culture on Sabouraud's agar plate and (b) Microscopic picture (x100) of the sporulating structure

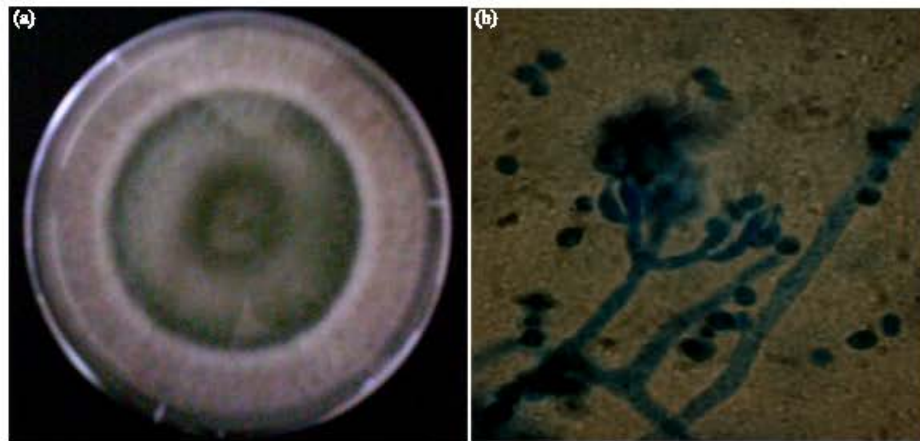


Fig. 4: *Trichoderma* sp.: (a) 3-day culture on Sabouraud's agar plate and (b) Microscopic picture (x100) of the sporulating structure

between 72 to 120 h of incubation. The fastest growth on the Sabouraud's and Czapek-Dox Agar was obtained for *Trichoderma* sp.

Growth on modified czapek-dox liquid medium: Figure 7 shows the mycelia weights of *Aspergillus niger* and *Trichoderma* sp. in modified Czapek-Dox liquid medium. The peak mycelium weight of both organisms was obtained at 48 h. Thereafter, there was a steady decline in the mycelia weight. A peak mycelia weight of 3.0 and 4.6 mg mL⁻¹ was obtained for *A. niger* whereas *Trichoderma* sp. respectively.

Cellulase activity

Protein yield and cellulase activity: Protein content and cellulase activity of culture filtrates of *A. niger* incubated

in modified Czapek-Dox liquid medium is shown in Fig. 8. The peak protein content and cellulase activity of *A. niger* was obtained at 96 and 72 h, respectively. Figure 9 shows the protein content and cellulase activity of culture filtrates of *Trichoderma* sp. incubated in modified Czapek-Dox liquid medium. Both the protein and cellulase activity of *Trichoderma* sp. peaked at 72 h. The peak protein and cellulase activity values of *A. niger* were 0.175 and 0.077 unit mL⁻¹, respectively. The cellulase activity of *Trichoderma* sp. was significantly higher than that of *A. niger*. *Trichoderma* sp. yielded a protein peak of 0.180 mg mL⁻¹ and peak cellulase activity of 0.108 unit mL⁻¹.

Mycelia weight and cellulase activity: The mycelia weights and cellulase activity of the culture filtrates of *A. niger* and *Trichoderma* sp. harvested at 24 h intervals

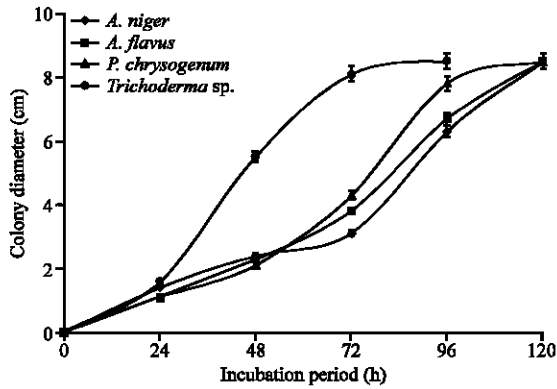


Fig. 5: Colony diameter of fungal isolates incubated at 25°C on Sabouraud's agar plates

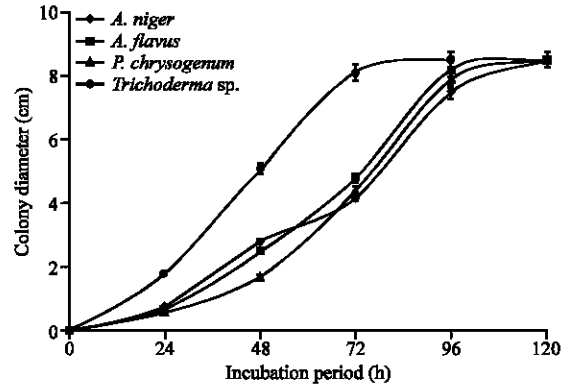


Fig. 6: Colony diameter of fungal isolates incubated at 25°C on Czapek-Dox agar plates

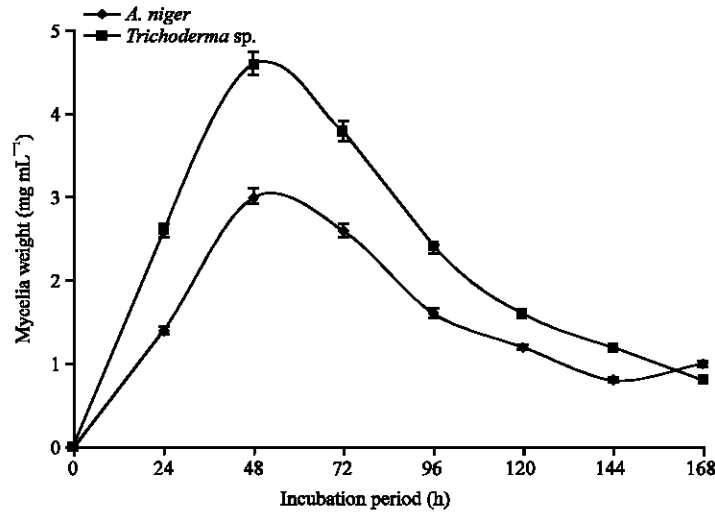


Fig. 7: Mycelia weights of *Aspergillus niger* and *Trichoderma sp.* incubated at 25°C in modified Czapek-Dox liquid medium

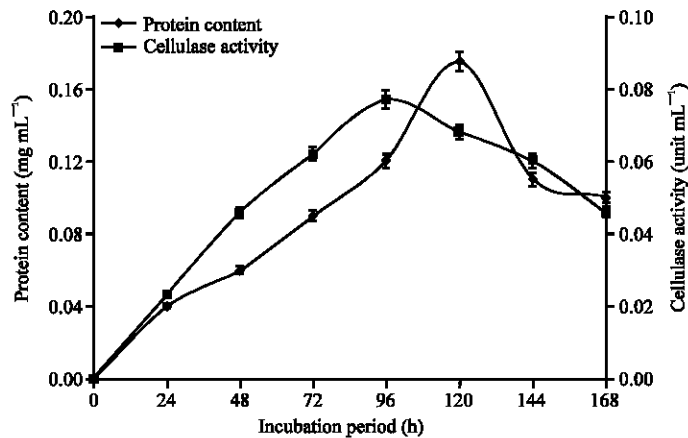


Fig. 8: Protein content and cellulase activity of culture filtrates of *A. niger* incubated at 25°C in modified Czapek-Dox liquid medium

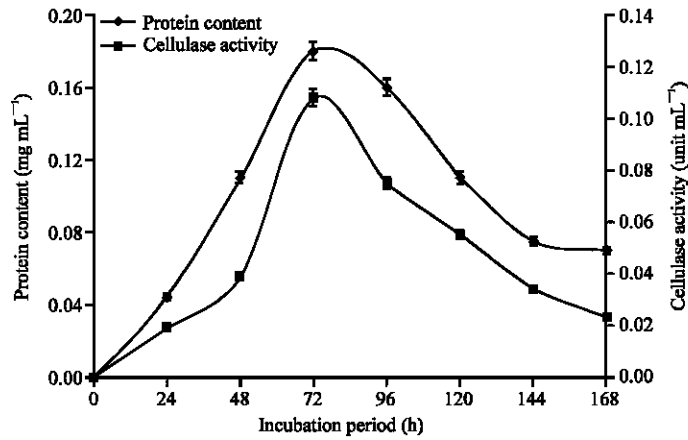


Fig. 9: Protein and cellulase activity of culture filtrates of *Trichoderma* sp. incubated at 25°C in modified Czapek-Dox liquid medium

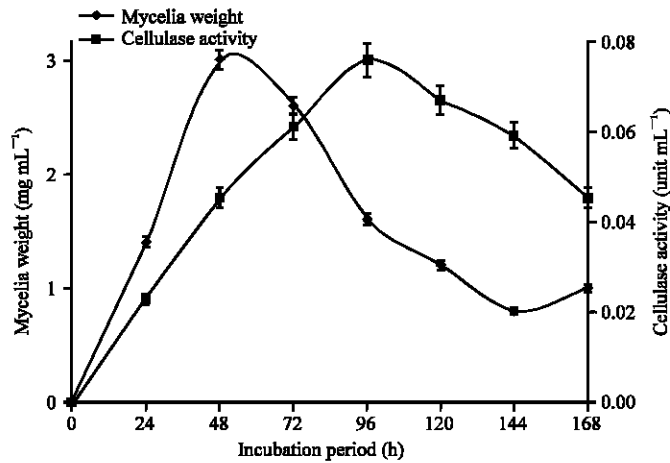


Fig. 10: Mycelia weight and cellulase activity of culture filtrates of *A. niger* incubated at 25°C in modified Czapek-Dox liquid medium

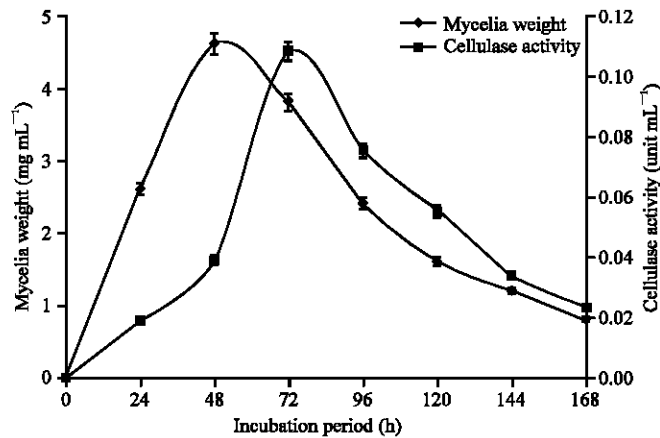


Fig. 11: Mycelia weight and cellulase activity of culture filtrates of *Trichoderma* sp. incubated at 25°C in modified Czapek-Dox liquid medium

over a period of 168 h are shown in Fig. 10 and 11, respectively. The peak mycelia weight for each organism was obtained at 48 h of incubation. The peak

cellulase activity for *A. niger* was obtained at 96 h whereas *Trichoderma* sp. gave its maximum cellulase activity at 72 h.

DISCUSSION

Wide variation in the nutritional requirements of microorganisms for growth and reproduction forms the basis for selective or differential media often engaged in microbiological studies (Harrigan and McCance, 1966). The ability of the isolated microfungi to thrive on modified Czapek-Dox agar containing cellulose as sole carbon source indicate that they can synthesize the enzymes required for the breakdown cellulose to meet their energy and carbon needs. The ability of some microfungi to grow on cellulosic materials was exploited in isolating the organisms from wood-wastes (Nwodo-Chinedu *et al.*, 2005).

The growth of the organisms on different media (Sabouraud's agar, Czapek-Dox agar, modified Czapek-Dox agar and modified Czapek-Dox liquid media) shows the versatility of the organisms to utilize different carbohydrate sources. However, these organisms utilized the carbon sources at different rates as indicated by their different rates of growth. The fastest of these organisms in terms of growth in all the media was *Trichoderma* sp. (Fig. 5-7). *Trichoderma* sp. belongs to a family of beneficial fungi commonly found in soils in nearly all parts of the world (Nederhoff, 2001). They are frequently the most prevalent culturable fungi in the soil where they basically live on plant debris and organic matter. Some strains are however parasitic on other fungi (Nederhoff, 2001). Fast growing strain of *Trichoderma* sp. such as the strain isolated in the present study can act as biological control agents, inhibiting the growth of phyto-pathogenic fungi that could destroy a large variety of crops (Cwdoza *et al.*, 2005). Two of the isolated microfungi, *Aspergillus flavus* and *A. niger*, belong to the genus *Aspergillus*. This genus has been documented as source of the most prevalent airborne moulds (Nwodo-Chinedu *et al.*, 2005). *Aspergillus flavus* is of great economic importance due to its ability to produce toxic and carcinogenic aflatoxins in agricultural commodities (Horn, 2006). *Aspergillus flavus* has, however, been safely used for many centuries in China in the production of various cheeses and soy sauce. *Aspergillus niger* is a filamentous ascomycete fungus that is ubiquitous in the environment and has been implicated in opportunistic infections of humans, yet industrial citric acid production by *Aspergillus niger* represents one of the most efficient, high-yield bioprocesses currently in use by industry (Baker, 2006). Strains of *Penicillium* sp. are perhaps best known for their use in pharmaceutical industry for the synthesis of penicillin and griseofulvin. Strains of *Penicillium*

chrysogenum has been reported to produce significant levels cellulolytic enzymes (Nwodo-Chinedu *et al.*, 2007).

There was a steady increase in the mycelia weight, protein released and cellulase activity in the culture media as the incubation progress until a specific period after which a decline set in (Fig. 7-10). The increase in the mycelia weight shows that the medium supports the growth of the organisms. Increase in protein level and cellulase activity of the culture filtrate shows that the organisms secrete extracellular proteins which have cellulolytic activity. Most cellulolytic fungi secrete hydrolytic enzymes for the breakdown of the polymers into their growth media; this largely accounts for the protein contents of the cell-free filtrates (Chinedu *et al.*, 2008).

There were however different peak periods for mycelia, protein and cellulase production by the two microfungi studied. The mycelia weight after reaching the peak value in both organisms steadily declined afterwards (Fig. 7, 10, and 11). This trend was also noted in the protein content and cellulase activity (Fig. 8, 9). This is expected since there was no inflow of nutrients into the culture medium all through the incubation period. The pattern is characteristic of typical batch culture fermentation. The protein content and cellulase activity of the culture filtrates at different periods indicated a correlation. This was best depicted in *Trichoderma* sp. where the peak period for both protein and cellulase production was at 72 h of incubation (Fig. 9). The finding supports the trend previously reported for different microfungi (Nwodo-Chinedu *et al.*, 2007; Chinedu *et al.*, 2008). In the case of *A. niger*, the protein peak was at 120 h whereas cellulase activity peak was at 96 h (Fig. 8). Though the peak periods are very close, the difference seems to imply that the organism produces other proteins which may not have cellulolytic activity. Cellulose is known to also induce the production of other cell wall hydrolyzing enzymes such as xylanases in *Aspergillus* sp. (De Vries and Visser, 2001). In both organisms, the peak mycelium weight was reached before the cellulase activity peak (Fig. 10, 11). This seems to imply that the organisms continued to produce the cellulolytic enzymes even at the declining phase of the organisms' growth. However, in each case, the cellulase activity peak was reached shortly after the peak mycelia weight and both continued to decrease afterwards.

The peak cellulase activity of the crude enzyme of *A. niger* and *Trichoderma* sp. was 0.077 and 0.108 unit mL⁻¹ respectively. This is considered very significant. It compares with the cellulase activity peak of 0.10 unit mL⁻¹ reported for a strain of *Penicillium chrysogenum* PCL 501 (Nwodo-Chinedu *et al.*, 2007).

In conclusion, the isolates of *A. niger* and *Trichoderma* sp. cultivated in basal medium containing cellulose as sole carbon source have been shown to produce extracellular proteins with significant cellulase activity. These filamentous fungi can thus serve as potential tools in the industrial saccharification of cellulose. Fast growing fungi such as the strain of *Trichoderma* sp. isolated in the present study can act as biological control agent, inhibiting the growth of phytopathogenic fungi that could destroy a large variety of crops.

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