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Isolation of Cellulolytic Microfungi Involved in Wood-Waste Decomposition: Prospects for Enzymatic Hydrolysis of Cellulosic Wastes

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ABSTRACT: Wood-wastes from dump-sites at Okobaba Saw-mills on the western part of the Lagos lagoon were examined for cellulolytic microorganisms. Cellulolytic microfungi were isolated from the wastes using minimal salt agar medium containing 0.2% (w/v) crystalline cellulose, sugarcane pulp, corn cob or saw-dust as sole carbon/energy source. The colonies of cellulolytic microfungi which appeared on the plates increased in size and number as the incubation period (days) increased.
Among the fungal isolates were two pathogenic Aspergilli (*A flavus* and *A fumigatus*), three different black Aspergilli (herein designated as *A.niger* I, *A.niger* II and *A.niger* III), *Botrytis cinerea*, *Fusarium* species and *Penicillium* species. Cell-free filtrates of 7 – day cultures of A.flavus, A.niger I, A.niger II, B. cinerea and P.species grown on the minimal salt broth supplemented with crystalline cellulose as sole carbon/energy source showed very significant CM–cellulase activity. P. species gave a very high value that was over 4 times the value for the closest organism, A.niger II. There is a good propect for cellulase production using the virgin strain of P. species isolated from the wood-wastes.

Introduction

Waste cellulosic materials which may be classified as agricultural, industrial or municipal wastes are produced in tremendous quantities in every part of Nigeria. These solid wastes are usually discarded indiscriminately or dumped at various sites where they are burnt, buried of left to decompose thereby causing enormous environmental pollution with serious health consequences (Ali *et al*, 1991, Abu *et al*, 2000). The saw-mills located at Okobaba, Ebute-Metta, Lagos, on the western part of Lagos Lagoon present a major source of point pollution. The wood–wastes (saw-dust, wood-shavings and leachates) are deposited on the shores of the lagoon from where they eventually find their way into the waters resulting in heavy organic contamination at the saw-mill site of the lagoon. This has been found to reduce biodiversity, particularly, of diatoms, which could negatively affect the aquatic food web (Nwankwo, 2004).

These waste materials could be turned from liabilities into assets. Organisms which utilize cellulosic materials for their carbon and energy source could be exploited for the conversion of these wastes into products that are beneficial to man. For instance, waste cellulosic materials provide ideal substrates for the growth of mushrooms while the fungi can in turn degrade the fibrous wastes and add fungal protein to the spent substrates (Belewu and Banjo, 1999; Banjo and Kuboye, 2000; Belewu and Afolabi, 2000). Recovery and re-use of waste cellulose has been a subject of

extensive investigations as a means of alleviating energy and food shortages (Ghose, 1977; Khan,1980). Cellulose is a renewable carbon source and accounts for the fermentation potential of the Ligno-cellulose materials. It can be degraded by a number of bacteria and fungi. These organisms can produce a coordinate battery of extracellular enzymes capable of degrading cellulose as well as some other plant cell-wall polymers (Grant and Long, 1981). Bioconversion, particularly, enzymatic hydrolysis of cellulosic materials to useful products has great potential. Of increasing interest is the production of industrial enzymes such as cellulases, xylanases, glucose oxidases etc from organisms grown on these materials (Abu *et al*, 2000).

Cellulase is a family of enzymes that hyrolyse. b(1→4) glycosidic bonds of native cellulose and other related cellooligosaccharide derivatives (Hitomi *et al*, 1994). Synergistic action of three principal types of the enzyme, namely, endo–1,4–b–glucanase (EC 3.2.1.4), cellobiohydrolase (EC 3.2.1.91) and b–D–glucosidase (EC 3.2.1.21) is required to accomplish the degradation of native hydrogen-bond-ordered cellulose by cellulolytic fungi (Wood and McCrae, 1986). Cellulase is the key enzymes for the conversion of cellulosic materials into simple sugars which can serve as feed-stock for the productiion of different chemicals and fuels via anaerobic fermentation (Bailey *et al*, 1975; Ryu and Mandels, 1980). The enzymes may eventually provide a better answer to the problems associated with cellulosic waste disposal (Roy *et al*, 1990).

The availability of huge amount of cellulosic materials in Nigeria underlines the need to explore the potentials of the natural decomposers of the plant cell-wall polymers for the transformation of these wastes into useful products. Strains of the viable organisms could be used for the production of cellulases and other cell-wall hydrolysing enzymes needed for industrial saccharification of cellulosic materials. Although bacteria and fungi can produce cellulolytic enzymes, the fungal enzymes are usually preferred because they are extracellular, adaptive and usually secreted in large quantities (up to 2% by weight) during growth in submerged batch liquid fermenters. This is in sharp contrast to many bacterial enzymes which exist as tight multi-enyme complexes, often membrane bound, from which it is difficult to recover individual active enzyme species (Petterson Cowling and Porath, 1963; Berry and Paterson,1990). Conseqently, our focus is on cellulolytic microfungi. We have assessed the potentials of some of the microfungi isolated from decomposing wood-wastes from Okobaba saw-mills, Ebute-Metta, Lagos for cellulase production.

Materials and Methods

Wood–wastes

Sample of decomposing wood-wastes were collected from dump-sites at Okobaba saw-mills, Ebute-Metta, Lagos. One gram of the sample was asceptically introduced into 9.0ml of sterile distilled water in a sterile tube and shaken vigorously. This was the stock culture used for the investigations.

Cellulosic wastes.

Cellulosic waste materials used in this study were prepared as follows:

(i) Sugarcane pulp: Peeled sugarcane (*Saccharum officinarum*) was purchased from vendors at Egbeda, Lagos. The fibrous pulp (obtained after chewing the sugarcane) was washed repeatedly in distilled water to remove residual simple sugars. This was sun-dried, ground with a grinder (Marlex Excella Mixer grinder Mumbai, India) and passed through a sieve with fine mesh to obtain a very fine powder.

(ii) Corn Cob: Fresh maize (*Zea may*s) were purchased from Oshodi market, Lagos. The cob (left after removing the grains) were cut into very small pieces, sun-dried and ground with the grinder. Fine powder was obtained through the sieve.

(iii) Saw dust: Fresh saw-dust of Gmelina aborea wood was collected from Cement saw-mills, Orile- Agege, Lagos and seived to obtain a very fine powder.

Other subsrates and Chemicals.

Potato Dextrose Agar (PDA), Sabouraud's Glucose Agar (SGA) and Crystalline cellulose (Avicel) were obtained from Merck, Darmstadt, Germany. Carboxymethyl-cellulose (CM52) was from whatman Ltd, Springfield mill, Maidstone, Kent, England. All other chemicals and reagents used were of analytical grade.

Isolation of Cellulolytic Fungi

Microfungi capable of degrading cellulose (and other cellulolytic materials) as sole carbon\energy source were isolated from the wood-wastes using a modification of minimal salt agar medium described by Kastner *et al*, (1994).

The medium contained per litre: Na_2HPO_4, 2.13g; KH_2PO_4, 1.3g; NH_4Cl, 0.5g; $MgSO_4.7H_2O$, 0.2g; Agar-agar, 20.0g; Crystalline cellulose (or any of the cellulosic materials), 2.0g. Trace element solution (1ml per litre) as described by Bauchop and Eslden (1960) was added. The pH was adjusted to 5.6 before autoclaving at 121ºC for15 minutes. The minimal salt agar plates were inoculated with the stock culture and incubated at 30ºC for 3–14 days. Colonies of cellulolytic fungi were sub-cultured on PDA or SGA plates, supplemented with 0.5% (w/v) ampicillin. Pure cultures of cellulolytic fungi were obtained by repeated subculturing on PDA until one strain of fungus per plate was obtained. The fungal isolates were maintained on PDA slants until needed.

Identification of the Fungal Isolates.
Isolated cellulolytic fungi were identified based on their colonial and cultural characteristics and, especially, on the morphology of their sporing structures. The morphology of the isolates, stained with lactophenol-cotton blue, was studied using a light microscope (Carl, Zeiss, Germany) and by the description given by Thom and Raper,(1945) Alexopoulus (1952), Talbot (1972) and Deacon (1980).

Enzyme Production.
The minimal salt broth containing 0.2% (w/v) crystalline cellulose as sole carbon/energy source was used for enzymes production. Fifty-millilitre-portions of the medium were dispensed into 160ml sterile media bottles and antoclaved at 121ºC for 15minutes. Three to Seven-day PDA plate cultures of the organisms were used for inoculation as follows: Plates of each cellulolytic fungal isolate was covered with 10 ml of 0.1% Tween 80 . Conidia and spores were collected using a sterile cotton swab and transferred carefully into a sterile tube. One millilitre of each suspension was inoculated into the 50ml portion of the sterile minimal salt broth in the media bottle and closed with sterile cotton wool. The cultures were incubated for 7 days at room temperature (25ºC – 30ºC) with shaking (for 60 seconds each day). Cultures were harvested by centrifugation at 6000xg for 15minutes and the cell-free culture supernatants used as crude enzyme source.

CM–Cellulase assay.
Reducing–sugar method described by Wood and McCrae (1977) was used for determining CM–cellulase activity. A mixture of 1.0ml of CM-cellulose solution (1%, w/v), 0.5ml of 0.2M acetic acid–NaOH buffer (pH 5.4) and 0.5ml of cell-free culture supernatant was inocubated at 37ºC for 1hour. The reaction was stopped by the addition of 2.0ml Somogyi reagent, and the reducing sugar determined by the method of Nelson (1944).
The protein content of the supernatant was determined by Lowry's method (1951) using BSA as standard. The reducing sugars liberated were expressed as glucose equivalent. Cellulolytic activity was expressed as µg glucose released per mg protein per min

Results and Discussion

Appearance of fungal colonies on minimal salt agar plates.
Colonies of cellulolytic fungi appeared on minimal Salt Agar plates containing Cellulose(MSA-CE),Sugarcane pulp (MSA–SC), Corn cob (MSA–CC) and Saw–dust (MSA–SD) as sole carbon/energy source at different rates (Figure 1, Table 1). The number and size of the colonies increased as incubation period (days) increased. The different rates of appearance of the cellulolytic fungal colonies on the minimal salt agar plates supplemented with the various cellulosic material could be a function of the complexity of the different carbon/energy sources.The organisms degraded and utilized these materials at different rates.Saw-dust, evidently the most complex of the carbon sources,was the hardest to degrade ,hence, least number of colonies were found on plates containing this material. Biodegradability of pure crystalline cellulose was the highest while that of sugarcane pulp, corn cob and saw-dust followed in decreasing fashion.

Table 1: Rate of appearance of fungal colonies on minimal salt agar plates containing Cellulose (MSA – CE), Sugar cane pulp (MSA – SC), Corn cob (MSA – CC) and Saw-dust (MSA – SD) respectively.

Media Relative Growth	Carbon Source	Incubation Period	No. of Colonies

		3-Day	7-Day	14-Day	
MSA-CE	Cellulose	26 ± 5	57 ± 6	139 ± 14	++++
MSA-SC	Sugarcane pulp	10 ± 3	36 ± 5	87 ± 5	+++
MSA-CC	Corn cob	5 ± 2	28 ± 5	59 ± 4	++
MSA-SD	Saw-dust	0	10 ± 4	21 ± 2	+
MSA	–	0	0	0	–

Cellulolytic Fungal Isolates

Eight different cellulolytic fungi were isolated from the wood-wastes. Five of them belonged to the genus, Aspergillus, while one each belonged to Botrytis, Fusarium and Penicillium respectively (Table 2). The isolates described in this report are filamentous fungi. The genus Aspergillus has been documented as source of the most prevalent airborne moulds (Thom and Paper, 1945) and the frequency of mycoses due to these fungi is rising world-wide (Vitale *et al*, 2002). Two of the isolated aspergilli (Aspergillus flavus and A. fumigateus) are notably pathogenic. These two fungi are encountered most frequently in invasive infections that occur in immunocompromised patients such as human immunodeficiency virus (HIV) infected patients, cancer patients, bone-marrow transplant and solid–organ transplant recipients (Denning, 1998; Kaiser *et al*, 1998 Mylonakis *et al*, 1998). The use of wood–wastes to 'sand–fill' swampy areas by coastal/ riverine inhabitants portends a great danger for people whose immunity are hampered by poor diets and diseases. A flavus (Fig. 2 A&B) produces high level of afflatoxins which causes afflatoxicosis in animals and man (Prescott, Harley and Klein, 2002).

A. fumigatus (Fig. 2 C&D) causes respiratory disease, aspergillosis,which also affects livestocks and man(Harrigan and and McCance, 1996).

The members of the fungal genus *Aspergillus* are commonly used for the production of polysaccharide–degrading enzymes. Some members of the black aspergilli are very important because they produce cell-wall hydrolysing enzymes which have industrial applications. Three of the isolated aspergilli(herein designated as *A. niger* I, *A. niger* II, and *A. niger* III) are black Aspergilli. *A. niger* group is very common in a variety of habitats. The heads are black or darkly coloured resulting in colonies that are black or dark brown. New molecular and biochemical techniques have been used to classify the black aspergilli into eight species (Kusters–Van Someren, Samson and Visser, 1991; Megnegneau, Debets and Hoekstra, 1993; Verga *et al*. 1994). We intend to go beyond the morphological techniques used in this study to appropriately classify the three distinct strains of black Aspergilli (Fig. 3 A-F) described in this report. *Botrytis cinera* (Fig. 4) is a saprophytic or plant parasitic fungus which causes 'grey mould' of many plants, and a similar spoilage of fruit and vegetable products (Harrigan and McCance, 1966). Fusarium species (Fig. 5) produces spindle-shaped or sickle-shaped multicellular macroconida. Fusarium solani has been shown to produce cellulase enzymes which act in synergism to solubilize cotton fiber (Wood, 1971). Some *Penicillium* species such as *P. notatum* and currently *P. chrysogenum* are used to synthezise the well-known antibiotics, penicillin and griseofulvin (Prescott, Harley and Klein, 2002). Others such as *P. funiculosum and P. pinophilum* have been found to synthesize efficient cellulase system capable of solubilizing cotton fibers (Selby, 1968; Wood and Mcrae, 1986). It is interesting to isolate a strain of cellulolytic *Penicillium* species from the wood–wastes with high cellulolytic activity.

Table 2: Colonial and microscopic charateristics of the cellulolytic fungal isolates.

FUNGAL ISOLATE	MACRO CULTURE (colonial characteristics)	MICROSCOPY (morphological features)
Aspergillus flavus	Yellow/dusty colonies	Septate hyphae, non-septate conidiosphore, columinar conidial head
Aspergillus fumigatus	Grey – Dark green/velvety colonies	Same as *A. flavus*
A. niger I	Large black head (thick black colonies)	Septate hyphae, non-septate conidiospore, mop-like conidial head.
A. niger II	Small black head (Black/dark brown colonies)	Same as *A. niger* I
A. niger III	Brown head (brown colonies	Same as *A. niger* II
Botrytis cinerea	Grey/fluffy – greenish colonies	Septate hyphae. Irregularly branched conidiosphores, clusters of grape-like conidial head.
Fusarium species	White/fluffy, spreading peripheral part, pinkish central part.	Spindle–or sickle–shaped multicellular macro-conidia.
Penicillium species	Blue-green or ash/ flaky colonies	Septate hyphae, branched conidosphore, a brush like conidial head

CM – cellulase activity.

The CM–cellulase activity of day 7 cultures supernatants of *A. flavus, A. niger* I, *A. niger* II, *B. cinerea* and *Penicillium* species showed significant activity (Table 3). P. Species gave a very high specific activity (266.67) that was over 4 times that of the closest organism, *A niger* II (61.67). From growth studies done in this laboratory(in press), the organisms attained optimal growth in the minimal salt broth between 5- 6 days. There was a sharp decline in the fungal growth thereafter. Thus, cellulase activity could be much higher before the 7-day incubation period used in this study.The strain of P.species we isolated from the wood–wastes could prove very useful in the production of cellulase needed for the saccharification of cellulosic waste materials.

Table 3: CM–cellulose activity of crude enzyme preparations from fungal isolates (A flavus, A niger 1, A niger 11, B cinerea and P. Species). Assay time was 60 minutes

FUNGUS	Protein (mg/ml)	Glucose released (mg/ml)	CM-CELLULASE ACTIVITY [ug glucose (mg protein) $^{-1}$min $^{-1}$]
A. flavus	0.7	1.8	42.86
A. niger I	1.5	4.7	52.22
A. niger II	1.0	3.7	61.67
B. cinerea	1.4	1.4	16.67
P. species	1.0	16.0	266.67

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