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Cellulolytic activities of wild type fungi isolated from decayed wood cuttings

Emoghene A. O[1]., Okungbowa, O. C[2]., Obayagbona, O. N[3]. and Jaboro, A. G[4]

[1]Department of Microbiology, Faculty of Life Sciences, University of Benin, Benin City, Nigeria. [2]Action Centre for Evaluation and Prevention of Environment-Borne Diseases, Suite 30, EDPA Shopping Complex, EDPA Housing Estate, Benin City, Nigeria. [3]Microbiology Laboratory, Edo Environmental Consults and Laboratory, Palm House Annex, Sapele Road, Benin City, Nigeria. [4]Precious Life Medical Center, Games village, Kaura District, Abuja, Nigeria.

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Abstract

The mycological profile of decayed wood cuttings sourced from a saw mill located at Uwasota Road, Benin City was investigated using serial dilution and pour plate techniques. The mean fungal counts ranged from 0.9 ×10^6 cfu/g to 2.7 ×10^6 cfu/g respectively. Four fungal species were identified; *Aspergillus niger*, *Aspergillus fumigatus*, *Mucor mucedo* and *Penicillium chrysogenum*. *P. chrysogenum* (97%) was the most prevalent isolate while *A. fumigatus* (70%) was the least occurring isolate. *P. chrysogenum* exhibited the highest cellulolytic potential amongst the fungal isolates while *M. mucedo* had the least cellulolytic activity during the screening test. *P. chrysogenum* had the maximal frequency of appearance (97%) amongst the fungal isolates. The protein content of the labelled cell free supernatants ranged from 0.07 mg/ml to 0.34 mg/ml. The released glucose values of the respective labelled cell free supernatants varied from 0.08 mg/ml to 0.86 mg/ml. The recorded cellulase activity of the labelled cell free extracts ranged from 19.05 µg glucose (mg protein) $^{-1}$ min $^{-1}$ to 64.71 µg glucose (mg protein) $^{-1}$ min $^{-1}$. *P. chrysogenum* exhibited the maximal cellulosic activity amongst the isolates studied.

Key Words: decayed wood cuttings, cellulolytic potential, cellulase activity, cell free supernatant

Correspondence: *Jaborograndy@gmail.com*

Introduction

Cellulose is the β-1, 4-polyacetal of cellobiose (4-0-β-D glucopyranosyl-D- glucose) (Harmsen *et al*., 2010). Lynd *et al.*, (2002) stated that cellulose is the most abundant component of plant biomass and it is found in nature almost exclusively in plant cell walls, although it is produced by some animals (i.e. tunicates) and a few bacteria. Despite great differences in composition and in the anatomical structure of cell walls across plant taxa, high cellulose content; typically in the range of approximately 35% to 50% of plant dry weight, is a unifying feature (Lynd *et al*., 1999). Lynd *et al*., (2002) reported that cellulolytic capability is well represented among the subdivisions of aerobic fungi. Within the approximately 700 species of Zygomycetes, only certain members of the genus *Mucor* have been shown to possess significant cellulolytic activity, although members of this genus are better known for their ability to utilize soluble substrates. Eriksson *et al.*, (1990) reported that cellulose biodegradation by fungi has generally been considered to involve only three types of hydrolytic enzymes: endoglucanases (EGs), cellobiohydrolases (CBHs), and β- glucosidase (BGL). EGs randomly cleave the internal β-1,4-glucosidic links, CBHs act on the free ends of cellulose polymer chains, and BGL hydrolyzes cellobiose and other water-soluble cellodextrins to glucose. Cellulases belong to a large group of glycosylhydrolases (GHs), which have been classified into several families based on amino acid sequence similarities (Henrissat, 1993; Henrissat & Bairoch, 1993; Henrissat & Davies, 1997).

Parihar *et al.*, (2012) stated that cellulosic materials have played an important role in everyday life as constituent of wood, paper, cloth, rayon film, plastic rope and fillers. The biological decomposition of cellulose is the most important process in nature. It constitutes the major necessary steps in maintaining the balance between the synthetic and degradative phenomenon in the carbon cycle (Parihar *et al.*, 2012).

Plant biomass is the only foreseeable sustainable source of fuels and materials available to humanity (Lynd *et al*., 1999). Cellulosic materials are particularly attractive in this context because of their relatively low

cost and plentiful supply. Thus, the aims and objectives of this study was to isolate and identify fungi from the decayed wood samples, screening of the purified fungal isolates for potential cellulolytic activity and the evaluation of cellulosic activity of the cell free supernatant prepared from the respective fungi isolates which had given positive results during the screening test.

Materials and Methods

Sample collection: Samples of decayed wood cuttings were collected from a Saw mill located at Uwasota road, Benin City, Edo State. The wood cuttings were placed into sterile polyethylene bags and taken to the laboratory.

Enumeration and isolation of heterotrophic wood fungi using general purpose medium: Ten (10) gram of the respective decayed wood samples were weighed, crushed and dissolved into 90 ml of sterile prepared peptone water diluent under aseptic conditions. Serial fold dilutions were then made up to 10^{-6} and aliquots of each dilution were cultured on plates of Potato Dextrose Agar (PDA) by pour plate method (Aneja, 2003). An antibiotic mixture made up of distilled water, 50 ml and Erythromycin: 500mg was prepared (Okungbowa, 2013) and 1 ml was pipetted onto each labelled plate already containing 1ml of the inoculum before the respective sterilized cool molten media were poured. This was done to discourage any bacterial contamination of the incubated agar plates. Plating was done in duplicates. The culture plates were swirled, allowed to solidify and incubated at ambient room temperature (28 ± 2^0C) for 5 days. The resultant fungal colonies were enumerated and recorded as colony forming units (cfu) per 10 g of each decayed wood (Aneja, 2003).

Characterization of the wood mycobiota: Discrete fungal colonies were purified by transferring a portion of the surface mycelia with the aid of a 5 mm sterile cork borer and sterile forceps to freshly prepared PDA plates with incorporated antibiotic solution as above by the pour plate method (Aneja, 2003). The plates were incubated at ambient temperatures (28 ± 2^OC) for 5 days. The cultural characteristics of the purified isolates were noted and the microscopic features of the fungal isolates were observed using the wet mount technique (Choi *et al*., 1999; Guan *et al*., 2007; Sharma, 2009). Purified cultures were stored in PDA slants. Both lactophenol cotton blue and distilled water were used respectively as mountants. The microscopic structures observed were recorded and compared to those stated by Barnett & Hunter (1972).

Screening of fungal isolates for cellulolytic activity: The cellulolytic activity of the fungal isolates were determined by their ability to grow and form clear zones around colonies on Czapek-dox agar with 0.1% sodium carboxymethyl cellulose (Doolotkeldieva & Bobusheva, 2011). The surface of the agar with the matured fungi colonies was mixed with 0.1 % Congo red indicator and incubated for 15 min at room temperature ($28\ ^OC \pm 2\ ^OC$). The dye was removed and 1M NaCl solution was then added and the agar plates were incubated for 10 min at room temperature. The ratio of the diameter of the clear zone to the diameter of the colony was determined using a meter ruler (Doolotkeldieva & Bobusheva, 2011). The cellulolytic ability of the decayed wood fungal isolates was further confirmed by sub culturing the isolates on Hutchinson agar. A piece of sterile ashless filter was then placed on the surfaces of the emergent colonies on the agar surface. To avoid desiccation, the plates were kept in a moist incubation chamber at 26–27 °C for 1–2 weeks. Cellulolytic activity was estimated by the extent of destruction of the strips of filter paper (Doolotkeldieva & Bobusheva, 2011).

Preparation of cell free supernatant: The cell free extract of the cellulolytic wood fungi was prepared using modified mineral salt broth (Doolotkeldieva & Bobusheva, 2011). Fifty (50) ml portions of the medium were dispensed into conical flasks and autoclaved at 121^0 C for 15 min. Three (3) to seven (7) day PDA plate culture of the respective pure fungal isolate was utilized for the inoculation process. The agar plates were covered with 10 ml of 0.1 % Tween 80. Fungal condida and spores were collected using a sterile cotton swab and transferred carefully into a sterile glass tube (Doolotkeldieva & Bobusheva, 2011). One (1) ml of each suspension was inoculated into conical flasks containing 50 ml of sterile modified mineral salt broth. The flasks were incubated at room temperature for 21 days. The flasks were shaken for 1min each day. The pH of the incubated broth was ascertained using a calibrated pH meter (Doolotkeldieva & Bobusheva, 2011). The rate of appearance of the submerged fungal mycelia was also ascertained (Doolotkeldieva & Bobusheva, 2011). The mycelia mass of the respective cellulolytic wood fungi was harvested and the cell free supernatants were also centrifuged at 6000 rpm for 15 min (Doolotkeldieva & Bobusheva, 2011). The centrifuged cell free supernatants were used as crude enzyme source.

Determination of the glucose content and cellulosic activity of the cell free supernatants: Reducing sugar method described by Wood and McCrae (1977) was used to determine the cellulase activity. A mixture of 1.0 ml of cellulose solution (1%w/v), 0.5 ml of 0.2M acetic acid-NaOH buffer (pH 5.4) and 0.5 ml of cell free culture supernatant was incubated at 37^0C for 1 hr. The reaction was stopped by the addition of 2.0 ml

Smogyi reagent and the reducing sugar content determined by the method of Nelson (1944). In the evaluation of the reducing sugar content, 3 ml of DNS was added to 0.1 ml of the cell free supernatant in a test tube and thoroughly mixed. The mixture was heated for 15 min in boiling water contained in a beaker. The test tubes were removed and 0.1 ml of 40% sodium potassium tartarate was added to stabilize the color. The samples were then diluted at 25 ml with distilled water and the absorbencies of the diluted samples were read at 570 nm against a reagent blank using a UV camp sec spectrophotometer. The reducing sugar liberated was expressed as glucose (mg/ml).

Determination of the protein content of the cell free supernatants: The protein content of the supernatant was determined by procedure described by Lowry (1951). About 0.4 ml of serum was added to 0.4ml of samples, mixed well and was incubated for 10 min at room temperature. About 0.2ml of Folin reagent was added and incubated for another 30 min. The absorbance was read at 750nm. Protein concentrations were obtained from a standard curve.

Results and Discussion

The mean fungal counts ranged from 0.9 $\times 10^6$ cfu/g for sample E to 2.7 $\times 10^6$ cfu/g for sample B respectively (Table 1). Four fungal species were identified from the decayed wood samples; *Aspergillus niger*, *Aspergillus fumigatus*, *Mucor mucedo* and *Penicillium chrysogenum*. (Table 2). *P. chrysogenum* (97%) was the most prevalent isolate while *A. fumigatus* (70%) was the least occurring isolate (Table 2). *P. chrysogenum* exhibited the highest cellulolytic utilizing potential amongst the fungal isolates while *Mucor* sp. had the least cellulolytic activity during the screen test (Table 3). *P. chrysogenum* had the maximal rate of appearance (99%) amongst the fungal isolates (Figure 1). The pH values of the labelled modified mineral salt broth had neutral values (7.0) at day 0, while at the 21st day, the flask containing *P. chrysogenum* was the most acidic (6.0) (Fig. 2).

Table 1: Mean heterotrophic fungal counts of the wood samples

Wood samples	Mean fungal count ($\times 10^6$ cfu/g)
A	1.4
B	2.7
C	1.0
D	2.3
E	0.9

KEY: A-E; wood samples

Table 2: Percentage frequency of occurrence ofthe fungal isolates

Fungal isolates	% occurrence
Aspergillus niger	83
Aspergillus fumigatus	70
Mucor mucedo	80
Pencillium chrysogenum	97

Table 3: Screening test for cellulolytic attributes of the fungal isolates

Fungal isolates	Activity
Aspergillus niger	+++
Aspergillus fumigatus	+++
Mucor mucedo	++
Pencillium chrysogenum	++++

KEY: +; Extent of ratio of the diameter of the clear zone to the diameter of the colony

The protein content of the labelled cell free supernatants ranged from 0.07 mg/ml to 0.34 mg/ml (Fig. 3). The released glucose values of the respective labelled cell free supernatants varied from 0.08 mg/ml to 0.86 mg/ml (Fig. 4). The recorded cellulase activity of the labelled cell free extracts ranged from 19.05 µg glucose (mg protein)$^{-1}$ min^{-1} to 64.71 µg glucose (mg protein)$^{-1}$ min^{-1} (Fig. 5)

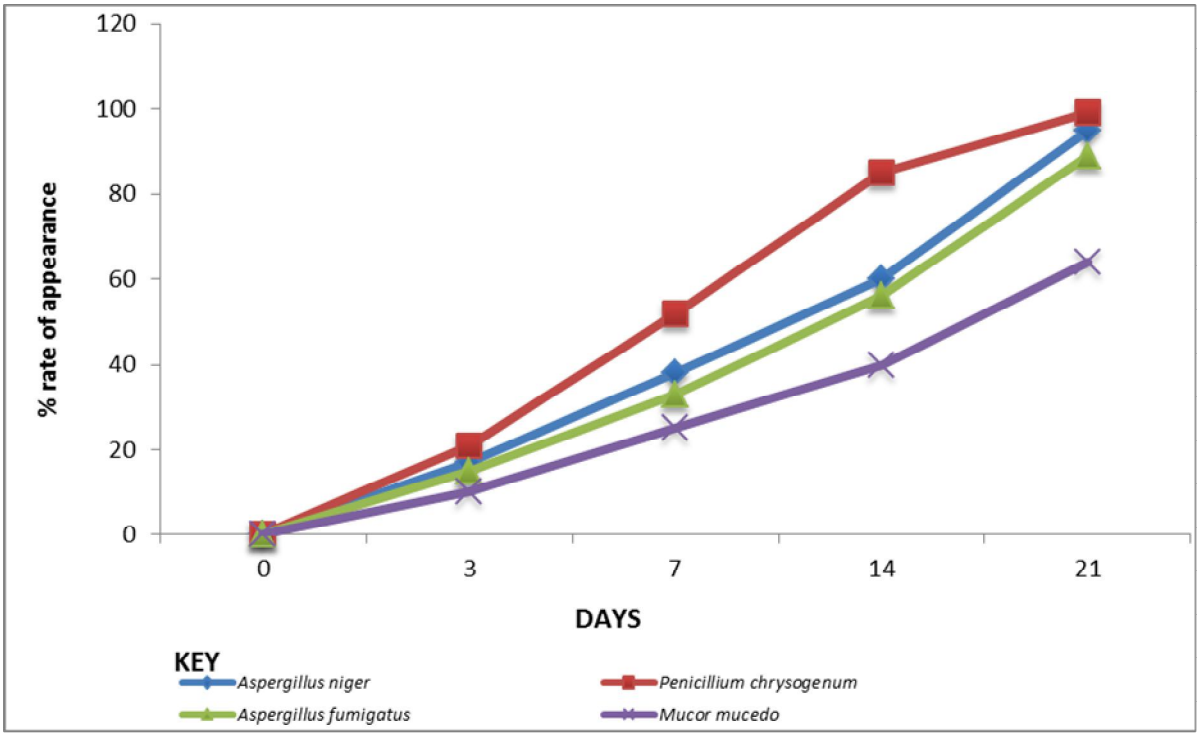

Fig. 1: Frequency (%) of fungal colonies on mineral salt agar containing cellulose

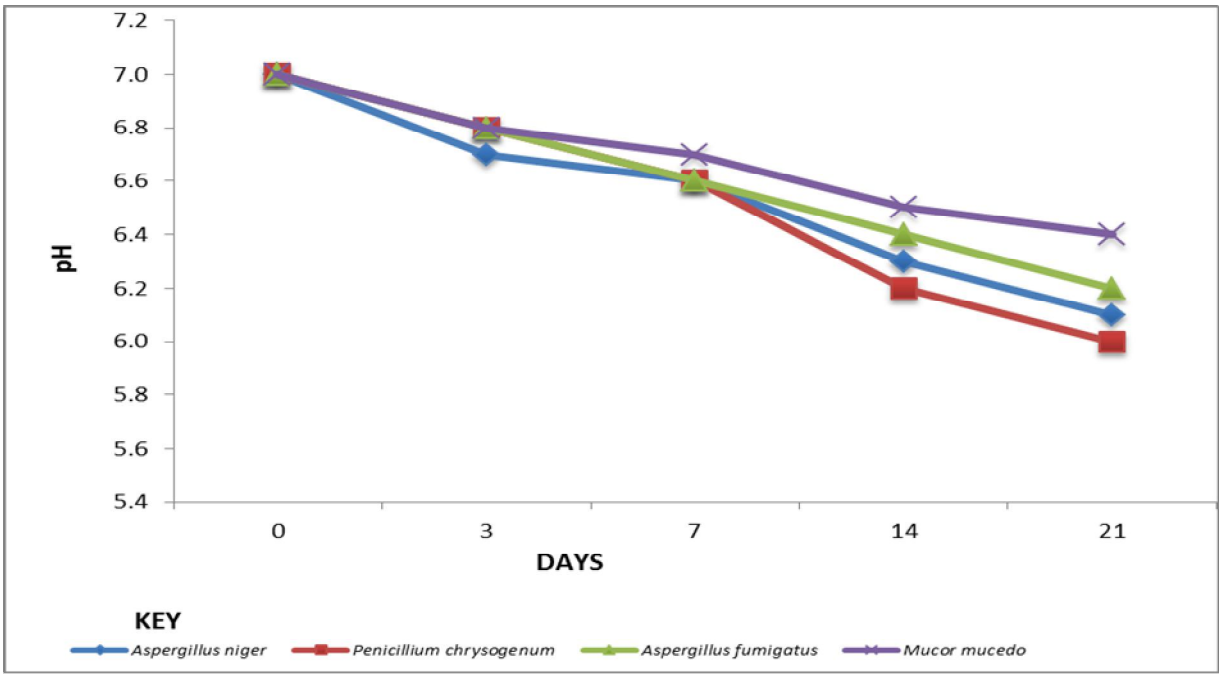

Fig. 2: pH values of the labelled modified mineral salt broth

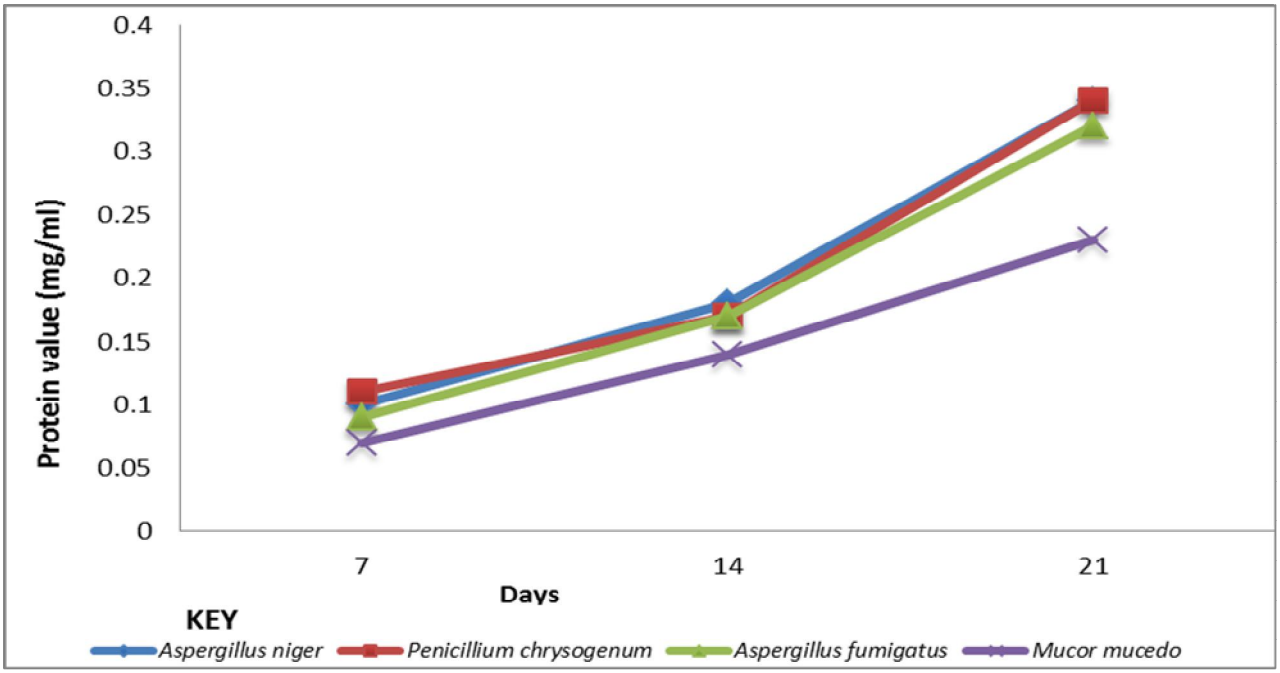

Fig. 3: Protein content of the labelled cell free supernatants

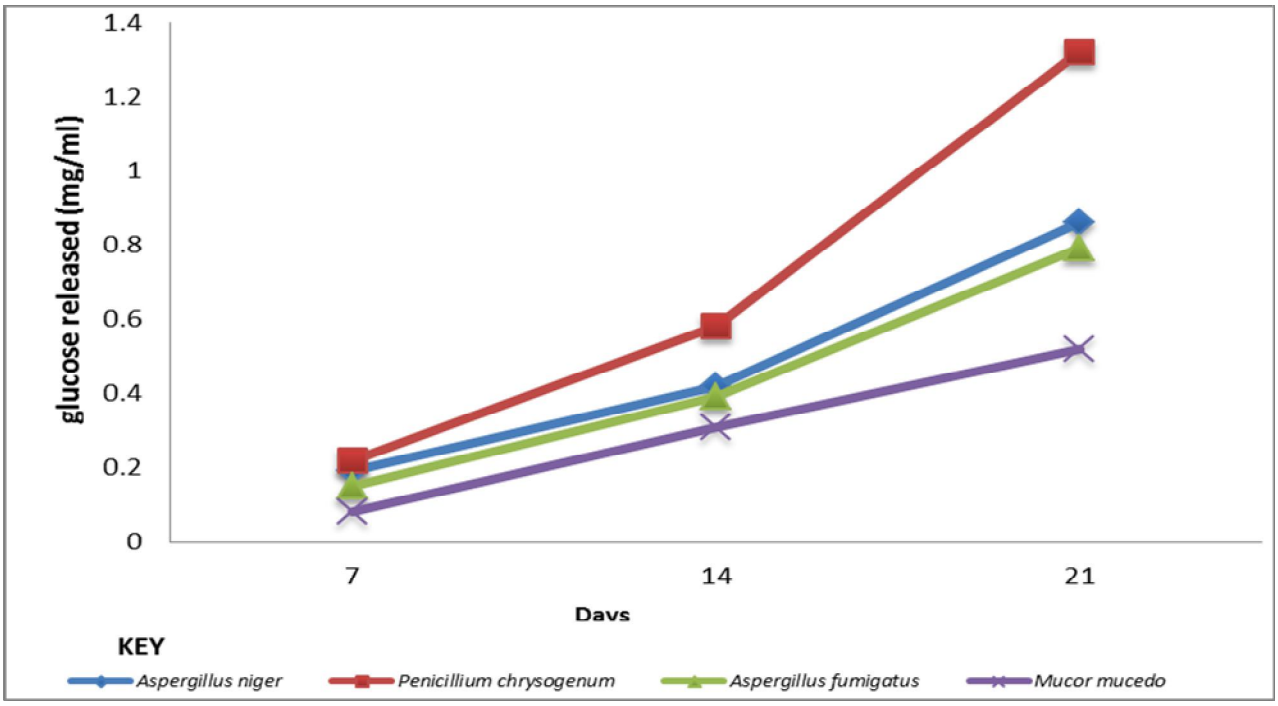

Fig. 4: Glucose content of the labelled cell free supernatants

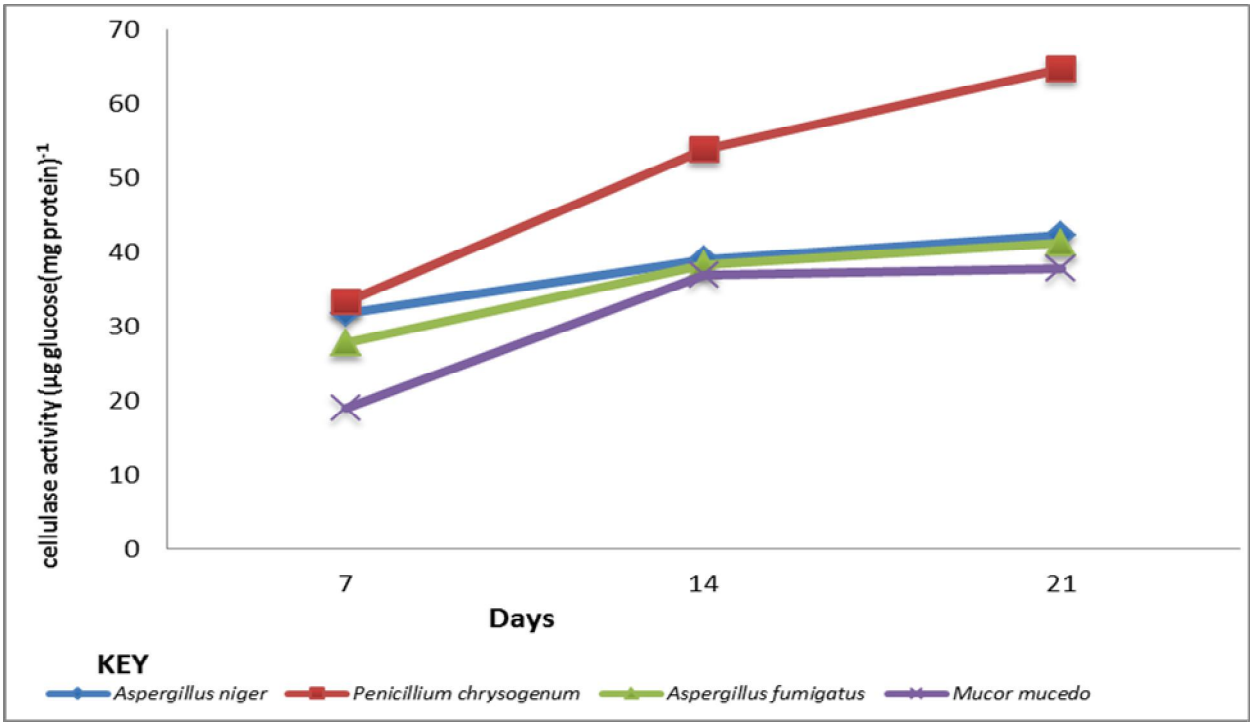

Fig. 5: Cellulase activity of the labelled cell free supernatants (Assay time was 60 min.).

The recovery of cellulolytic *A. niger*, *A. fumigatus* and *P. chrysogenum* (Tables 2 and 3) from the decayed wood samples was not surprising as these terrestrial fungi are known to cause soft rot which is form of wood decay (Sin *et al*., 2002). Kirk *et al*., (2001), reported that most of the commercially available cellulases including β-glucosidase were produced from *Penicillium, Aspergillus* and *Trichoderma* genera. The ability of the isolated microfungi to thrive on modified Czapek-dox agar and mineral salt agar containing sodium carboxymethyl cellulose and cellulose respectively as sole carbon source (Fig. 1, Table 3) could indicate that they can synthesize the enzymes necessary for the breakdown of cellulose to meet their energy and carbon needs. The ability of some microfungi to grow on cellulosic materials has been exploited in the isolation of several microfungi from wood wastes (Nwodo-Chinedu *et al*., 2005). The isolation of *M. mucedo*, whose cellulolytic activity was comparatively lesser than the other isolates (Table 3) is interesting and could suggest that members of *Mucorales* are capable of growing on a variety of lignocellulosic substrates such as palms, wood and bamboo. The isolation and identification of *P. chrysogenum*, *A.niger*, *A. fumigatus* and *M. mucedo* was in agreement with earlier reports of Damaso *et al*., (2012) and Sahab *et al*., (2012) who recovered these mitosporic cellulolytic fungi from diverse sources. This trend is also similar to a report by Nwodo-Chinedu *et al*., (2010) who isolated cellulolytic strains of *A. niger*, *A. flavus*, *P. chrysogenum* and *Trichoderma* sp. from a wood waste dump site at Covenant University, Ota, Ogun State, Nigeria. However, this observation is at variance with the report of Sun *et al*., (2002), who identified white rot cellulolytic fungi; *Trametes versicolor*, *Phanerochaete chrysosporium* and other cellulolytic fungi which included *Curvularia senegalensis*, *Spacicodes* sp. and *Anthostomella* sp. from several decaying lignocellulosic substrates.

The gradual decrease in the pH readings (Fig. 2) of the respective broth containing both the modified mineral salt medium and fungal cultures could be reflective of a steady increase in metabolic activities as the incubation period (21 days) progressed. This observation was also collaborated by a report of Parihar *et al*., (2012) which stated the formation of cellobionic acid and gluconic acid in the course of cellulose biodegradation. The lowest pH value was recorded for the flask containing *P. chrysogenum* (Fig. 2). This could suggest that the rate of metabolic activities associated this isolate was higher in comparison to other fungal isolates incubated under the same conditions. The observed cellulolytic activity of the cell free extracts (Fig. 5) is not surprising, as most cellulolytic fungi secrete hydrolytic enzymes for the breakdown of the polymers into their growth media (Chinedu *et al*., 2008). This phenomenon might also account for the protein content of the cell free supernatants (Fig. 3). *P. chrysogenum* apart from exhibiting the highest cellulolytic activity during the screening test (Table 3), had the highest % rate of appearance; 99% at day 21 (Fig. 1). These observations are indicative of the potency of the cellulase(s) elaborated by this isolate. The cell free supernatant derived from the broth culture of this fungus displayed the highest cellulosic activity as assayed

in terms of protein content, released glucose and cellulase activity (Figs. 3, 4 and 5). These trends could reflect the extracellular nature and intense activity of *P.chrysogenum* cellulase(s). This phenomenon is similar to the report of Dootlotkeldieva & Bobusheva (2011), who stated that amongst wild strains of cellulolytic fungi identified from soil and plant materials, *Penicillium* sp. was the most cellulolytic.

Conclusion

Several cellulolytic molds; *A.niger*, *A. fumigatus*, *M. mucedo* and *P. chrysogenum* were isolated and identified from several decayed wood cuttings. *P.chrysogenum* displayed the highest cellulolytic potential amongst the fungal species. As a consequence of this cellulolytic capability, these fungi or their purified cellulases can be potentially applied in the industrial saccharification of cellulosic residues to produce fermentable sugars such as glucose.

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