Journal of Environmental Biology
ISSN : 0254-8704
http : //www.geocities.com/j_environ_biol/

J. Environ. Biol.
26 (1), 37- 42 (2005)
j_environ_biol@yahoo.com

Biodegradation of crude petroleum and petroleum products by fungi isolated from two oil seeds (melon and soybean)

A. A .Adekunle and T. F. Oluyode
Department of Botany and Microbiology, University of Lagos, Yaba, Lagos, Nigeria.

(Received: 19 August, 2002 ; Accepted: 09 November, 2002)

Abstract : *Crude petroleum oil degrading fungi were isolated from two oil seeds, Cucumeropsis mannii (melon) and Glycine max (soybean) seeds in the presence and absence of petroleum fumes. An assessment of the relative ability of each fungus to degrade crude petroleum, diesel and kerosene on minimum salt solution was done using change in optical density read on spectrophotometer. Twenty-one fungal species (14 genera) were isolated alltogether during this experiment. These include eight species of Aspergillus; one species each of Botryodiplodia, Bipolaris, Cladosporium, Cunnighamella, Dreschlera, Fusarium, Helminthosporium, Macrophomina, Mucor, Paeciliomyces, Penicillium, Rhizopus and Talariomyces. It was evident that most of the fungi tested were able to biodegrade the crude petroleum oil, though at different rates. Bipolaris had a low rate of biodegradation of the petroleum oil of all the fungal species isolated Botryodiplodia theobromae had the highest degrading ability on the crude oil, while Aspergillus flavus had the least after 40 days of incubation. Aspergillus flavus had the highest ability to biodegrade diesel while A. niger had the least ability. In kerosene, Macrophomina phaseolina had the highest ability while A. niger had the least ability to biodegrade it. There was fluctuation in the growth pattern of the fungi in the petroleum oil medium. The implication of these are discussed.*

Key words **:** *Oilseeds, Crude petroleum, Fungi, Oil pollution, Biodegradation.*

Introduction

Many species of bacteria, fungi and algae have enzymatic capability to use petroleum hydrocarbons as food (Amund *et al*, 1987; Amund and Igiri, 1990; Wang *et al*, 1994). Fungi play an important role in the hydrocarbon utilization of soil samples and several fungi such as *Aspergillus fumigatus* and *Penicillium* sp were found to exhibit greater hydrocarbon biodegrading ability than bacteria such as *Arthrobacter, Brevibacterium, Flavobacterium, Micrococcus* and *Pseudomonas* (Cerniglia and Perry, 1973). It was demonstrated by Barth a and Atlas (1973) that fungi can degrade the petroleum hydrocarbons into much smaller non-environmental hazardous fractions than bacteria.

Most reports on the biodegradation or bioremediation using fungi has being mainly on fungi isolated from soil or aquatic environment (Colombo *et al*, 1996). Some fungi are known to be pathogenic on oilseeds such as *Cucumeropsis mannii* and *Glycine max* (soybean) seeds (Oyeniran, 1980). It has been shown that fungal infection of these seeds led to deterioration of the vegetable oil extracted from the oil seeds by changing the free fatty acid content, peroxide and saponification values, and also reducing the quantity of oil present in the seeds (Kuku, 1979; Adekunle and Uma, 1996). Adekunle and Uma (1996) showed that the pathogenic fungi isolated from melon seeds contain lipase enzyme which helped in the degradation of this oil seed components. The ability of the lipase or other enzymes produced by the fungi to degrade the hydrocarbons in the oilseed can also be tried to degrade the hydrocarbon in the petroleum crude oil. There is no documented report on the biodegradation of petroleum oil by fungi isolated from oil seeds.

The aim of this paper is to investigate and document the capability of fungal species isolated from two oil seeds (melon and soybean) to utilize and degrade hydrocarbons in crude petroleum oil and some petroleum products such as diesel and kerosene.

Materials and Methods

Collection of seed samples : Soybean (*Glycine max*) and "Egusi" malon (*Cucumeropsis mannii*) seeds used were collected from two different markets (Tejuosho and Lawanson markets) in Lagos State, Nigeria (Long. 8^{0}N Lat. 4^{0}E). Diseased seeds were separated from the healthy seeds. The visually diseased seeds were used in this experiment. The oil seeds were sampled from the markets thrice every month for 3 months. Four hundred seeds of each plant species were collected from each market at every sampling period.

Isolation, identification and screening for crude petroleum oil degrading fungi : Sixty diseased seeds from each market per sampling day, each of melon and soybean seeds, were surface sterilized. This was done by leaving the diseased seeds in a solution of common bleach (sodium hypochloride) and sterile distilled water in a ratio of 3:2 for one minute. They were then rinsed with three changes of sterile distilled water. These sterilized seeds were used for isolation. To isolate fungi from the oil seeds under petroleum crude oil fumes, the modified method of Amund *et al* (1987) was adopted. Thirty-two filter papers (Whatman No. 1001125) were sterilized in the autoclave and then allowed to dry in the oven at 40°C for 15 minutes. Sixteen of the dried filter papers were dipped in 250ml petroleum crude oil contained in a 500ml beaker for about 15 seconds with the help of a sterile forcep, and drained. The petroleum crude oil was obtained from the microbiology laboratory of Chevron Petroleum Plc, Lagos, Nigeria. Each of the sixteen crude oil treated filter papers were placed on the cover of twelve petri dishes containing solidified potato dextrose agar (PDA) and the sterilized diseased oil seeds (seven seeds per plate) under sterile conditions. Eight plates contained melon seeds and another eight contained soybean seeds. The aim of the petroleum fumes was to supply the fungi with hydrocarbons through vapour transfer by supplying petroleum fumes. The other sixteen plates, eight each of melon and soybean seeds were without the "oiled" filter papers and they served as control. All the plates were incubated at room temperature (28 – 31°C) in the incubator, and observed daily for fungal growth. This process was repeated 5 times per sample from the two markets. The developing fungal colonies were sub cultural aseptically into fresh PDA plates to get pure culture of isolates. A part of each pure culture was then aseptically transferred into sterile agar slants which had previously been prepared in 14ml McCartney bottles, and served as stock culture.

To identify the fungi, light microscopic examination was carried out and also cultural characteristics such as colour of the fungal colony, number of days taken for the fungus to reach maximum diameter (9 cm) of the petris dish, and the texture of fungal growth were noted. The morphological and cultural features of each fungus was compared with descriptions given by Talbot (1971), Deacon (1980), Domoschet *et al* (1980) and Bryce (1992) for identification. Some mycologist within the Department of Botany and Microbiology, University of Lagos were consulted for confirmatory identification of the fungi.

Confirmatory test for hydrocarbon utilization potential of the fungi : The estimation of hydrocarbon utilizers were obtained using enrichment procedure as described by Nwachukwu (2000). A minimal salt solution (MSS) containing 2.0g of Na_2HPO_4, 0.17g of K_2SO_4, 4.0g of NH_4NO_3, 0.53g of KH_2PO_4 and 0.10g of $MgSO_4$. $7H_2O$ was dissolved in 1000ml distilled water. Thirty-six test tubes were sterilised, plugged with cotton wrapped in aluminium foil, and placed on test-tube racks. There were 4 test-tube racks, containing 9 test-tubes each. In each test-tube 10ml of the minimal salt solution (MSS) was added and each of the 4 racks had either 2ml of petroleum crude oil, diesel or kerosene, and the fourth rack served as control, with only the MSS in its 9 test-tubes.

Eight fungi (choice was based on frequency of occurrence on seeds) which were isolated from both melon and soybean seeds in the presence or absence of crude oil were inoculated into each test-tube on the rack. The last tube on each rack served as a control, it was not inoculated with any fungus. Each of the test-tubes were plugged with sterile cotton wool wrapped with aluminium foil so as to ensure maximum aeration and prevent cross contamination. All the test-tubes were then inoculated at the room temperature in an incubator for 40 days. Constant shaking of the test-tubes was ensured to facilitate oil/cell phase contact. The ability to degrade petroleum crude, diesel and kerosene (based on the growth rate of the organisms on the MSS medium) was measured every 5 days using the visual method which was based on the turbidity of the MSS medium. The turbidity was measured using the spectrophotometer at a wavelength of 520nm. This experiment was repeated twice. Results were statistically analysed using T-test, anova (F-test) and Duncan multiple range test as described by Parker (1979).

Results and Discussion

Table 1 shows some of the fungal species isolated from both melon and soybeans in the presence or absence of petroleum fumes. Twenty-one fungal species (14 genera) were isolated altogether during this experiment. The fungi isolated from soybean in the presence of petroleum fumes were *Aspergillus flavus*, *A. fumigatus*, *A. niger*, *Fusarium solani*, *Helminthosporium*, *Macrophomina phaseolina*, *mucor racemosa*,

Table – 1 : Fungi isolated from melon and soybean seeds with or without crude petroleum oil fumes.

Melon seeds without petroleum fumes	Melon with petroleum fumes	Soybean without petroleum fumes	Soybean with petroleum fumes
Aspergillus flavus	*Aspergillus flavus*	*Aspergillus flavus*	*Aspergillus flavus*
Aspergillus fumigatus	*Aspergillus niger*	*Aspergillus fumigatus*	*Aspergillus fumigatus*
Aspergillus japonicus	*Aspergillus wentii*	*Aspergillus niger*	*Fusarium solani*
Aspergillus niger	*Cladosporium* sp	*Aspergillus oryzae*	*Helminthosporium* sp
Aspergillus vesicolor	*Helminthosporium* sp	*Bipolaris* sp	*Macrophomina haseolina*
Aspergillus tamarii	*Macrophomina phaseolina*	*Dreschlera* sp	*Mucor racemosa*
Aspergillus wentii	*Rhizopus sp*	*Fusarium solani*	*Paeciliomyces* sp
Botryodiplodia theobramae		*Helminthosporium* sp	*Talariomyces* sp
Cunnighamella sp		*Mucor racemosa*	
Mucor racemosa		*Pemicillium pinophylum*	
Penicillium chrysogenium		*Rhizopus* sp	
Pencillium pinophyllum		*Talariomyces* sp	
Rhizopus sp			
Talariomyces sp			

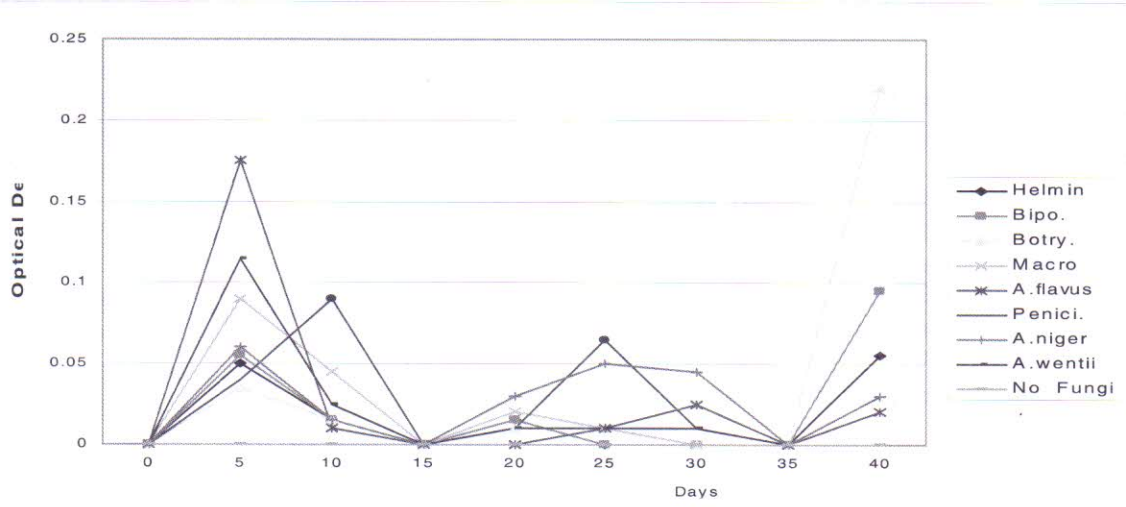

Fig. 1 : The growth pattern of fungi in minimal salt solution.

Paeciliomyces, *Rhizopus* and *Talariomyces* while those in the absence of fumes were *Aspergillus flavus*, *A. fumigatus*, *A. niger*, *A. oryzae*, *Bipolaris*, *Dreschlera*, *Fusarium solani*, *Helminthosporium*, *Mucor*, *Pencilliulm pinophylum*, *Rhizopus* and *Talariomyces*. The fungi isolated melon seeds in the absence of petroleum fumes were *A. flavus*, *A. niger*, *A. wentii*, *Cladosporium*, *Helminthosporium*, *Macrophomina*, *Paeciliomyces* and *Rhizopus*; while those isolated in the presence of fumes were *A. flavus*, *A. fumigatus*, *A. japonicus*, *A. niger*, *A. versicolor*, *A. tamarii*, *A. wentii*, *Botrydiplodia theobromae*, *Cunnighamella*, *Mucor*, *Pencillium chrysogenum*, *Penicillium pinophylum*, *Rhizopus* and *Talariomyces*. More fungal species were isolated from the diseased oilseeds incubated without the petroleum fumes than those incubated with petroleum fumes. Fourteen fungal species were isolated from melon seeds without the petroleum fumes, which was the highest number of species isolated from the four set of seed treatment. The eight most frequent fungi were *A. flavus*, *A. niger*, *A. wentii*, *Bipolaris*, *Botryodiplodia theobromae*, *Helminthosporium*, *Macrophomina phaseolina* and *Pencilium chrysogenum*. *A. favus*, *A. fumigatus*, *A. niger* and *Rhizopus* were isolate from the oilseeds with or

without petroleum fumes. Two fungi, *Cladosporium* and *Paeciliomyces* were isolated from the oilseeds in the presence of the petroleum funes but not without the fumes (Table 1). *Bipolaris* was only isolated from seeds without fumes.

The growth pattern of fungi in the minimal salt solution is represented in Fig. 1. Figs. 2-4 show the growth pattern of fungi in the MSS and crude petroleum or petroleum products. In Fig. 1-4 there was fluctuation in the growth pattern of each fungus. It was observed that the 15th and 35th day of incubation recorded zero optical density for the growth of each fungus. On the 40th day of incubation, the MSS with *Botryodiplodia* had the highest optical density while *Pencillium* and A. *niger* had the least at zero optical density. The growth pattern of each fungus had different maximum growth peaks. *Botryodiplodia* had a maximum peak on the 30th day at an optical density of 2.0 and *Bipolaris* had the lowest peak of 0.17 on the 25th day of incubation. On the 40th day *Macrophomina* had the highest optical density at 1.8 and *Aspergillus niger* had the lowest optical density of 0.18 for kerosene. Fig. 3 shows the growth pattern of fungi in diesel and MSS. There were different growth peaks for each of the fungus. On the 40th day of incubation *A. flavus* had the highest peak at an optical density of 0.58, followed by *Bipolaris* and *A. niger* with the lowest of 0.14. In Fig. 4 the *A. niger* and *Botroyodiplodia* had maximum growth peaks of 2.0 on the 30th and 40th day respectively in crude petroleum and MSS. *Aspergillus flavus* had the lowest optical density of 0.26.

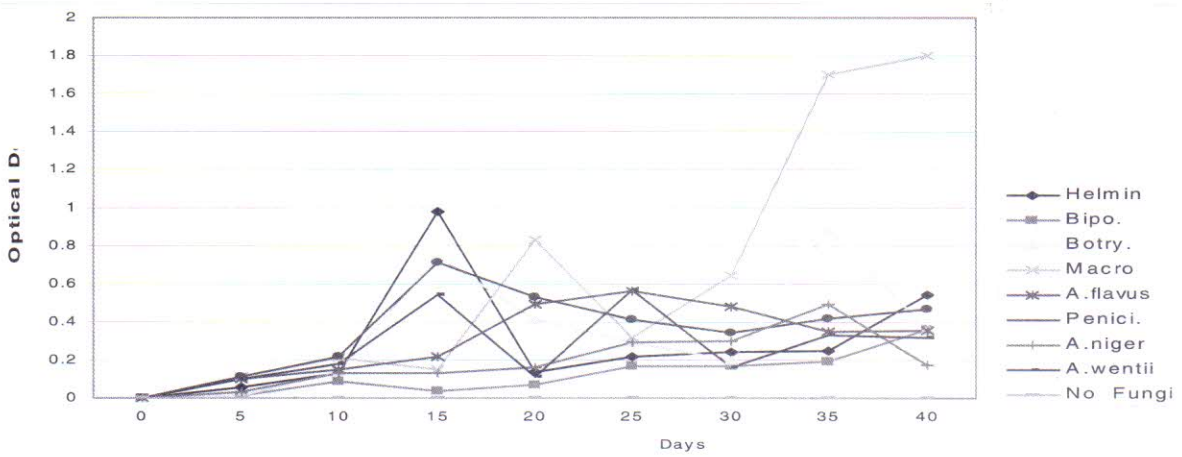

Fig. 2 : The qrowth pattern of fungi in kerosene and minimal salt solution.

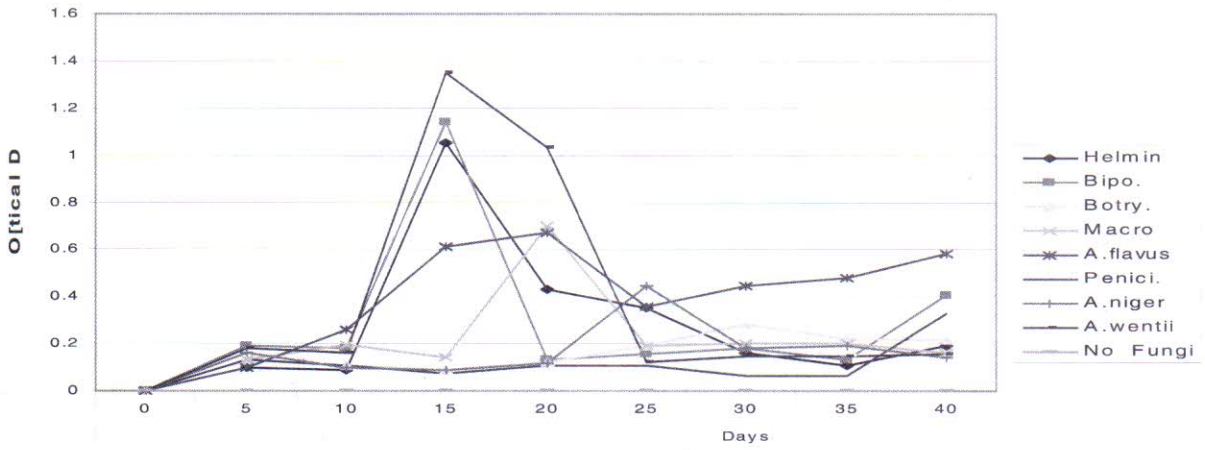

Fig. 3 : The growth pattern of fungi in diesel and minimal salt solution.

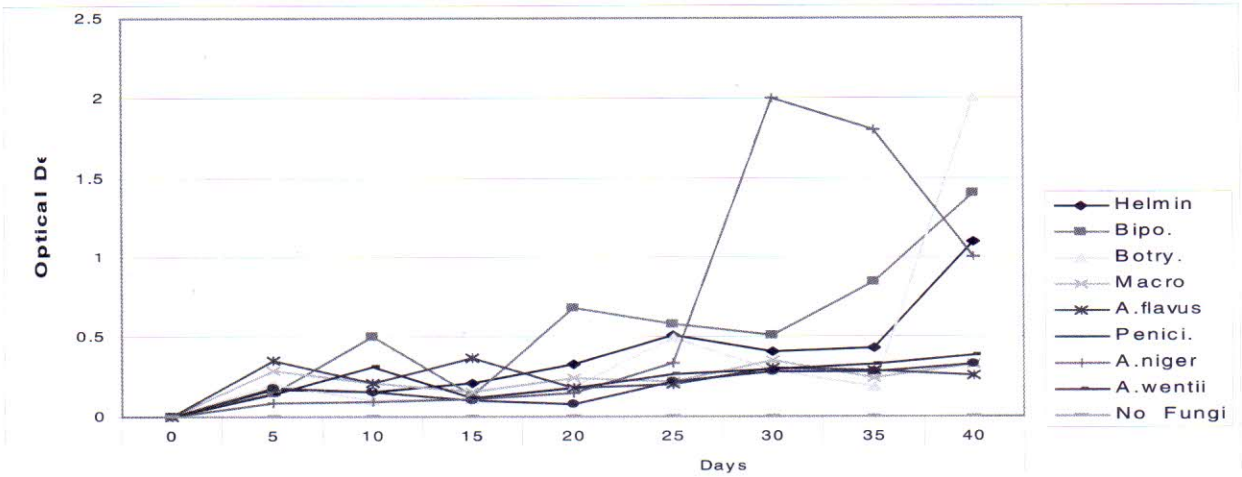

Fig. 4 : The growth pattern of fungi in crude oil and minimal salt solution

Discussion

The results of this work indicates that many fungal species isolated from the oilseeds were capable of degrading petroleum hydrocarbons. Zobell (1946) in his review noted more than 100 species representing 30 microbial genera capable of utilising hydrocarbons. Since then several workers like Bartha and Atlas (1973); Amund and Akangbou (1993); Cooney *et al* (1998) and Hughes amd Mekenzie (1998) have listed and shown that upto 22 genera of bacteria, 1 algal genus and 14 genera of fungi isolated from the soil and aquatic environment are able to biodegrade petroleum hydrocarbon. Colombo *et al* (1996) reported the biodegradation of lignolytic fungi which were isolated from soil. Here fourteen genera (21 species) of fungi isolated from oilseeds are being implicated to biodegrade crude petroleum and petroleum products such as diesel and kerosene. This therefore is probably the first report of hydrocarbon utilizers isolated from oilseeds, melon and soybean seeds to be specific.

It is of interest that *Botyodiplodia* and *Talariomyces* are shown probably for the first time in literature to have the ability to biodegrade crude petroleum. It is not surprising to have occurrence of some fungi, such as *Aspergillus flavus, A. fumigatus, Penicillium chrysogenum* and *Cladosporium* to be associated with crude oil degradation during oil pollution. This report supports earlier works by Hughes and Mckenzie (1998) who reported these four fungi to be rich in hydrocarbon assimilation. The low rate growth rate of Bipolaris in the MSS broth with the petroleum oil might be an indication that Bipolaris was poor in biodegrading the petroleum oil. This is further proved in the fact that Bipolaris was only isolated from seeds without petroleum fumes.

The difference or fluctuation in growth pattern of fungi investigated in the study might be that each fungus degraded the crude petroleum and its products at different rates. The rate of biodegradation of each fungus might be dependent on the type of enzymes involved and quantity of enzymes secreted as suggested by Keeler (1991). Shaw (1995) found that micro-organisms break down hydrocarbons and use the energy to synthesis cellular components. Studies by Prince (1993) estimated between 70-90% of crude oil is biodegraded. Though there were also nutrients in the minimal salt solution, more of the nutrient was probably present in the oil which stimulated the growth of each fungus. There is a demonstration here that fungi which could biodegrade oils of the oilseed can do same with the petroleum hydrocarbons.

In conclusion, the result here shows that fungi isolated from the oilseeds can be exploited in the biodegradation of crude petroleum oil spill and bioremediation of the environment.

References

Adekunle, A. A. and Ngwanma, U. Uma : Lipase activity of fourteen fungi on Cucumeropsis mannii seeds. *Nigerian Journal of Botany*, **9**, 35-40 (1996).

Amund, O. O. and C. N. Igiri, : Biodegradation of petroleum hydrocarbons under tropical estuarine conditions. *World J. Microbial Biotechnol.*, **6**, 225-262, (1990).

Amund, O. O. and T. S. Akangbou : Microbial degradation of four Nigerian crude oils in an estuarine microsmi. *Lett. Appl. Microbial.*, **16,** 118-121, (1993).

Amund, O. O., A. A. Adebowale and E. O. Ugoji : Occurrence and characteristics of hydrocarbon utilising bacteria in Nigerian soils contaminated with spent motor oil. *Indian Journal of Microbiology,* **27**, 63-67, (1987).

Bartha, R and R. M. Atlas : Biodegradation of oil in seawater: Limiting factors and artificial stimulation. *In*: The microbial degradation of oil pollutants (*Eds.*: D.G Ahern and S.P. Meyers,). Centre for wetland resources, Louissiana State University, Baton Rouge. pp 147-152, (1973).

Bryce, K. : The fifth kingdom. Mycologue publications, Ontario. 412pp, (1992).

Cerniglia, C. E. and J. J. Perry : Hydrocabon Utilisation by *Cladosporium resinae In*: The microbial degradation of oil pollutants (*Ed.* D.G. Ahern and S. P. Meyers.) Centre for wetland resources, Louissiana State University, Baton Rouge. pp 25-32 (1973).

Colombo, J.C., M Cobello and A. M Arambari : Biodegradation of aliphatic and aromatic hydrocarbons by natural soil microflora and ligolytic fungi. *Environmental pollution,* **40**, 335-362 (1996).

Cooney, J. J./ M. N. Doolittle and M. E.Miller : Marine fungi are potential agents for bioremediation of oil spills. *Biodegradation and biodeterioration symposium*, Leeds, 620pp (1998).

Damoschet, K. H., W. Gams, and T. Anderson : Compendium of *soil fungi.* Vol. 1. Academic press, London. 859pp (1980).

Deacon, J. W. : Introduction of modern mycology. Blackwell scientific publications, London. 197pp.

Hughes, D. E. and P. Mekenzie, (1998). The microbial degradation of oil in the sea. *Proceedings R. Society*, **189**, 375-390 (1980).

Keeler, R. : "Bioremediation" Healing the environment naturally. *R & D Magazine,* **2**, 34-40 (1991).

Kuku, F. O. : Some biodeterioration effects of lipolytic moulds on vegetable oils. Reports of Nigerian stored products research institute, *technical report*, **6**, 23-29 (1979).

Nwachukwu, S. C. U. : Enhanced rehabilitation of tropical aquatic environments polluted with crude petroleum using Candida utilis. *Journal of Environmental Biology*, **21(3),** 241-250 (2000).

Oyeniran, J. O. : The role of fungi in the deterioration of tropical stored products. Rep Nigerian stored products research institute, *occasional paper series*, **2**, 1-25 (1980).

Parker, R. E. : Introductory statistics for biology. 2nd edition. Edward Arnold, London. 112pp (1979).

Prince, R. C. : Petroleum spills bioremediation in marine environments. *Critical reviews in microbiology,* **19,** 217-242 (1993).

Shaw, B. : Microbes safety, effectively bioremediate oil fields pits. *Oils and gas Journal,* **4,** 85-88 (1995).

Talbot, O. H. K. : An introduction to mycology. Leonard Hill, London. 252pp (1971).

Wang, Z., M. Fingas and G. Sergy, : Study of 22 year old arrow oil samples using biomaker compounds by GC/MS. *Environ. Sc. Technol.*, **28**, 1733-1746 (1994).

Zobell, C. E. : Action of micro organism in hydrocarbons. *Bacteriol. Rev.*, **10**, 1-49 (1946).

Correspondence to:
Dr. A. A .Adekunle
Department of Botany and Microbiology, University of Lagos, Yaba, Lagos, Nigeria
E-mail : aaded@yahoo.com