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Antimicrobial agent production by fungi isolates from petroleum product contaminated soil

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ABSTRACT

Crude Cell-free extract of eight(8) fungal isolates gained from a petroleum effluent contaminated soil in Calabar, Cross River State Nigeria, were tested for antimicrobial activity against the native hydrocarbon degrading bacteria (Bacillus species, micrococcus species) and some clinical isolates (pseudomonas aeroginosa, Escherichia coli and staphylococcus aureus) as well, using Agar well diffusion technique. The results indicated that three of the obtained isolates (Penicillium sp, Aspergillus sp., and Cladosporium) demonstrated anti-microbial activity against some of the microbes tested (80%), with the diameter of zones of inhibition ranging from 20mm-34mm. Crude cell-free extract of Penicillium sp (F2) exhibited activity against two of the native hydrocarbon utilizers (Bacillus sp., and micrococcus sp.), while that of Aspergillus sp. (F8) exhibited a broad spectrum activity on one of the hydrocarbon utilizing bacteria (Micrococcus sp) and two of the clinical isolates (Escherichia coli and pseudomonas aeruginosa) staphylococcus aureus was resistant to all cell free extracts. These results provided grounds for further work to investigate the repressive effects of these metabolites as it affects bioremediation involving organisms.

Keywords: Antimicrobial, Fungi, Isolates, Petroleum, Contaminated, Soil

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INTRODUCTION

In the beginning of the 20th century the idea of growth inhibition of one microorganism present in the vicinity of other came into existence, later, it was discovered and demonstrated that growth inhibition of microorganism was mediated by secretion of toxic metabolite by another organism (Dubey). Indeed, these compounds are mostly biosynthesized by bacteria, fungi, algae, corals, sponges, plants and lower animals (Demain et al.,). A large number of chemical substances have the ability to inhibit the growth and metabolism of microorganisms or kill them (Zheng). Hence antimicrobial agents refers to chemical agents used for the control of microorganism, whether on inanimate objects e.g. disinfectant or on animal host in the treatment of disease (Itah et al.).

The spectrum of antimicrobial activity of the active substance is very important for the competition in nature. In the soil, where most antimicrobial producing microorganisms are found, life is competitive. The inhabitant must compete for carbon, nitrogen and phosphate necessary for their growth. Successful competition may be ensured by inhibition of the growth of other organisms through the production and secretion of substances interfering with their metabolism (Lancini et al.). These substances owe their selective toxicity to the fundamental differences between prokaryotes and eukaryotes cells {Dietera et al.}. Antimicrobial agents were originally used to refer only to

substances extracted from fungi or other microorganism but has come to also include the many synthetic and semi-synthetic agents which are obtained by modification of side chains (Okafor).

The search for and development of new antimicrobial agents is an expensive and time consuming venture.(Shogi). Nevertheless, several antimicrobials have been discovered, each sufficiently, well-characterized for one to be certain, it differs chemically and biologically from others. Despite the wide variety of known antimicrobial less than 1% of antimicrobial agents have any medical or commercial value, thus it varies between countries as preciously unknown shortcomings are discovered even in the existing ones (Gelderblom). Therefore, there is the need to search for new antimicrobial agents to meet new challenges, such as the emergence of a new disease and evolution of high rate of multiple resistances to known antimicrobials by microorganisms (Vasavada, et al.).

MATERIALS AND METHODS

Materials

The following criterion dehydrated cultured media (Sabouraud) Dextrose Agar, Malt Extract- Yeast Extract Agar, Bushnell Hass, Brain Heart Infusion medium, Nutrient Broth) procured from hardy Diagnostic USA and Titan Biotech, India were used for this study and prepared according to manufacturers instructions.

Reagents

Analytically graded alcohol, dilute hydrochloric acid (Hcl) and Lactophenol cotton blue procured from Sigma Aldrich, USA and Titan Biotech India were also used in this study.

Methods

Sample Collection

Petroleum effluent contaminated surface and sub-surface soil samples were collected from mechanic workshops at Etta Agbo in Calabar South of Cross River State Nigeria into sterile plastic containers and transported to microbiology laboratory for analysis. Microbial analyses were carried out within twenty-four hours after collection of samples. Clinical bacterial isolates were obtained from the Microbiology Laboratory, General Hospital Mary Slessor, Calabar. Their identities were confirmed before use by microscopic examination and biochemical tests. They were Staphylococcus aureus, Pseudomonas aeruginosa, and Escherichia coli.

Enumeration of Culturable Heterotrophic Fungi

10gm of the soil sample were diluted in 90ml of sterile distilled water. Ten-fold serial dilution was carried out, 0.1ml of 10^{-3} and 10^{-4} dilution were planted out in duplicate unto Sabouraud Dextrose Agar and Malt Extract-Yeast Extract Agar using a speed plate technique supplemented with 50mg/ml of streptomycin to inhibit the growth of bacteria, also the Ph of the medium was adjusted to 5.8 to encourage the growth of the fungi. The plates were incubated at room temperature (28^0C) for 96 hours. At the end of the incubation period, the number of colonies present in each plate was determined and the average count obtained.

Enumeration of Culturable Hydrocarbon utilizing Fungi

Hydrocarbon utilizing fungi were enumerated by viable plate count, using spread plate technique. Bushnell Hass Agar supplemented with 50ug/ml of streptomycin was prepared and pH adjusted to 5.8, then 0.1ml of 10^{-3} and 10^{-4} dilutions were inoculated unto the medium in duplicate, at same time sterile whattman No. 1 filter paper was saturated with 1ml of crude oil and place aseptically on the lid of the inoculated plate (Atlas and Bartha). The plates were taped round with masking tape. This was done to provide the crude oil as the soul source of carbon and energy for the growth of the organisms on the surface through vapour phase transfer. The plates were then incubated at room temperature for 4-7 days after which fungal count was recovered.

Isolation and Purification of Fungi Isolates

The fungal colonies which developed were isolated and purified by repeated subculturing onto a fresh Sabouraud Dextrose Agar plates supplemented with antibacterial agent (50ug/ml of streptomacin) without adjusting the pH.

Characterization and Identification of Fungi Isolates

Pure isolates of the fungi were presumptively identified macroscopically on the basis of their cultural, morphological and physiological characteristic and microscopically using lactophenol staining technique.

Identification of the isolates was accomplished by using the dichotonomous key and picture key of known fungi class.

Screening for Antimicrobial Agent Production

Selected isolates were studied for their ability to produce antimicrobial, in broth culture and by direct inoculation. Dense inoculants of selected strains were inoculated into test tubes containing 10ml of Brain Heart infusion broth with pH of 5.8; the inoculated media were incubated at 28^0C for 7 days with constant agitation. At the end of the incubation, the cultures were washed by centrifugation at 4000g for 30mins (Kanlayani et al) after which the crude supermatant were assayed using the Agar well diffusion technique. The entire surface of Mueller-Hinton plate was inoculated via spread plate technique using sterile Swab stick, sterile precipitin tubes were used to bore the media and 0.5ml portion of the crude supermatant was immediately introduced into the well and then incubated at 37^0C for 24hours

RESULTS

Petroleum effluent contaminated soil samples were analysed. The result of the enumeration of heterotrophic fungi and hydrocarbon utilizing fungi are presented in table A. samples showed an average heterotrophic fungi count of $6.3x10^4$ cfu/ml and an average hydrocarbon utilizers count 4.5104 cfu/ml with and an average of 68.6% as hydrocarbon utilizers.

Table B shows the results of the characterization and identification of fungi isolates. It showed that the fungi generally isolated were Pennicillium sp (F2), Aspergillus sp (F8), Cladosporium sp, (F3) unidentified sp (F4) Aspergillus sp (F5), Aspergillus sp (6) unidentified sp (F7) and unidentified sp (F1). The screen test result by direct inoculation is presented in table 4.3. It shows that only the following isolates Peniccilium sp (F2),

Cl;addosporium sp (F3) and Aspergillus sp (F8) which constitute 37.5% of the entire isolates, where promising with very little zone of clearance observed on the plates with the test organisms.

The spectrum of antimicrobial activity of the crude cell-free extract is presented in table D. Crude cell-free extract of all the isolates produced various degrees of zone of inhibition against the test organism. This shows that Penicillium sp (F2) has an antagonistic activity on the two hydrocarbon utilizing bacteria i.e. Bacillus sp (30mm), Micrococcus sp (20mm) and no effect on the clinical isolates while Aspergillus sp (F10) inhibited growth of Micrococcus sp (20mm) and Escherichia coli (34mm) which are clinical isolates with Cladosporium sp (F3) inhibiting only Escherichia coli (25mm). a clinical isolate while Staphylococcus aureus was resistance to all the isolates.

DISCUSSION

This study was aimed at investigating the antagonistic activity of obtained fungi isolates from petroleum effluent polluted environment against native hydrocarbon degrading bacteria and some clinical bacteria isolates. The antimicrobial agent producers' isolates were species of Peniccillium, Aspergillus and Cladosporium (Table A). The results suggests the presence of Metabolites from 37.5% (i.e. 3 out of 8 of the fungi isolates that shows various degree of antagonistic effect against the test isolate with only 25% (i.e. 2 out of 8) antagonizing the native hydycarbon utilizing bacteria (Table B). this was evidenced by the clear zone of inhibition produced by the bacteria around the tested fungi extract. The best in-vitro antimicrobial activity (34mm) was exhibited Aspergillus sp ((F8) against Escherichia coli. (Table B). This was followed by Penicillium sp (F2) (30mm) against Bacillus sp and then Cladosporium sp (F3) (25mm) against Escherichia coli. This observation is in agreement with the work of Atalla et al., (1993) which had previously reported the ability of these organisms (Penicillium, Aspergillus, and Cladosporium) to produce antimicrobials in broth culture against gram-positive and gram-negative bacteria. Ding et al (2008) also reported that Cladosporium sp (F3) was able to produce Phenylacetic acid and P-hydroxyphenylethl which are strong antimicrobial compound. Also Furtado (2002) reported that Aspergillus sp was able to produce 3,4-dimethoxylphenol and 1,2,5-trimethoxybenzine which are also strong antmicrobial agents. This is in agreement with the result above (Table B), which showed that Penicillium sp (F2) exhibited antimicrobial activity against Bacillus sp, Micrococcus sp, while Aspergillus sp (R8) was effective against Micrococcus sp Pseudomonas aeruginosa and Escherichia coli. Furthermore, from Table B, it shows that Staphylococcus aureus was resistant to the crude cell-free extract of all the fungi isolates tested (Table D). This observation was not surprising as Staphylococcus aureus is the most frequently identify antimicrobial drug resistance pathogen in hospitals (Livermore, 2000). Aspergillus sp (F8)

exhibited broad spetrum activity against the test organisms, though it inhibited only one of the hydrocarbon degrades (Micrococcus sp). However crude cell-free extract of the isolates produce better zones of inhibition than were obtained by direct inoculation screening procedure were little or no zone of clearance were observed during the screening process, this is probably due to the fact that more of the antimicrobial substances were infused into the growing medium as secondary metabolites and were concentrated during the extraction process.

Table A, Enumeration of culturable heterotrophic and hydrocarbon utilizing fungi

Sample code	Heterotrophic fungi	Hydrocarbon utilizing Fungi count cfu/ml	Percentage of Hydrocarbon utilizing fungi
FA1	$15.5X10^3$	$10X10^3$	64.5
FA2	$11X10^4$	$8X10^4$	72.7
Average Count Cfu/ml	$6.3x10^4$	$4.5x10^4$	68.6

Table B Characterization and identification of fungi isolates

Isolate Laboratory code	Colour of aerial hyphae	Colour of substrate hyphae	Nature of hyphae	Shape and kind of hyphae	Presence of special structure	Appearance of conidiophores	Characteristics of spore head	Probable organism
F3	Gray-brown	Dark-olive	Septate and branched	Ellipsoid conidia	Prominent scars on the spores	Long branching chain of conidiophores	Branching chain of conidia	Cladospodium sp
F8	White	Gray-green	Septate and branched	Oval coidia	Root cell present	Long and erect non-conidiophores	Long chains of conidia	Aspergillus sp
F2	_	Green	Septate and erect	Round conidia	_	Long and erect conidiophores	Brush-like chain of conidia	Penicillium sp
F5	Yellow	Black	Septate and Branched	Spherical conidia	Root cell present	Long and erect non- septate conidiophores	Long chains of conidia	Aspergillus sp
F6	Yellow	Green	Septate and Branched	Spherical conidia	Root cell present	Long and erect non- septate conidiophores	Long chains of conidia	Aspergillus sp
F1	Cotton White	_	_	_	_	_	_	Unidentified
F4	Green	Blossom Green	_	_	_	_	_	Unidentified
F7	Yellow	Green	_	_	_	_	_	Unidentified

Table C Screen Test Results of the Fungi Isolates by direct Inoculation

Laboratory	Diameter of zone of inhibition (mm) of test organisms				
	Bacillus sp	Micrococcus sp	Staphylococcus aureus	Escherichia coli	Pseudomonas sp
(F1) Unidentified sp	ND	ND	ND	ND	ND
(F2) Penicillium sp	D	D	ND	ND	ND
Cladosporium sp	ND	ND	ND	D	ND
(F4) Unidentified sp	ND	ND	ND	ND	ND
(F5) Aspergillus sp	ND	ND	ND	ND	ND
(F6) Aspergillus sp	ND	ND	ND	ND	ND
(F7) Unidentified sp	ND	ND	ND	ND	ND
(F8) Aspergillus sp	ND	D	ND	D	D

ND = Not Detected
D = Detected

Also, the fungi isolates were observed to exhibit different morphological appearance on the two media used in growing them (i.e. SDA and ME-YEA). This is probably due to the media composition; from the results it shows that Malt Extract – Yeast Extract Agar is easier for macrocospic examination and identification of fungi than Sabouraud Dextrose Agar. Also, the pH regulation of the media resulted in having a particular fungi strain dominating each plate.

Table D Spectrum of Antimicrobial Activity of Crude Cell-Free

Supernatant	Diameter of zone of inhibition (mm) of test organisms				
Isolate Laboratory code	Bacillus sp	Micrococcus sp	Staphylococcus aureus	Escherichia coli	Pseudomonas sp
Unidentified sp (F1)	ND	ND	ND	ND	ND
Penicillium sp (F2)	30mm	20mm	ND	ND	ND
Cladosporium sp (F3)	ND	ND	ND	25mm	ND
Unidentified sp (F4)	ND	ND	ND	ND	ND
Aspergillus sp (F5)	ND	ND	ND	ND	ND
Aspergillus sp (F6)	ND	ND	ND	ND	ND
Unidentified sp (F7)	ND	ND	ND	ND	ND
Aspergillus sp (F8)	ND	20mm	ND	34mm	20mm

ND = Not Detected

CONCLUSION

In the soil where most antimicrobial substance producing Microorganisms are found, life is competitive and changes in composition of microbial community due to secondary metabolites have been reported (Lancini and Perenti, 1982). Thus 66.7% of the antimicrobial substances producing fungi isolated (Penicillium sp and Aspergillus sp) and the test isolates (Bacillus sp and Micrococcus sp) are efficient hydrocarbon utilizers (Amadi et al.. 1991). Based on the results obtained from this study, it shows that lots of antagonistic activity occurs amongst the hydrocarbon degraders since the inhabitants must compete for carbon, nitrogen and phosphate necessary for their growth. Successful competition may be ensured by inhibition of the growth of other organism through the production and secretion of substances interfering with their metabolism. This implies that bioremediation involving micro-organisms do not occur as swiftly as assumed to be.

Hence, the hydrocarbon degradation activity of other organism will be greatly repressed, thus resulting in having an environment which remain polluted for a very long time before complete degradation is achieved because faster degradation is best achieved by a consortium of micro-organisms and not a single micro-organism as reported by Amadi and Antai (1991). Hence, more investigation should be carried out in hydrocarbon polluted soil so as to assay and identify the active substance which interferes with the metabolism of other hydrocarbon degraders and develop a better strain that will be resistant to the secondary metabolite and degrade hydrocarbon effectively.

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