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قسم التقنيات البتروكيمياوية

Petroleum biodegradation capacity of fungi isolated from petroleum –contaminated soil in Wasit Governorate

بحث تخرج مقدم الى قسم التقنيات البتروكيمياوية وهو جزء من متطلبات نيل درجة الدبلوم في التقنيات البتروكيمياوية

من قبل الطلاب:
حيدر محمد فيصل
علي قحطان علي
حسن حيدر كاظم

أشراف
د. اسراء جبار شمخي

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بسم الله الرحمن الرحيم

(وَيَسْأَلُونَكَ عَنِ الرُّوحِ قُلِ الرُّوحُ مِنْ أَمْرِ رَبِّي وَمَا أُوتِيتُم مِّنَ الْعِلْمِ إِلَّا قَلِيلًا)

صدق الله العلي العظيم

سورة الاسراء : الآية (85)

نحمد الله عز وجل الذي وفقنا في اتمام هذا البحث العلمي والذي أعطانا الصحة والعافية والعزيمة . فالحمدلله حمدا كثيرا .

أهداء ألى:

الشمعة التي انارة طريق حياتي

(عائلتي الكريمة)

الشكر و التقدير

اتقدم بجزيل الشكر والتقدير الى الاستاذة الفاضلة المشرفة على البحث الدكتورة اسراء جبار شمخي على كل ما قدمته لنا من توجيه ومعلومات قيمة ساهمت في اثراء موضوع دراستنا في جوانبه المختلفه .

كما نتقدم بجزيل الشكر الى رئاسة قسم التقنيات البتروكيمياوية واساتذته الأفاضل والى اعضاء لجنة المناقشة الموقرة.

والى كل من ساندي في مشوار دراستي.

Abstract

This study assayed oil-degrading potential of fungi isolated from crude-oil pollution soils. The samples were collected aseptically from four different sites with crude-oil pollution in Wassit province. These samples were analyzed for fungi loads and oil-degrading fungi using potato dextrose agar and mineral salt medium respectively. The biodegradation of crude oil was observed spectrophotometrically using the broth culture of the fungal isolates for a period of 15 days on mineral salt medium. The fungi were identified based on the microscopic and macroscopic features of the hyphal mass, nature of the fruiting bodies and the morphology of cells and spores. The five (5) fungi identified from the contaminated soils include; *Aspergillus fumigatus, Aspergillus niger, Trichoderma viridae, Candida albicans*, and *Aspergillus flavus*. All fungi showed degradation of the crude oil, with *Trichoderma viridae*, and *Aspergillus flavus* demonstrating best degradation ability. *Trichoderma viridae* exhibited highest degradation (66.2%) while *Aspergillus flavus* exhibited least degradation (40%). The measurement of the rate of biodegradation of crude oil by the three fungi was further confirmed using Gas Chromatography and Mass Spectrophotometer (GC-MS). The GC-MS analysis showed that the fungi degraded the hydrocarbon compounds when compared to that of the control. The result obtained revealed that oil-degrading fungi can be isolated from crude-oil pollution sites and they are competent microflora for the biodegradation of crude oil polluted soils. They can be used as a better approach to restoring oil contaminated environments through bioremediation process.

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Chapter one

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1.1 Introduction

The common problems associated with petroleum industry are accidental and deliberate discharge of oil spills. Oil spillage is known to be a major environmental problem in Nigeria, most especially in the Niger-Delta region, attracting considerable attention in the recent years. Many approaches (physical, chemical and biological methods) have been employed in order to reduce or eliminate the effect of oil spillage on the environment and living organisms. However, most of these efforts have limitations in their applications, either as being too expensive or posing threats to the ecosystem. The most promising of many researches carried out to deal with large-scale oil spills is the use of microorganisms to provide an effective alternative (Singh *et al.*, 2001). This approach is referred to as 'bioremediation', and it is one of the most rapidly growing areas of environmental microbiology, which has been used for cleaning up pollutants. This is because of its low cost, safety and its public acceptability (Grazyna *et al*., 2001). Microbial degradation represents the major route responsible for the ecological recovery of oil spills (Johnsen, *et al*., 2005).

Many species of bacteria and fungi isolated from oil spilled sites have been shown in recent years to have the abilities to use petroleum hydrocarbon as sole source of carbon and energy (Olukunle, et al., 2012; Boboye et al., 2010 and Ojo, 2006). Fungi have been found to be better degraders of petroleum than traditional bioremediation techniques including bacteria (Batelle, 2000). Although, hydrocarbon degraders may be expected to be readily isolated from a petroleum oil-associated environment, the same degree of expectation may be anticipated for microorganisms isolated from a totally unrelated environment (Ojo,2006).

Aims of the study:

1- Isolation and identification of fungi isolates from the soil.

2- Identification of fungi that capable of degrading crude oil.

1.2 Literatures Review

1.2.1 Petroleum

Petroleum is a complex mixture of liquid and solid hydrocarbons; whose composition also varies with the source. The components are paraffin hydrocarbon, saturated alicyclic hydrocarbon and aromatic hydrocarbon (Atlas and Bartha, 1998). Petroleum can be accidentally or deliberately released into the environment leading to serious pollution problems (Thouand *et al.*, 1999; Okoh *et al*., 2002; Okoh 2006).

Petroleum hydrocarbon pollution is hazardous to the ecosystem and public health (Iheoma, *et al*., 2015). It is because of toxicity of petroleum hydrocarbon that immediate and urgent attention should be given with view to cleaning up the spill. Crude oil negatively affects soil agricultural productivity and soil organisms; Crude oil has also been shown to reduce plant germination and productivity (Agbogidi, *et al*., 2005). A study by Wokocha, Emeodu and Ihenko (2011) also reported that crude oil pollution increase soil acidity. High soil acidity is not good for plants because it can destroy plants and impotent soil organisms.

Still small releases of petroleum hydrocarbons into aquifers can lead to concentrations of dissolved hydrocarbons far in excess of regulatory limits. These pollution problems often result in huge disorder of both the biotic and abiotic components of the ecosystems (Mueller et al. 1992), more so that some hydrocarbon components have been known to belong to a family of carcinogenic and neurotoxic organ pollutants (Hallier-Soulier et al., 1999). The processes leading to the eventual removal of hydrocarbon pollutants from the environment has been extensively documented and involves the trio of physical, chemical and biological alternatives (Okoh, 2006).

1.2.2 Fungi

1.2.2.1 *Aspergillus niger*

Aspergillus niger is one of the most common species of the *Aspergillus* genus. It can cause a disease that called black mould on certain vegetables and fruits such as onions, apricots, grapes, and peanuts, and is a common food contaminant (Wucherpfennig *et al*., 2011).

Aspergillus niger is less likely to cause human disease than some other *Aspergillus* species. In extremely rare instances, humans may infect but this is due to a serious of lung disease, aspergillosis, that can occur. Aspergillosis is frequent among workers of horticultural that inhale dust of peat, that will can be rich in spores of *Aspergillus* (Reiss *et al*., 2011).

Aspergillus niger is one of the most common causes of ear infections, otomycosis, which can cause temporary hearing loss, pain, and, in severe cases, it can cause damage to the ear canal and tympanic membrane (Schuster et al., 2002).

1.2.2.2 *Aspergillus flavus*

Aspergillus flavus belong to the kingdom Fungi, Division Ascomycota. It was a saprotrophic and pathogenic fungus distribution. *A. flavus* produce toxic compounds known as mycotoxins, which toxic to mammals (Agrios and george, 2005). It produced four major aflatoxins (B1, B2, G1, and G2). The production of toxins was result of particular strains of *A. flavus* Aflatoxin B1 is the most toxic and potent hepatocarcinogenic natural compound characterized. *A. flavus* also produces other toxic compounds including sterigmatocystin, cyclopiazonic acid, kojic acid, β-nitropropionic acid, aspertoxin, aflatrem, gliotoxin, and aspergillic acid (Hedayati *et al*., 2007).

A. flavus was also an opportunistic pathogen for human and animal, causing aspergillosis in immunocompromised individuals. *A. flavus* is found in soils as saprophytic fungi and causes disease on many important agriculture crops (Amaike and Nancy, 2011). The hypha was branching growth thread-like branching and produces mycelia. Hyphae were septate and hyaline. The mycelium secreted degradative enzymes or proteins that can break down complex nutrients. Conidiophore was rough and colorless and the Phialides were both uniseriate (arranged in one row) and biseriate. The conidiospores were asexual spores producing during reproduction (Alexopoulos, 1996).

1.2.2.3 *Aspergillus fumigatus*

Colonies of *A. fumigatus* on sabouraud dextrose agar supplemented with chloramphenicol, appear powdery, the color at the first seem to be white then turning to dark greenish and changed to gray, reversed side of the colonies appeared pale yellow to tan with growth conditions at 37°C for 7 days of incubation (Dheeb, 2013). Conidial heads are typically columnar and uniseriate row of phialides on the upper two thirds of the vesicle. Conidiophore stipes are short, smooth-walled and have conical-shaped terminal vesicles. Conidia are produced in basipetal succession forming long chains and are globose to sub globose, green and rough-walled to echinulate.

1.2.2.4 *Candida*

Yeasts are a type of fungus. This group of fungi were the most common cause of fungus infections in the world (Manolakaki *et al*.2010). Most people have or are exposed to this group of fungus but not sick. *Candida* cause opportunistic infection, when the host's immune defences were compromised in any way they have the potential to overgrow and cause infection due to HIV infection, anticancer

therapy and treatment with immunosuppressive drugs used in organ transplantation (Kourkoumpetis and Themistoklis,2011).

Candida albicans was the most common kind of *Candida*, Infection with this fungus was called candidiasis or thrush, some species in this group can be found in the human gut, blood infection, vaginal infection in women, genital infection in man. It is cause opportunistic infection for the person antibiotics for long time and People with diabetes or HIV are more likely to get a yeast infection. *Candida* can be found on almost all healthy skin and other areas of the body these are the nose, throat, lungs, digestive system and the vagina and can cause disease in the mouth it is called thrush (oropharyngeal candidiasis). When this fungus causes disease in the female reproductive organs it is called vaginal candidiasis (Darwazeh *et al*., 1990).

1.2.2.5 *Trichoderma viridae*

Among thousands of fungal genera, the genus *Trichoderma* is a diverse group of free-living fungi in the phylum Ascomycetes, class Sordariomycetes, order Hypocreales, family Hypocreaceae, commonly present in all soils (Mohan, 2017). These ascomycetes fungi are opportunistic, a virulent plant symbionts inhabiting root ecosystems and parasites on other groups of fungi (Schuster and Schmoll,2010; Digamber,2017). *Trichoderma* is one of the broadest impacts on mankind. Some of its species, chiefly *T. harzianum, T. viride*, *T. asperllum*, and *T. orientale* are known as antimicrobial producers of enzymes, anthraquinone, and recombinant proteins; some are used for the production of secondary metabolites (Jeleń *et al*., 2014),

Chapter two

Chapter two

2. Materials and Methods:

2.1 Chemicals and apparatus:

2.1.1 Chemicals:

The chemical and biological materials used in this analysis are listed in the (Table 2.1).

Table (2.1): The chemicals that used in this stud

Chemical	Company	Country
Agar	BDH	England
Aniline blue(cotton blue)	Fluka	Germany
Bromo Cresol Purple	BDH	England
Casein	Fluka	Germany
Chloramphenicol	SDI	Iraq
Glucose	BDH	England
Glycerol	BDH	England

2.1.2 Apparatus

The apparatus used in this study are listed in table (2.2).

Table (2.2): The apparatus that used in this study

Equipment	Company	Country
Autoclave	Takizawa - Japan	Japan
Compound Microscope	Olympus optical co. LTD	Japan
Distillator	Gesell schaft fur	Germany
Hot Plate and Magnetic Stirrer	Gallenkamp	England
Incubator	Gallenkamp	England
Laminar flow hood	K & K Scientific Supplier	Korea
pH-Meter	Bio Red	Italy
Sensetive Balance	Gallenkamp	England
Separating funnel	Rogo sampaic	France

2.2 Methods:

2.2.1 Culture media

All the used media were dissolved into D.W. and sterilized by autoclave at 121°C for 15 minutes below 15 psi according to the manufacturers' instructions.

2.2.1.1 Potato Dextrose Agar

It was prepared for routine fungal cultivation, and identification according to the manufacturer company (Richardson and Warnock, 1993).

2.2.1.2 Sabouraud's Dextrose Agar

It was prepared according to the manufacturer company for routine fungal cultivation with add chloramphenicol (0.05 g / L) (Shamqi *et al*., 2014).

2.3 Collection of soil samples

Four soil samples were collected from different locations of Wassit fileds (petroleum-contaminated soil) at a depth within (4-5 cm) using a metal spatula that was sterilized every time with 70% alcohol. The samples were kept in new polythene bags, sealed, and transported to the laboratory immediately for the mycological examination (Ashok *et al.,* 2015). Several methods are available for the isolation of fungi; however, one of the commonest methods reported in literature is the serial dilution of sample (Iqbal *et al*., 2017). This technique is simple, cost-effective, and appropriate to handle large samples (Fig 2.1).

Figure (2.1): Soil sample collection position

2.4 Isolation of fungi from soil

Dissolving 10 g of soil samples were placed in a 250 ml conical flask containing 90 ml sterile distilled water is preparing the stock solution. The flask was shaken on an electric shaker to get a homogenous suspension next, a serial dilution of sample was prepared as (10^{-1}, 10^{-2} and 10^{-3}). One milliliter of each of the dilutions is uniformly distributed on a petri dish that contains potato dextrose agar. It was incubated at 28 ± 1°C for seven days (Mutia and Prilya, 2017). After growing various colonies on medium-sized potato dextrose agar plates, fungal cultures were then transmitted and subculture to have a pure culture.

2.5 Identification of fungi isolates by Microscopic and macroscopic examination:

Depending on the morphology of the colony and microscopic examination (Conventional methods) according to (Samuels and Hebbar, 2015). The technique of slide culture was used to distinguish *Trichoderma* species accurately. The precise arrangement of the conidiophores and the manner in which spores are formed must be observed (conidial ontogeny). One plate of potato dextrose agar was used to prepare the slide culture:

1. A small agar block (7 x 7 mm) was cut out using a sterile blade.

2. The block flipped up onto the surface of the agar plate.

3. The four sides of the agar block were inoculated with spores or mycelia fragments of the fungus to be grown.

4. A flamed coverslip was placed centrally upon the agar block.

5. The plate was incubated at 26°C until growth and sporulation have occurred.

6. Coverslip was removed from the agar block.

7. A drop of 95% alcohol applied as a wetting agent.

8. The coverslip was lowered gently onto a small drop of lacto phenol cotton blue on a clean glass slide.

9. The slide can be left overnight to dry and later sealed with fingernail Polish (Ellis *et al*., 2007).

2.6 Determination of Physicochemical Characteristics of petroleum soil contaminated

The temperature was determined in situ using a held-held Mercury thermometer. The pH of the soil was determined by making a dilution of the soil sample with deionized water in the ratio of water soil of 2:1 (Stewart *et al*., 1947).

Chapter three

Chapter three

Results and Discussion

3.1 Fungal isolation

The yeast diagnosis by API 20 Candida kit showed *Candida albicans* showed (Fig 3.1).

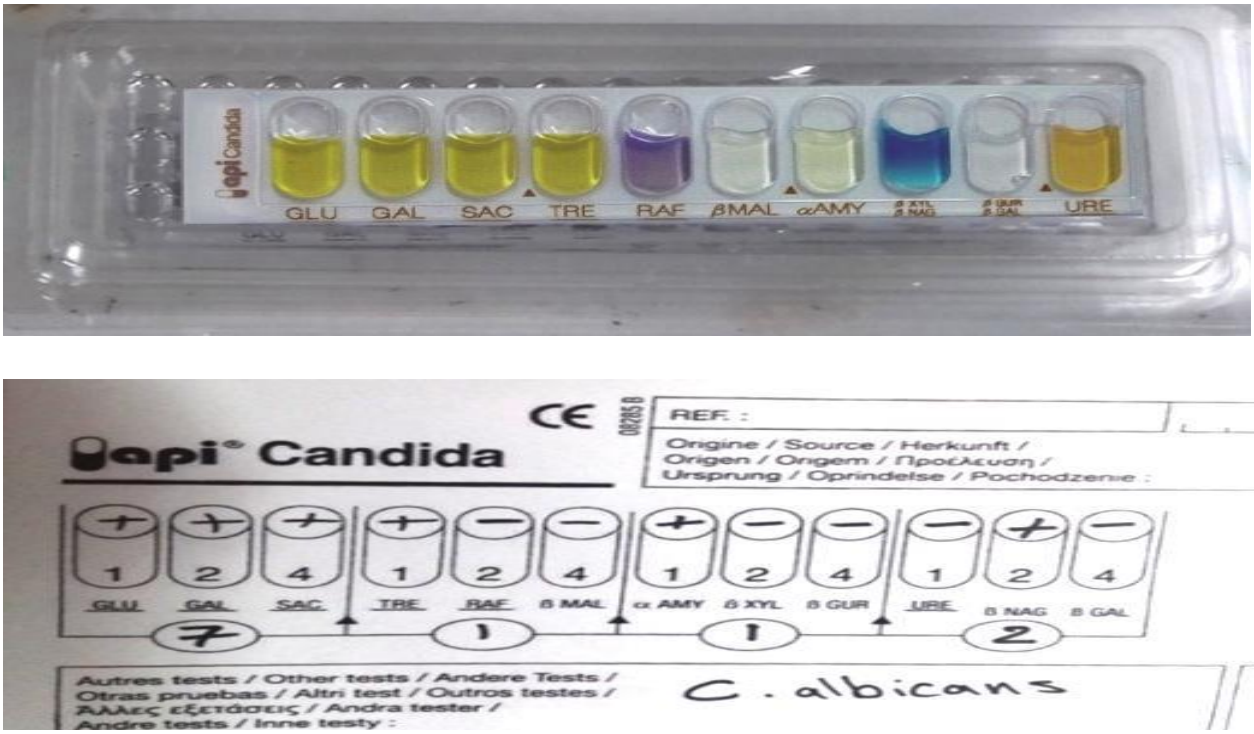

Figure (3.1): Identification of *Candida albicans* by Api Candida Kit

Also, Microscopic and macroscopic features of the hyphal cells and morphology of cells and spores revealed diverse microbial community, such as *C. albicans*, and three species of *Aspergillus*: *A. niger*, *A. flavus*, and *A. fumigatus* were obtained from crude-oil pollution soils, and *T. viridae*, as shown in (Table 3.1), (Fig 3.2).

Table (3.1): suspected fungi from petroleum soils contaminated

Isolate sample	Cultural appearance	Microscopic features	Name of Organisms
1	Brown mycelia growth	An upright conidiophores that terminates in a debate swelling bearing phialides at the apex or radiating from the entire surface , conidia are 1- celled and globe	***A spergillus Fumigatus***
2	Black mycelia And fully extended In the growth Mealime	An upright conidiophores that terminates in a debate swelling bearing phialides at the apex or radiating from the entire surface, conidia are 1- celled and globe.	***A niger***
3	Green mycelia growth	A branched conidiophore hyaline, not verticillates phialides single, conidia hyaline 1- celled ovoid borne in smell terminal cluster.	***Trichoderms viridae***
4	Greenish mould	My celium are not extensive conidia are 1- celled , ovoid to fusoid , forming short chains by budding which are producel on my celium epically or laterally .	
5	Yellow mycelia growth	An upright conidiophores that terminates in a debate swelling bearing phialides at the apex or radiating from the entire surface, conidia are 1- celled and globe.	***A spergillus flavus***

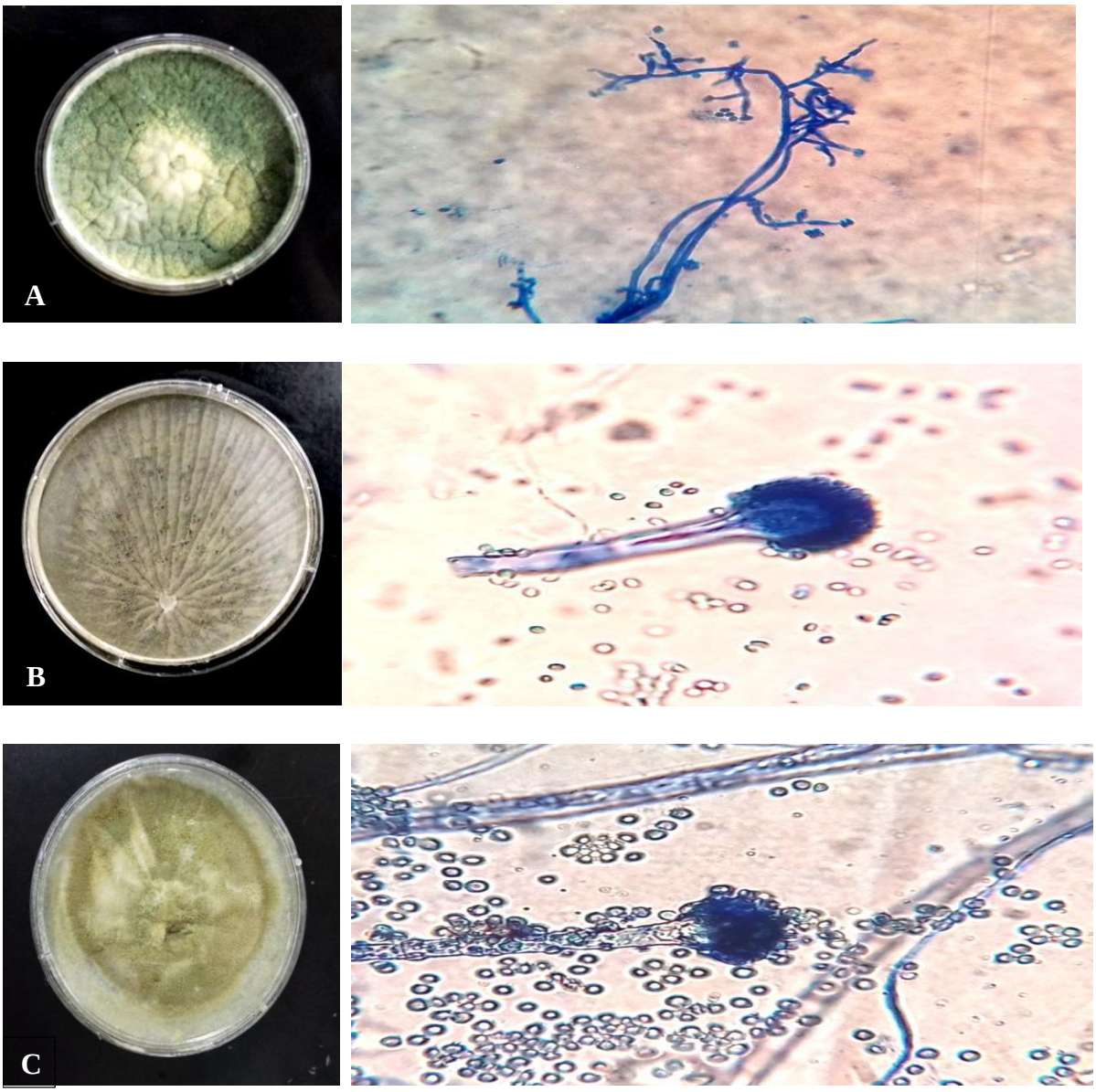

Figure (3.2): Fungi species grown on SDA at 35±2°C after 5 days of incubation and microscopic examination: (A) *Trichoderma viridae* (B) *Aspergillus fumegatus* (C) *Aspergillus flavus*.

The fungi isolated from the soil samples are *Bdellospora helicoides, Aspergillus fumigatus, Aspergillus niger, Trichoderma viridae, Pleurothecium recurvatum, Streptothrix atra, Gonadobotricum apiculata, Candida albicans, Aspergillus flavus, Helminthosporium velutinum, Botrytis cinerea, Zoophage*

nitospora, Varicosporium elodeae, Articulospora inflata, Neurospora crassa and *Thysarophora longispora* (Obire,1988; Arotupin, and Akinyosoye, 2001, and Ekundayo, 2004).

Fungi spp. had the best growth in the mineral salt broth after 15 days of incubation. From the Gas Chromatography and Mass Spectrophotometric analysis on the one isolates, (Fig 3.3) shows the gas chromatogram of undegraded (incubated control) crude oil.

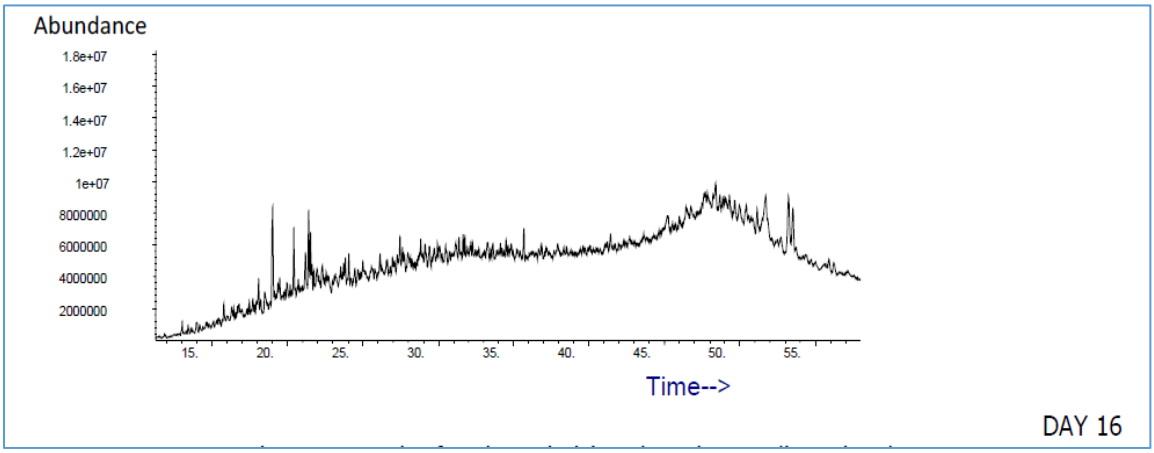

Figure (3.3): Chromatograph of undegraded (incubated control) crude oil

Gas chromatographic patterns of crude oil degraded by *T. viridae* after 16 days of incubation, (Fig 3.4). It injection was used with helium as carrier gas. The oven temperature was initially set at 35°c for 2 minutes and increased at a rate of 5°c/min to 280°c and ran for 60 minutes.

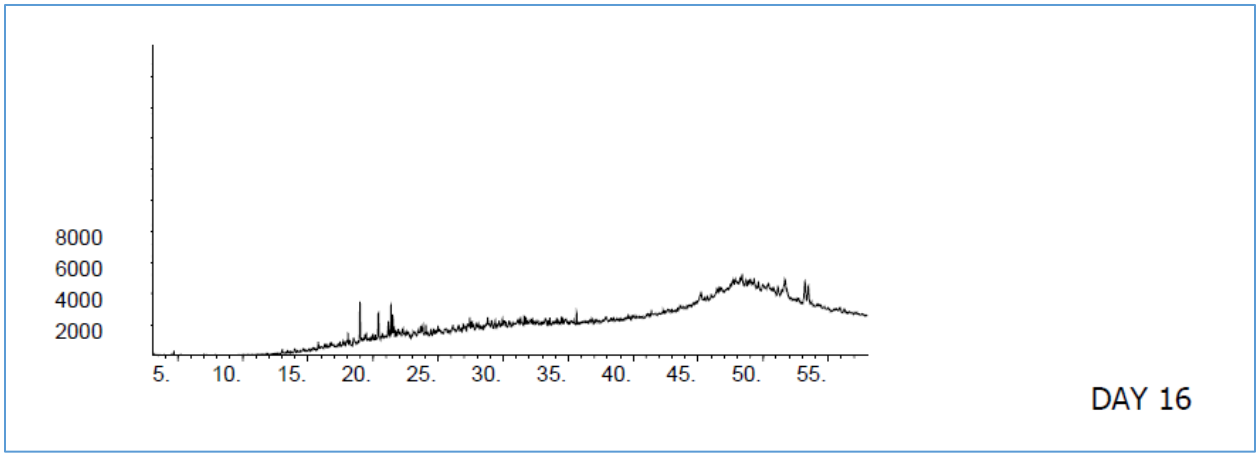

Figure (3.4): Chromatograph of degraded crude oil by *T. viridae.*

The study observed that the fungi were slow in attacking or degrading the crude oil on the 4th, 8th and 12th day of incubation but were later able to degrade the oil effectively. This is due to the fact that the fungi are slow growers, so it took them longer time to start attacking the crude oil. It was also observed that after 4, 8 and 12 days of incubation the organisms did not show any significant degradation but after the 16th day of incubation, it was observed that *T. viridae* degraded the crude oil best from other fungi.

All the fungal isolates showed varying degree of biodegradation of crude oil in Mineral salt medium indicating that they all utilize crude oil as their main source of carbon. An interesting demonstration generated in this work shows an increase in rates of fungal growth in the media containing crude oil which might be due to the fact that the fungi used crude oil as a substrate for their growth using extra cellular enzymes to break down the recalcitrant hydrocarbon molecules, by dismantling the long chains of hydrogen and carbon, thereby, converting crude oil into simpler forms or products that can be absorbed for the growth and nutrition of the fungi as shown by Adekunle and Adebambo, (2007). It also agreed with the

work of Akinde and Obire (2008), which revealed the presence of potential hydrocarbon degraders in cow dung.

Conclusion

It can be concluded from this study that the rate of biodegradation of petroleum hydrocarbon in petroleum-contaminated soils. The fungi isolated and tested for biodegradation were very effective and since they were isolated from petroleum-contaminated soils. The organisms *Aspergillus* spp, *Trichoderma viridie* and *Candidia albicans* may be pointed out as potential fungi to degrade petroleum Hydrocarbons.

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